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(54) Title: DEATH DOMAIN CONTAINING RECEPTOR 4

(57) Abstract

The present invention relates to novel Death Domain Containing Receptor–4 (DR4) proteins which are members of the tumor necrosis factor (TNF) receptor family. In particular, isolated nucleic acid molecules are provided encoding the human DR4 proteins. DR4 polypeptides are also provided as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of DR4 activity and methods for using DR4 polynucleotides and polypeptides.

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Death Domain Containing Receptor 4

Background of the Invention

Field of the Invention

The present invention relates to a novel member of the tumor necrosis factor family of receptors. More specifically, isolated nucleic acid molecules are provided encoding human Death Domain Containing Receptor 4, sometimes herein "DR4". DR4 polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of DR4 activity and methods for using DR4 polynucleotides and polypeptides.

Related Art

Many biological actions, for instance, response to certain stimuli and natural biological processes, are controlled by factors, such as cytokines. Many cytokines act through receptors by engaging the receptor and producing an intra-cellular response.

For example, tumor necrosis factors (TNF) alpha and beta are cytokines which act through TNF receptors to regulate numerous biological processes, including protection against infection and induction of shock and inflammatory disease. The TNF molecules belong to the "TNF-ligand" superfamily, and act together with their receptors or counter-ligands, the "TNF-receptor" superfamily. So far, nine members of the TNF ligand superfamily have been identified and ten members of the TNF-receptor superfamily have been characterized.

Among the ligands there are included TNF-α, lymphotoxin-α (LT- α, also known as TNF-β), LT-β (found in complex heterotrimer LT-α2-β), FasL, CD40L, CD27L, CD30L, 4-lBBL, OX40L and nerve growth factor (NGF). The superfamily of TNF receptors includes the p55TNF receptor, p75TNF receptor, TNF receptor-related protein, FAS antigen or APO-1, CD40, CD27, CD30,

4-lBB, OX40, low affinity p75 and NGF-receptor (Meager, A., *Biologicals, 22*:291-295 (1994)).

Many members of the TNF-ligand superfamily are expressed by activated T-cells, implying that they are necessary for T-cell interactions with other cell types which underlie cell ontogeny and functions. (Meager, A., *supra*).

Considerable insight into the essential functions of several members of the TNF receptor family has been gained from the identification and creation of mutants that abolish the expression of these proteins. For example, naturally occurring mutations in the FAS antigen and its ligand cause lymphoproliferative disease (Watanabe-Fukunaga, R., *et al., Nature 356*:314 (1992)), perhaps reflecting a failure of programmed cell death. Mutations of the CD40 ligand cause an X-linked immunodeficiency state characterized by high levels of immunoglobulin M and low levels of immunoglobulin G in plasma, indicating faulty T-cell-dependent B-cell activation (Allen, R.C. *et al., Science 259*:990 (1993)). Targeted mutations of the low affinity nerve growth factor receptor cause a disorder characterized by faulty sensory innovation of peripheral structures (Lee, K.F. *et al., Cell 69*:737 (1992)).

TNF and LT-α are capable of binding to two TNF receptors (the 55- and 75-kd TNF receptors). A large number of biological effects elicited by TNF and LT-α, acting through their receptors, include hemorrhagic necrosis of transplanted tumors, cytotoxicity, a role in endotoxic shock, inflammation, immunoregulation, proliferation and anti-viral responses, as well as protection against the deleterious effects of ionizing radiation. TNF and LT-α are involved in the pathogenesis of a wide range of diseases, including endotoxic shock, cerebral malaria, tumors, autoimmune disease, AIDS and graft-host rejection (Beutler, B. and Von Huffel, C., *Science 264*:667-668 (1994)). Mutations in the p55 Receptor cause increased susceptibility to microbial infection.

Moreover, an about 80 amino acid domain near the C-terminus of TNFR1 (p55) and Fas was reported as the "death domain," which is responsible for

transducing signals for programmed cell death (Tartaglia *et al., Cell 74*:845 (1993)).

Apoptosis, or programmed cell death, is a physiologic process essential to the normal development and homeostasis of multicellular organisms (H. Steller, *Science* 267, 1445-1449 (1995)). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer, neurodegenerative disorders, and acquired immune deficiency syndrome (C.B. Thompson, *Science* 267, 1456-1462 (1995)). Recently, much attention has focused on the signal transduction and biological function of two cell surface death receptors, Fas/APO-1 and TNFR-1 (J.L. Cleveland, *et al., Cell* 81, 479-482 (1995); A. Fraser, *et al., Cell* 85, 781-784 (1996); S. Nagata, *et al., Science* 267, 1449-56 (1995)). Both are members of the TNF receptor family which also include TNFR-2, low affinity NGFR, CD40, and CD30, among others (C.A. Smith, *et al., Science 248*, 1019-23 (1990); M. Tewari, *et al.*, in *Modular Texts in Molecular and Cell Biology* M. Purton, Heldin, Carl, Ed. (Chapman and Hall, London, 1995). While family members are defined by the presence of cysteine-rich repeats in their extracellular domains, Fas/APO-1 and TNFR-1 also share a region of intracellular homology, appropriately designated the "death domain", which is distantly related to the Drosophila suicide gene, reaper (P. Golstein, *et al., Cell 81*, 185-6 (1995); K. White *et al., Science* 264, 677-83 (1994)). This shared death domain suggests that both receptors interact with a related set of signal transducing molecules that, until recently, remained unidentified. Activation of Fas/APO-1 recruits the death domain-containing adapter molecule FADD/MORT1 (A.M. Chinnaiyan, *et al., Cell 81*, 505-12 (1995); M. P. Boldin, *et al., J. Biol Chem 270*, 7795-8 (1995); F.C. Kischkel, *et al., EMBO 14*, 5579-5588 (1995)), which in turn binds and presumably activates FLICE/MACH1, a member of the ICE/CED-3 family of pro-apoptotic proteases (M. Muzio *et al., Cell 85*, 817-827 (1996); M.P. Boldin, *et al., Cell 85*, 803-815 (1996)). While the central role of Fas/APO-1 is to trigger cell death, TNFR-1 can signal an array of diverse biological activities-many of which stem from its ability to activate NF-kB

(L.A. Tartaglia, *et al., Immunol Today 13*, 151-3 (1992)). Accordingly, TNFR-1 recruits the multivalent adapter molecule TRADD, which like FADD, also contains a death domain (H. Hsu, *et al., Cell 81*, 495-504 (1995); H. Hsu, *et al., Cell 84*, 299-308 (1996)). Through its associations with a number of signaling molecules including FADD, TRAF2, and RIP, TRADD can signal both apoptosis and NF-kB activation (H. Hsu, *et al., Cell 84*, 299-308 (1996); H. Hsu, *et al., Immunity 4*, 387-396 (1996)).

Recently a new apoptosis inducing ligand was discovered. Wiley, S.R. et al., refer to the new molecule as TNF-related apoptosis-inducing ligand or ("TRAIL") (*Immunity 3*:673-682 (1995)). Pitti, R.M. et al., refer to the new molecule as Apo-2 ligand or ("Apo-2L"). For convenience, it will be referred to herein as TRAIL.

Unlike FAS ligand whose transcripts appear to be largely restricted to stimulated T-cells, significant levels of TRAIL are seen in many tissues, and it is constitutively transcribed by some cell lines. It has been shown that TRAIL acts independently from FAS ligand (Wiley, S.R., et al. (1995)), *supra*). Studies by Marsters, S.A. et al., have indicated that TRAIL activates apoptosis rapidly, within a time frame that is similar to death signaling by FAS/Apo-1L but much faster than TNF-induced apoptosis (*Current Biology, 6*:750-752 (1996)). All work to date suggest that the receptor for TRAIL is not one of the many known TNF-receptors.

The effects of TNF family ligands and TNF family receptors are varied and influence numerous functions, both normal and abnormal, in the biological processes of the mammalian system. There is a clear need, therefore, for identification and characterization of such receptors and ligands that influence biological activity, both normally and in disease states. In particular, there is a need to isolate and characterize the receptor for the newly discovered TRAIL ligand.

Summary of the Invention

The present invention provides for isolated nucleic acid molecules comprising, or alternatively consisting of, nucleic acid sequences encoding the amino acid sequence shown in SEQ ID NO:2 or the amino acid sequence encoding the cDNA clone deposited as ATCC Deposit No. 97853 on January 21, 1997.

The present invention also provides vectors and host cells for recombinant expression of the nucleic acid molecules described herein, as well as to methods of making such vectors and host cells and for using them for production of DR4 polypeptides or peptides by recombinant techniques.

The invention further provides an isolated DR4 polypeptide having an amino acid sequence encoded by a polynucleotide described herein.

The present invention also provides diagnostic assays such as quantitative and diagnostic assays for detecting levels of DR4 protein. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of DR4, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors.

Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes. Cellular response to TNF-family ligands include not only normal physiological responses, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis-programmed cell death-is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes. Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers, autoimmune disorders, viral infections, inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include

AIDS, neurodegenerative disorders, myelodysplastic syndromes, ischemic injury, toxin-induced liver disease, septic shock, cachexia and anorexia.

Thus, the invention further provides a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR4 polypeptide an effective amount of an agonist capable of increasing DR4 mediated signaling. Preferably, DR4 mediated signaling is increased to treat and/or prevent a disease wherein decreased apoptosis is exhibited.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR4 polypeptide an effective amount of an antagonist capable of decreasing DR4 mediated signaling. Preferably, DR4 mediated signaling is decreased to treat and/or prevent a disease wherein increased apoptosis is exhibited.

Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below. Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR4 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By the invention, a cell expressing the DR4 polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Brief Description of the Figures

Figure 1 shows the nucleotide and deduced amino acid sequence of DR4. It is predicted that amino acids from about 1 to about 23 constitute the signal peptide, amino acids from about 24 to about 238 constitute the extracellular domain, amino acids from about 131 to about 229 constitute the cysteine rich domain, amino acids from about 239 to about 264 constitute the transmembrane domain, and amino acids from about 265 to about 468 constitute the intracellular domain of which amino acids from about 379 to about 422 constitute the death domain.

Figure 2 shows the regions of similarity between the amino acid sequences of DR4, human tumor necrosis factor receptor 1 (SEQ ID NO:3), human Fas protein (SEQ ID NO:4), and the death domain containing receptor 3 (DR3) (SEQ ID NO:5). Residues that match the consensus are shaded.

Figure 3 shows an analysis of the DR4 amino acid sequence. Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown, as predicted for the amino acid sequence depicted in Figure 1 (SEQ ID NO:2) using the default parameters of the recited computer programs. In the "Antigenic Index - Jameson-Wolf" graph, amino acid residues 35-92, 114-160, 169-240, 267-298, 330-364, 391-404, and 418-465 in Figure 1 (SEQ ID NO:2) correspond to the shown highly antigenic regions of the DR4 protein.

Figure 4 shows the nucleotide sequences of related nucleic acid fragments HTOIY07R (SEQ ID NO:6) and HTXEY80R (SEQ ID NO:7).

Figures 5A and 5B show the ability of DR4 to induce apoptosis in the cell lines MCF7 and 293. Figure 5C shows the ability of death protease inhibitors z-VAD-fmk and CrmA to inhibit the apoptotic action of DR4.

Figure 6A shows the ability of a soluble extracellular DR4-Fc fusion to block the apoptotic inducing ability of TRAIL. Figure 6B shows the inability of

soluble extracellular DR4-Fc fusion to block the apoptotic inducing ability of TNF-alpha.

Detailed Description of the Preferred Embodiments

The present invention provides isolated nucleic acid molecules comprising, or alternatively consisting of, a nucleic acid sequence encoding the DR4 polypeptide whose amino acid sequence is shown in SEQ ID NO:2, or a fragment of the polypeptide. The DR4 polypeptide of the present invention shares sequence homology with human TNFR-I, DR3 and Fas ligand (Figure 2). The nucleotide sequence shown in SEQ ID NO:1 was obtained by sequencing cDNA clones such as HCUDS60, which was deposited on January 21, 1997 at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia, 20110-2209, and given Accession Number 97853. The deposited clone is contained in the pBK plasmid (Stratagene, LaJolla, CA).

Nucleic Acid Molecules

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the

art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

By "isolated" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example, recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for purposed of the invention, as are native or recombinant polypeptides which have been substantially purified by any suitable technique such as, for example, the single-step purification method disclosed in Smith and Johnson, *Gene 67*:31-40 (1988).

Using the information provided herein, such as the nucleic acid sequence set out in SEQ ID NO:1, a nucleic acid molecule of the present invention encoding a DR4 polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the gene of the present invention has also been identified in cDNA libraries of the following tissues: amniotic cells, heart, liver cancer, kidney, leukocyte, activated T-cell, K562 plus PMA, W138 cells, Th2 cells, human tonsils, and CD34 depleted buffy coat (cord blood).

The DR4 gene contains an open reading frame encoding a mature protein of about 445 amino acid residues whose initiation codon is at position 19-21 of the nucleotide sequence shown in SEQ ID NO.1, with a leader sequence of about 23 amino acid residues (*i.e.*, a total protein length of 468 amino acids), and a deduced molecular weight of about 50 kDa. In this context "about" includes the particularly recited size, larger or smaller by several (5, 4, 3, 2, or 1) amino acid residues, at either terminus or at both termini.

Of known members of the TNF receptor family, the DR4 polypeptide of the invention shares the greatest degree of homology with human TNFRl and DR3

polypeptides shown in Figure 2, including significant sequence homology over the multiple Cysteine Rich domains.

In addition to the sequence homology exhibited between DR4 and other death domain containing receptors, DR4 has been shown to bind to TRAIL and to induce apoptosis when transiently expressed. MCF7 human breast carcinoma cells and 293 cells were transiently transfected with a DR4 expressing construct, as described in Example 5. As shown in Figures 5A and 5B a substantial proportion of transfected cells underwent the morphological changes characteristic of apoptosis. As anticipated, deletion of the death domain abolished the ability of DR4 to engage the death pathway. As can be seen in Figure 5C, DR4-induced apoptosis was efficiently blocked by inhibitors of death proteases including z-VAD-fmk, an irreversible broad spectrum caspase inhibitor and CrmA, a cowpox virus encoded serpin that preferentially inhibits apical caspases such as FLICE/MACH-1 (caspase-8). Since TNFR-1, CD-95 and DR3-induced apoptosis is also attenuated by these same inhibitors, it is likely that the downstream death effector molecules are similar in nature.

To determine if DR4 was capable of binding TRAIL, the extracellular ligand binding domain of DR4 was expressed as a fusion to the Fc region of human IgG (DR4-Fc). TRAIL selectively bound to DR4-Fc but not to corresponding extracellular domains of TNFR-1 or CD-95, also expressed as Fc fusions, data not shown. Additionally, DR4-Fc did not bind either TNF alpha or Fas ligand under conditions where both of these ligands bound their cognate receptors.

The ability of TRAIL to induce apoptosis in MCF7 cells was specifically blocked by DR4-Fc but not influenced by TNFR1-Fc, CD95-Fc or Fc alone (Figure 6A). Further, as expected, TNF alpha-induced apoptosis was inhibited by TNFR-1-Fc but not by DR4-Fc, CD95-Fc or Fc alone (Figure 6B).

Taken together, the data described above indicate that DR4 is a death domain containing receptor with the ability to induce apoptosis and is a receptor for TRAIL a known apoptosis inducing ligand.

As indicated, the present invention also provides the mature form(s) of the DR4 protein of the present invention. According to the signal hypothesis, proteins secreted by mammalian cells have a signal or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Most mammalian cells and even insect cells cleave secreted proteins with the same specificity. However, in some cases, cleavage of a secreted protein is not entirely uniform, which results in two or more mature species on the protein. Further, it has long been known that the cleavage specificity of a secreted protein is ultimately determined by the primary structure of the complete protein, that is, it is inherent in the amino acid sequence of the polypeptide. Therefore, the present invention provides a nucleotide sequence encoding the mature DR4 polypeptide having the amino acid sequence encoded by the cDNA contained in the host identified as ATCC Deposit No. 97853, and as shown in SEQ ID NO:2. By the mature DR4 protein having the amino acid sequence encoded by the cDNA contained in the host identified as ATCC Deposit No. 97853, is meant the mature form(s) of the DR4 protein produced by expression in a mammalian cell (*e.g.*, COS cells, as described below) of the complete open reading frame encoded by the human cDNA contained in the vector in the deposited host. As indicated below, the mature DR4 having the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 97853, may or may not differ from the predicted "mature" DR4 protein shown in SEQ ID NO:2 (amino acids from about 24 to about 468 in SEQ ID NO:2) depending on the accuracy of the predicted cleavage site based on computer analysis. In this context "about" includes the particularly recited size, larger or smaller by several (5, 4, 3, 2, or 1) amino acid residues, at either terminus or at both termini.

Methods for predicting whether a protein has a secretory leader as well as the cleavage point for that leader sequence are available. For instance, the method of McGeoch (*Virus Res.* *3*:271-286 (1985)) and von Heinje (*Nucleic Acids Res.* *14*:4683-4690 (1986)) can be used. The accuracy of predicting the cleavage

points of known mammalian secretory proteins for each of these methods is in the range of 75-80%. von Heinje, *supra*. However, the two methods do not always produce the same predicted cleavage point(s) for a given protein.

In the present case, the predicted amino acid sequence of the complete DR4 polypeptide of the present invention was analyzed by a computer program ("PSORT"). (*See* K. Nakai and M. Kanehisa, *Genomics 14*:897-911 (1992)), which is an expert system for predicting the cellular location of a protein based on the amino acid sequence. As part of this computational prediction of localization, the methods of McGeoch and von Heinje are incorporated. The analysis by the PSORT program predicted the cleavage sites between amino acids 23 and 24 in SEQ ID NO:2. Thereafter, the complete amino acid sequences were further analyzed by visual inspection, applying a simple form of the (-1,-3) rule of von Heine. von Heinje, *supra*. Thus, the leader sequence for the DR4 protein is predicted to consist of amino acid residues 1-23, underlined in SEQ ID NO:2, while the predicted mature DR4 protein consists of about residues 24-468.

As one of ordinary skill would appreciate, due to the possibility of sequencing errors, as well as the variability of cleavage sites for leaders in different known proteins, the predicted DR4 receptor polypeptide encoded by the deposited cDNA comprises about 468 amino acids, but may be anywhere in the range of 458-478 amino acids; and the predicted leader sequence of this protein is about 40 amino acids, but may be anywhere in the range of about 30 to about 50 amino acids. It will further be appreciated that, the domains described herein have been predicted by computer analysis, and accordingly, that depending on the analytical criteria used for identifying various functional domains, the exact "address" of, for example, the extracelluar domain, intracellular domain, death domain, cysteine-rich motifs, and transmembrane domain of DR4 may differ slightly. For example, the exact location of the DR4 extracellular domain in SEQ ID NO:2 may vary slightly (*e.g.*, the address may "shift" by about 1 to about 20 residues, more likely about 1 to about 5 residues) depending on the criteria used to define the domain. In this context "about" includes the particularly recited size, larger or smaller by

several (5, 4, 3, 2, or 1) amino acid residues, at either terminus or at both termini. In any event, as discussed further below, the invention further provides polypeptides having various residues deleted from the N-terminus and/or C-terminus of the complete DR4, including polypeptides lacking one or more amino acids from the N-termini of the extracellular domain described herein, which constitute soluble forms of the extracellular domain of the DR4 polypeptides.

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution.

However, a nucleic acid molecule contained in a clone that is a member of a mixed clone library (*e.g.*, a genomic or cDNA library) and that has not been isolated from other clones of the library (*e.g.*, in the form of a homogeneous solution containing the clone without other members of the library) or a chromosome isolated or removed from a cell or a cell lysate (*e.g.*, a "chromosome spread", as in a karyotype), is not "isolated" for the purposes of this invention. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Isolated nucleic acid molecules of the present invention include DR4 DNA molecules comprising, or alternatively consisting of, an open reading frame (ORF)

shown in SEQ ID NO:1 and further include DNA molecules which comprise, or alternatively consist of, a sequence substantially different than all or part of the ORF whose initiation codon is at position 19-21 of the nucleotide sequence shown in SEQ ID NO:1 but which, due to the degeneracy of the genetic code, still encode the DR4 polypeptide or a fragment thereof. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

In another aspect, the invention provides isolated nucleic acid molecules encoding the DR4 polypeptide having an amino acid sequence encoded by the cDNA contained in the plasmid deposited as ATCC Deposit No. 97853 on January 21, 1997. Preferably, these nucleic acid molecules will encode the mature polypeptide encoded by the above-described deposited cDNA. The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in SEQ ID NO:1 or the nucleotide sequence of the DR4 cDNA contained in the above-described deposited plasmid, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated DNA molecules and fragments thereof, have uses which include, but are not limited to, as DNA probes for gene mapping by *in situ* hybridization of the DR4 gene in human tissue by Northern blot analysis.

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By fragments of an isolated DNA molecule having the nucleotide sequence shown in SEQ ID NO:1 or having the nucleotide sequence of the deposited cDNA (the cDNA contained in the plasmid deposited as ATCC Deposit No. 97853) are intended DNA fragments at least 20 nt, and more preferably at least 30 nt in length, and even more preferably, at least about 40, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, or 1500 nt in length, which are useful as DNA probes as discussed above. Of course, DNA fragments corresponding to most, if not all, of the nucleotide sequence shown in SEQ ID NO:1 are also useful as DNA probes. By a fragment

about 20 nt in length, for example, is intended fragments which include 20 or more bases from the nucleotide sequence in SEQ ID NO:1. In this context "about" includes the particularly recited size, larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini.

Representative examples of DR4 polynucleotide fragments of the invention include, for example, fragments that comprise, or alternatively consist of, a sequence from about nucleotide 19 to 87, 88 to 732, 88 to 138, 139 to 189, 190 to 240, 241 to 291, 292 to 342, 343 to 705, 343 to 393, 394 to 444, 445 to 495, 496 to 546, 547 to 597, 598 to 648, 649 to 699, 700 to 732, 733 to 810, 733 to 771, 772 to 810, 811 to 1422, 811 to 861, 862 to 912, 913 to 963, 964 to 1014, 1015 to 1065, 1066 to 1116, 1117 to 1167, 1153 to 1284, 1153 to 1203, 1204 to 1254, 1255 to 1284, 1168 to 1218, 1219 to 1269, 1270 to 1320, 1321 to 1371, and 1372 to 1422 of SEQ ID NO:1, or the complementary strand thereto, or the cDNA contained in the deposited plasmid. In this context "about" includes the particularly recited ranges, larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini.

The present invention is further directed to polynucleotides comprising, or alternatively consisting of, isolated nucleic acid molecules which encode domains of DR4. In one aspect, the invention provides polynucleotides comprising, or alternatively consisting of, nucleic acid molecules which encode beta-sheet regions of DR4 protein set out in Table I. Representative examples of such polynucleotides include nucleic acid molecules which encode a polypeptide comprising, or alternatively consisting of, one, two, three, four, five or more amino acid sequences selected from the group consisting of amino acid residues from about 8 to about 17, amino acid residues from about 53 to about 60, amino acid residues from about 87 to about 103, amino acid residues from about 146 to about 155, amino acid residues from about 161 to about 166, amino acid residues from about 214 to about 221, amino acid residues from about 240 to about 252, amino acid residues from about 257 to about 264, amino acid residues from about 274 to about 283, amino acid residues from about 324 to about 329, amino acid

residues from about 349 to about 354, amino acid residues from about 363 to about 369, amino acid residues from about 371 to about 376, amino acid residues from about 394 to about 399, and amino acid residues from about 453 to about 458 in SEQ ID NO:2. In this context "about" includes the particularly recited value and values larger or smaller by several (5, 4, 3, 2, or 1) amino acids. Polypeptides encoded by these polynucleotides are also encompassed by the invention.

In specific embodiments, the polynucleotide fragments of the invention encode a polypeptide which demonstrates a DR4 functional activity. By a polypeptide demonstrating a DR4 "functional activity" is meant, a polypeptide capable of displaying one or more known functional activities associated with a complete (full-length) or mature DR4 polypeptide. Such functional activities include, but are not limited to, biological activity (*e.g.*, ability to induce apoptosis in cells expressing the polypeptide (*see, e.g.*, Example 5), antigenicity (ability to bind (or compete with a DR4 polypeptide for binding) to an anti-DR4 antibody), immunogenicity (ability to generate antibody which binds to a DR4 polypeptide), ability to form multimers, and ability to bind to a receptor or ligand for a DR4 polypeptide (*e.g.*, TRAIL; Wiley *et al.*, *Immunity 3*, 673-682 (1995)).

The functional activity of DR4 polypeptides, and fragments, variants derivatives, and analogs thereof, can be assayed by various methods.

For example, in one embodiment where one is assaying for the ability to bind or compete with full-length (complete) DR4 polypeptide for binding to anti-DR4 antibody, various immunoassays known in the art can be used, including but not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and

immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

In another embodiment, where a DR4 ligand is identified (*e.g.*, TRAIL), or the ability of a polypeptide fragment, variant or derivative of the invention to multimerize is being evaluated, binding can be assayed, *e.g.*, by means well-known in the art, such as, for example, reducing and non-reducing gel chromatography, protein affinity chromatography, and affinity blotting. See generally, Phizicky, E., *et al., Microbiol. Rev. 59*:94-123 (1995). In another embodiment, physiological correlates of DR4 binding to its substrates (signal transduction) can be assayed.

In addition, assays described herein (see Examples 5 and 6), and those otherwise known in the art may routinely be applied to measure the ability of DR4 polypeptides and fragments, variants derivatives, and analogs thereof to elicit DR4 related biological activity (*e.g.*, ability to bind TRAIL (see *e.g.*, Example 6), ability to induce apoptosis in cells expressing the polypeptide (see *e.g.*, Example 5) *in vitro or in vivo*). For example, biological activity can routinely be measured using the cell death assays performed essentially as previously described (Chinnaiyan *et al., Cell 81*:505-512 (1995); Boldin *et al., J. Biol. Chem. 270*:7795-8 (1995); Kischkel *et al., EMBO 14*:5579-5588 (1995); Chinnaiyan *et al., J. Biol. Chem. 271*:4961-4965 (1996)) and as set forth in Example 5 below. In one embodiment involving MCF7 cells, plasmids encoding full-length DR4 or a candidate death domain containing receptor are co-transfected with the pLantern reporter construct encoding green fluorescent protein. Nuclei of cells transfected with DR4 will exhibit apoptotic morphology as assessed by DAPI staining.

Other methods will be known to the skilled artisan and are within the scope of the invention.

Preferred nucleic acid fragments of the present invention include a nucleic acid molecule encoding a member selected from the group: a polypeptide comprising, or alternatively consisting of, the DR4 extracellular domain (amino acid residues from about 24 to about 238 in SEQ ID NO:2); a polypeptide comprising, or alternatively consisting of, the DR4 cysteine rich domain (amino acid residues from about 131 to about 229 in SEQ ID NO:2); a polypeptide comprising, or alternatively consisting of, the DR4 transmembrane domain (amino acid residues from about 239 to about 264 in SEQ ID NO:2); a fragment of the predicted mature DR4 polypeptide, wherein the fragment has a DR4 functional activity (*e.g.*, antigenic activity or biological activity); a polypeptide comprising, or alternatively consisting of, the DR4 intracellular domain (amino acid residues from about 265 to about 468 in SEQ ID NO:2); a polypeptide comprising, or alternatively consisting of, the DR4 receptor extracellular and intracellular domains with all or part of the transmembrane domain deleted; a polypeptide comprising, or alternatively consisting of, DR4 receptor death domain (predicted to constitute amino acid residues from about 379 to about 422 in SEQ ID NO:2); a polypeptide comprising, or alternatively consisting of, one, two, three, four or more, epitope bearing portions of the DR4 receptor protein. In additional embodiments, the polynucleotide fragments of the invention encode a polypeptide comprising, or alternatively consisting of, any combination of 1, 2, 3, 4, 5, 6, 7, or all 8 of the above-encoded polypeptide embodiments. As above, with the leader sequence, the amino acid residues constituting the DR4 receptor extracellular, transmembrane and intracellular domains have been predicted by computer analysis. Thus, one of ordinary skill would appreciate that the amino acid residues constituting these domains may vary slightly (*e.g.*, by about 1 to 15 residues) depending on the criteria used to define the domain. Polypeptides encoded by these nucleic acid molecules are also encompassed by the invention.

It is believed one or both of the extracellular cysteine rich motifs of the DR4 polypeptide disclosed in SEQ ID NO:2 is important for interactions between DR4 and its ligands (*e.g.*, TRAIL). Accordingly, specific embodiments of the

invention are directed to polynucleotides encoding polypeptides which comprise, or alternatively consist of, the amino acid sequence of one or both of amino acid residues 131 to 183, and/or 184 to 229 of SEQ ID NO:2. In a specific embodiment the polynucleotides encoding DR4 polypeptides of the invention comprise, or alternatively consist of both of the extracellular cysteine rich motifs disclosed in SEQ ID NO:2. Polypeptides encoded by these polynucleotides are also encompassed by the invention.

In additional embodiments, the polynucleotides of the invention encode functional attributes of DR4. Preferred embodiments of the invention in this regard include fragments that comprise, or alternatively consist of, one, two, three, four, or more of the following functional domains: alpha-helix and alpha-helix forming regions ("alpha-regions"), beta-sheet and beta-sheet forming regions ("beta-regions"), turn and turn-forming regions ("turn-regions"), coil and coil-forming regions ("coil-regions"), hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions and high antigenic index regions of DR4.

Certain preferred regions in these regards are set out in Figure 3, but may, as shown in Table I, be represented or identified by using tabular representations of the data presented in Figure 3. The DNA*STAR computer algorithm used to generate Figure 3 (set on the original default parameters) was used to present the data in Figure 3 in a tabular format (See Table I). The tabular format of the data in Figure 3 may be used to easily determine specific boundaries of a preferred region.

The above-mentioned preferred regions set out in FIG. 3 and in Table I include, but are not limited to, regions of the aforementioned types identified by analysis of the amino acid sequence set out in SEQ ID NO:2. As set out in FIG. 3 and in Table I, such preferred regions include Garnier-Robson alpha-regions, beta-regions, turn-regions, and coil-regions (columns I, III, V, and VII in Table I), Chou-Fasman alpha-regions, beta-regions, and turn-regions (columns II, IV, and VI in Table I), Kyte-Doolittle hydrophilic regions (column VIII in Table I),

Hopp-Woods hydrophobic regions (column IX in Table I), Eisenberg alpha- and beta-amphipathic regions (columns X and XI in Table I), Karplus-Schulz flexible regions (column XII in Table I), Jameson-Wolf regions of high antigenic index (column XIII in Table I), and Emini surface-forming regions (column XIV in Table I). Among highly preferred polynucleotides in this regard are those that encode polypeptides comprising, or alternatively consisting of, regions of DR4 that combine several structural features, such as several (*e.g.*, 1, 2, 3, or 4) of the same or different region features set out above.

The data representing the structural or functional attributes of DR4 set forth in Figure 3 and/or Table I, as described above, was generated using the various modules and algorithms of the DNA*STAR set on default parameters. In a preferred embodiment, the data presented in columns VIII, XII, and XIII of Table I can be used to determine regions of DR4 which exhibit a high degree of potential for antigenicity. Regions of high antigenicity are determined from the data presented in columns VIII, XII, and/or XIII by choosing values which represent regions of the polypeptide which are likely to be exposed on the surface of the polypeptide in an environment in which antigen recognition may occur in the process of initiation of an immune response.

Table I

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Met	1	.	.	B	.	.	.	.	0.12	.	.	.	-0.10	0.90
Ala	2	.	.	.	.	.	.	C	-0.08	*	*	.	0.25	1.08
Pro	3	.	.	.	.	.	.	C	0.42	*	*	.	0.10	0.86
Pro	4	.	.	.	.	.	T	C	-0.04	*	*	.	1.05	1.69
Pro	5	A	.	.	.	.	T	.	0.31	.	*	F	1.00	1.24
Ala	6	A	.	.	.	.	T	.	0.10	.	*	F	1.00	1.10
Arg	7	A	.	.	.	.	T	.	0.34	.	*	.	0.10	0.58
Val	8	.	.	B	B	.	.	.	-0.03	.	*	.	-0.30	0.37
His	9	.	.	B	B	.	.	.	-0.52	.	*	.	-0.30	0.37
Leu	10	.	.	B	B	.	.	.	-1.12	.	*	.	-0.60	0.17
Gly	11	.	.	B	B	.	.	.	-1.12	.	*	.	-0.60	0.18
Ala	12	.	.	B	B	.	.	.	-2.09	.	*	.	-0.60	0.14
Phe	13	.	.	B	B	.	.	.	-1.54	.	*	.	-0.60	0.12
Leu	14	.	.	B	B	.	.	.	-1.72	.	.	.	-0.60	0.18
Ala	15	.	.	B	B	.	.	.	-0.91	.	.	.	-0.60	0.27
Val	16	.	.	B	B	.	.	.	-0.78	.	.	.	-0.60	0.51
Thr	17	.	.	B	B	.	.	.	-0.53	.	.	F	-0.45	0.95
Pro	18	.	.	.	B	.	.	C	-0.13	.	.	F	0.05	0.93
Asn	19	.	.	.	.	.	T	C	0.09	.	.	F	0.60	1.69
Pro	20	.	.	.	.	.	T	C	0.09	.	.	F	0.60	1.18
Gly	21	.	.	.	.	T	T	.	0.64	.	.	F	0.65	0.77
Ser	22	.	.	.	.	.	T	C	0.61	.	.	F	0.45	0.64
Ala	23	.	.	.	.	.	.	C	0.51	.	.	F	0.25	0.41
Ala	24	.	.	.	.	.	T	C	0.51	.	.	F	0.45	0.60
Ser	25	.	.	B	.	.	T	.	0.13	.	.	F	0.85	0.78
Gly	26	A	.	.	.	.	T	.	-0.11	.	.	F	0.85	0.78
Thr	27	A	.	.	.	.	T	.	-0.40	.	.	F	0.85	0.78
Glu	28	A	A	.	.	.	.	.	-0.40	.	.	F	0.45	0.58
Ala	29	A	A	.	.	.	.	.	-0.12	.	.	.	0.30	0.60

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Ala	30	A	A	.	.	.	.	.	-0.03	.	.	.	0.30	0.60
Ala	31	A	A	.	.	.	.	.	0.01	.	.	.	0.30	0.53
Ala	32	A	A	.	.	.	.	.	0.37	.	.	.	-0.30	0.71
Thr	33	A	.	.	.	.	T	.	-0.49	*	.	F	1.00	1.40
Pro	34	A	.	.	.	.	T	.	-0.19	.	.	F	1.00	1.03
Ser	35	.	.	B	.	.	T	.	0.06	.	.	F	0.40	1.07
Lys	36	.	.	B	.	.	T	.	0.34	.	.	F	0.25	0.73
Val	37	.	.	B	B	.	.	.	0.63	.	.	F	-0.15	0.64
Trp	38	.	.	B	B	.	.	.	0.36	.	.	F	-0.15	0.64
Gly	39	.	.	B	B	.	.	.	0.22	*	*	F	-0.15	0.32
Ser	40	.	.	.	.	.	.	C	0.63	*	*	F	-0.05	0.43
Ser	41	.	.	.	.	.	T	C	-0.30	*	*	F	0.45	0.80
Ala	42	.	.	.	.	.	T	C	0.56	*	*	F	1.05	0.57
Gly	43	.	.	.	.	.	T	C	0.63	*	*	F	1.35	0.73
Arg	44	.	.	B	.	.	T	.	1.09	*	*	F	1.49	0.84
Ile	45	.	.	B	.	.	.	.	1.04	*	*	F	1.78	1.63
Glu	46	.	.	B	.	.	.	.	1.00	*	*	F	2.12	1.63
Pro	47	.	.	B	.	.	T	.	1.24	*	*	F	2.51	0.83
Arg	48	.	.	.	.	T	T	.	1.70	*	*	F	3.40	1.17
Gly	49	.	.	.	.	T	T	.	1.24	*	*	F	3.06	1.32
Gly	50	.	.	.	.	T	T	.	1.54	*	*	F	2.57	0.84
Gly	51	.	.	.	.	.	T	C	0.73	*	*	F	2.03	0.44
Arg	52	.	.	.	.	.	T	C	0.73	*	*	F	1.39	0.36
Gly	53	.	.	B	.	.	T	.	0.31	*	*	F	0.85	0.57
Ala	54	.	.	B	.	.	T	.	0.36	.	*	F	0.85	0.83
Leu	55	.	.	B	.	.	.	.	0.10	.	*	F	0.65	0.57
Pro	56	.	.	B	.	.	.	.	0.10	.	*	F	-0.25	0.57
Thr	57	.	.	B	.	.	.	.	-0.01	.	*	F	-0.25	0.55
Ser	58	.	.	B	.	.	T	.	0.30	.	.	F	0.10	1.16
Met	59	.	.	B	.	.	T	.	0.54	.	.	F	0.40	1.02
Gly	60	.	.	B	.	.	T	.	1.14	.	.	F	0.25	0.70
Gln	61	.	.	.	.	T	T	.	1.06	.	.	F	0.65	0.81

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
His	62	.	.	.	.	.	.	C	0.78	.	*	F	0.40	1.10
Gly	63	.	.	.	.	.	T	C	1.19	.	*	F	0.60	1.12
Pro	64	.	.	.	.	.	T	C	1.20	.	*	F	1.20	1.27
Ser	65	.	.	.	.	.	T	C	1.66	.	*	F	1.05	0.94
Ala	66	.	.	B	.	.	T	.	1.07	.	*	F	1.30	1.86
Arg	67	.	.	B	.	.	.	.	0.76	*	*	.	1.29	1.22
Ala	68	.	.	B	.	.	.	.	1.21	*	*	.	1.48	0.90
Arg	69	.	.	B	.	.	T	.	0.83	.	*	.	2.17	1.74
Ala	70	.	.	B	.	.	T	.	0.92	.	*	F	2.51	0.90
Gly	71	.	.	.	.	T	T	.	1.17	.	*	F	3.40	1.37
Arg	72	.	.	.	.	.	T	C	0.84	.	*	F	2.71	0.69
Ala	73	.	.	.	.	.	T	C	1.54	*	.	F	2.48	1.06
Pro	74	.	.	.	.	.	T	C	1.22	*	.	F	2.70	2.10
Gly	75	.	.	.	.	.	T	C	1.22	*	.	F	2.62	1.66
Pro	76	.	.	.	.	.	T	C	1.68	*	*	F	2.24	1.66
Arg	77	.	.	.	.	.	.	C	1.57	*	.	F	2.60	2.10
Pro	78	.	A	B	.	.	.	.	1.57	*	.	F	1.94	3.68
Ala	79	.	A	B	.	.	.	.	1.48	*	.	F	1.68	2.40
Arg	80	.	A	B	.	.	.	.	1.61	*	*	F	1.42	1.64
Glu	81	.	A	B	.	.	.	.	1.93	*	*	F	1.16	1.64
Ala	82	A	A	.	.	.	.	.	1.01	*	*	F	0.90	3.19
Ser	83	A	.	.	.	.	T	.	1.33	*	*	F	1.30	1.34
Pro	84	A	.	.	.	.	T	.	1.07	*	*	F	1.30	1.52
Arg	85	A	.	.	.	.	T	.	0.92	*	*	F	1.00	1.12
Leu	86	A	.	.	.	.	T	.	0.97	.	*	.	0.85	1.13
Arg	87	A	.	.	B	.	.	.	1.24	.	*	.	0.75	1.46
Val	88	A	.	.	B	.	.	.	0.84	*	*	.	0.75	1.08
His	89	A	.	.	B	.	.	.	1.10	.	*	.	-0.15	1.13
Lys	90	A	.	.	B	.	.	.	0.29	*	*	F	0.90	1.16
Thr	91	.	.	B	B	.	.	.	0.24	*	*	F	0.00	1.35
Phe	92	.	.	B	B	.	.	.	-0.72	*	*	.	-0.30	0.74
Lys	93	.	.	B	B	.	.	.	-0.72	*	*	.	-0.30	0.27

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Phe	94	.	.	B	B	.	.	.	-1.03	*	.	.	-0.60	0.14
Val	95	.	.	B	B	.	.	.	-1.93	*	.	.	-0.60	0.16
Val	96	.	.	B	B	.	.	.	-2.43	.	*	.	-0.60	0.06
Val	97	.	.	B	B	.	.	.	-2.54	.	*	.	-0.60	0.06
Gly	98	.	.	B	B	.	.	.	-2.59	.	*	.	-0.60	0.06
Val	99	.	.	B	B	.	.	.	-2.74	.	.	.	-0.60	0.15
Leu	100	.	.	B	B	.	.	.	-2.74	*	.	.	-0.60	0.15
Leu	101	.	.	B	B	.	.	.	-2.10	*	.	.	-0.60	0.11
Gln	102	.	.	B	B	.	.	.	-1.54	*	.	.	-0.60	0.23
Val	103	.	.	B	B	.	.	.	-1.50	.	.	.	-0.60	0.37
Val	104	.	.	B	.	.	T	.	-1.23	.	.	.	-0.20	0.61
Pro	105	.	.	B	.	.	T	.	-1.01	*	.	F	0.25	0.35
Ser	106	A	.	.	.	.	T	.	-0.51	*	.	F	-0.05	0.48
Ser	107	A	.	.	.	.	T	.	-1.40	*	*	F	0.25	0.94
Ala	108	A	.	.	.	.	.	.	-0.50	.	*	F	0.05	0.43
Ala	109	A	.	.	.	.	.	.	-0.46	.	*	.	0.50	0.63
Thr	110	A	.	.	.	.	.	.	-0.28	.	*	.	-0.10	0.39
Ile	111	A	.	.	.	.	.	.	0.02	.	*	.	-0.10	0.53
Lys	112	.	.	B	.	.	.	.	0.32	.	*	.	0.50	0.87
Leu	113	.	.	B	.	.	.	.	0.61	.	*	F	1.05	1.04
His	114	.	.	B	.	.	.	.	0.31	.	*	F	1.30	1.99
Asp	115	.	.	.	.	.	T	C	0.28	*	*	F	1.80	0.70
Gln	116	.	.	.	.	T	T	.	0.86	.	*	F	1.65	0.84
Ser	117	.	.	.	.	T	T	.	0.81	.	.	F	2.50	0.89
Ile	118	.	.	.	.	T	T	.	1.62	.	.	F	2.25	0.92
Gly	119	.	.	.	.	.	.	C	1.37	.	.	F	1.00	0.92
Thr	120	.	.	.	.	.	.	C	1.37	.	.	F	0.45	0.72
Gln	121	.	.	B	.	.	.	C	1.33	.	.	F	0.65	1.79
Gln	122	.	.	B	.	.	.	.	1.33	.	.	F	0.20	2.46
Trp	123	.	.	B	.	.	.	.	2.01	.	.	.	0.05	2.28
Glu	124	.	.	.	.	.	.	C	1.54	.	.	.	0.25	2.04
His	125	.	.	.	.	.	.	C	1.51	.	.	.	0.10	0.97

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Ser	126	.	.	.	.	.	T	C	1.51	.	.	F	0.45	0.91
Pro	127	.	.	.	.	T	T	.	0.70	.	.	F	1.55	0.91
Leu	128	.	.	.	.	T	T	.	0.32	.	.	F	0.65	0.55
Gly	129	.	.	.	.	T	T	.	0.11	.	.	F	0.65	0.22
Glu	130	.	.	.	.	T	.	.	-0.07	.	.	F	0.45	0.22
Leu	131	.	.	B	.	.	.	.	-0.11	*	.	.	0.18	0.42
Cys	132	.	.	B	.	.	.	.	-0.20	*	.	F	1.21	0.42
Pro	133	.	.	B	.	.	T	.	0.58	*	*	F	1.69	0.32
Pro	134	.	.	.	.	T	T	.	1.03	.	*	F	1.47	0.53
Gly	135	.	.	.	.	T	T	.	0.73	.	*	F	2.80	1.94
Ser	136	.	.	.	.	.	T	C	1.54	*	.	F	2.32	1.68
His	137	.	.	.	.	.	.	C	2.32	*	.	F	2.48	1.88
Arg	138	.	.	B	.	.	.	.	2.32	*	.	F	2.34	3.72
Ser	139	.	.	B	.	.	.	.	2.19	*	.	F	2.40	4.29
Glu	140	.	.	.	.	T	.	.	1.94	*	.	F	2.86	3.12
Arg	141	.	.	.	.	T	T	.	1.58	*	.	F	3.40	1.61
Pro	142	.	.	.	.	T	T	.	1.61	.	*	F	2.91	0.64
Gly	143	.	.	.	.	T	T	.	1.61	.	*	F	2.57	0.60
Ala	144	.	.	.	.	T	T	.	1.24	.	*	.	2.08	0.60
Cys	145	.	.	.	.	T	.	.	0.93	.	*	.	1.41	0.21
Asn	146	.	.	B	.	.	.	.	0.82	.	*	.	0.84	0.30
Arg	147	.	.	B	.	.	.	.	0.69	*	.	.	1.01	0.52
Cys	148	.	.	B	.	.	T	.	0.18	*	.	F	1.83	0.96
Thr	149	.	.	B	.	.	T	.	0.42	*	.	F	1.70	0.44
Glu	150	.	.	B	.	.	T	.	0.84	*	.	F	1.53	0.22
Gly	151	.	.	B	.	.	T	.	0.53	*	.	F	0.76	0.65
Val	152	.	.	B	B	.	.	.	0.42	.	*	F	0.19	0.65
Gly	153	.	.	B	B	.	.	.	0.50	.	.	.	-0.13	0.61
Tyr	154	.	.	B	B	.	.	.	0.51	.	.	.	-0.60	0.62
Thr	155	.	.	B	B	.	.	.	0.51	.	.	F	-0.30	1.12
Asn	156	.	.	.	B	.	.	C	0.86	.	.	F	0.20	1.81
Ala	157	.	.	.	.	T	T	.	0.90	.	.	F	0.80	1.86

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Ser	158	.	.	.	.	T	T	.	0.54	.	.	F	0.80	1.06
Asn	159	.	.	.	.	T	T	.	0.20	.	.	F	0.35	0.57
Asn	160	.	.	.	.	T	T	.	-0.16	*	.	F	0.35	0.57
Leu	161	.	A	B	.	.	.	.	-0.97	*	.	.	-0.60	0.23
Phe	162	.	A	B	.	.	.	.	-0.59	.	.	.	-0.60	0.12
Ala	163	.	A	B	.	.	.	.	-0.96	.	.	.	-0.60	0.11
Cys	164	.	A	B	.	.	.	.	-1.27	*	.	.	-0.60	0.07
Leu	165	.	.	B	.	.	T	.	-1.86	.	.	.	-0.20	0.12
Pro	166	.	.	B	.	.	T	.	-1.71	*	.	.	-0.20	0.12
Cys	167	.	.	.	.	T	T	.	-0.97	*	.	.	0.20	0.12
Thr	168	A	.	.	.	.	T	.	-0.68	.	.	.	0.10	0.30
Ala	169	A	.	.	.	.	.	.	-0.01	.	.	.	0.50	0.26
Cys	170	A	.	.	.	.	T	.	0.80	.	.	.	0.70	0.80
Lys	171	A	.	.	.	.	T	.	1.01	.	.	F	1.15	0.96
Ser	172	A	.	.	.	.	T	.	1.68	.	*	F	1.30	1.65
Asp	173	A	.	.	.	.	T	.	2.10	.	*	F	1.30	5.33
Glu	174	A	A	.	.	.	.	.	2.39	.	*	F	0.90	5.22
Glu	175	A	A	.	.	.	.	.	2.84	.	*	F	1.24	5.22
Glu	176	A	A	.	.	.	.	.	2.13	.	*	F	1.58	4.83
Arg	177	.	A	.	.	T	.	.	2.12	.	.	F	2.32	1.50
Ser	178	.	.	.	.	.	T	C	1.81	.	.	F	2.86	1.25
Pro	179	.	.	.	.	T	T	.	1.50	*	.	F	3.40	1.04
Cys	180	.	.	.	.	T	T	.	1.61	*	.	F	2.61	0.77
Thr	181	.	.	.	.	T	T	.	1.61	*	.	F	2.67	1.12
Thr	182	.	.	.	.	T	.	.	1.19	*	*	F	2.38	1.16
Thr	183	.	.	.	.	T	T	.	0.90	.	.	F	2.49	3.13
Arg	184	.	.	.	.	T	T	.	0.44	.	.	F	2.40	2.19
Asn	185	.	.	.	.	T	T	.	1.11	.	.	F	2.50	0.81
Thr	186	.	.	.	.	T	T	.	0.76	*	.	F	2.25	0.98
Ala	187	.	.	.	.	T	.	.	1.11	*	.	.	1.65	0.27
Cys	188	.	.	.	.	T	.	.	1.21	*	.	.	1.40	0.33
Gln	189	.	.	B	.	.	.	.	0.76	*	.	.	0.75	0.36

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Cys	190	.	.	B	.	.	.	.	0.44	.	.	.	0.50	0.35
Lys	191	.	.	B	.	.	T	.	0.06	.	*	F	0.85	0.94
Pro	192	.	.	.	.	T	T	.	0.76	.	.	F	0.65	0.47
Gly	193	.	.	.	.	T	T	.	1.42	.	*	F	1.74	1.72
Thr	194	.	.	B	.	.	T	.	1.42	.	*	F	1.68	1.38
Phe	195	.	.	B	.	.	.	.	2.09	.	*	F	1.82	1.49
Arg	196	.	.	.	.	T	.	.	1.74	.	*	F	2.56	2.42
Asn	197	.	.	.	.	T	T	.	1.37	.	*	F	3.40	2.25
Asp	198	.	.	.	.	T	T	.	1.71	.	*	F	3.06	2.63
Asn	199	.	.	.	.	.	T	C	1.42	.	*	F	2.52	2.32
Ser	200	A	.	.	.	.	T	.	1.46	.	*	F	1.98	1.43
Ala	201	A	.	.	.	.	.	.	1.46	.	*	.	1.14	0.46
Glu	202	A	.	.	.	.	.	.	1.50	*	.	.	0.80	0.56
Met	203	A	.	.	.	.	.	.	0.83	*	.	.	1.11	0.83
Cys	204	A	.	.	.	.	T	.	0.53	*	.	.	1.62	0.44
Arg	205	.	.	.	.	T	T	.	0.52	*	.	.	2.33	0.34
Lys	206	.	.	.	.	T	T	.	0.77	*	.	F	2.49	0.50
Cys	207	.	.	.	.	T	T	.	0.10	*	.	F	3.10	0.92
Ser	208	.	.	.	.	T	.	.	0.49	*	*	F	2.59	0.25
Thr	209	.	.	.	.	T	.	.	1.27	*	*	F	1.98	0.19
Gly	210	.	.	.	.	T	.	.	0.81	*	.	F	1.67	0.71
Cys	211	.	.	B	.	.	T	.	0.17	*	*	F	1.16	0.53
Pro	212	.	.	.	.	T	T	.	-0.02	*	*	F	1.25	0.36
Arg	213	.	.	.	.	T	T	.	0.32	*	*	F	0.65	0.27
Gly	214	.	.	B	.	.	T	.	-0.22	*	*	.	0.85	1.01
Met	215	.	.	B	B	.	.	.	0.17	*	*	.	0.30	0.48
Val	216	.	.	B	B	.	.	.	0.83	*	*	.	0.79	0.49
Lys	217	.	.	B	B	.	.	.	0.38	*	*	.	0.98	0.83
Val	218	.	.	B	B	.	.	.	-0.04	*	*	F	1.32	0.45
Lys	219	.	.	B	B	.	.	.	0.09	.	*	F	1.51	0.88
Asp	220	.	.	B	.	.	.	.	0.40	.	*	F	1.90	0.68
Cys	221	.	.	B	.	.	.	.	0.96	.	*	F	0.81	0.96

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Thr	222	.	.	.	.	.	T	C	0.91	.	*	F	1.62	0.65
Pro	223	.	.	.	.	T	T	.	0.88	.	*	F	1.63	0.65
Trp	224	.	.	.	.	T	T	.	0.83	.	*	F	0.54	0.84
Ser	225	A	.	.	.	.	T	.	0.17	.	.	F	1.00	1.01
Asp	226	A	A	.	.	.	.	.	-0.02	.	.	F	0.45	0.35
Ile	227	A	A	.	.	.	.	.	0.26	*	.	.	-0.30	0.25
Glu	228	A	A	.	.	.	.	.	0.51	*	.	.	0.30	0.25
Cys	229	.	A	B	.	.	.	.	0.80	*	.	.	0.60	0.30
Val	230	A	A	.	.	.	.	.	0.80	*	*	.	0.60	0.74
His	231	A	A	.	.	.	.	.	0.46	*	*	.	0.60	0.58
Lys	232	A	A	.	.	.	.	.	1.34	*	.	F	0.60	1.06
Glu	233	.	A	.	.	T	.	.	1.00	*	.	F	1.30	2.30
Ser	234	.	.	.	.	T	T	.	1.63	*	.	F	1.70	1.68
Gly	235	.	.	.	.	T	T	.	2.49	*	.	F	1.70	1.14
Asn	236	.	.	.	.	T	T	.	1.63	*	.	F	1.40	1.06
Gly	237	.	.	.	.	.	T	C	1.30	*	.	F	0.45	0.55
His	238	.	.	.	B	.	.	C	0.44	.	.	.	-0.40	0.59
Asn	239	.	.	.	B	.	.	C	-0.14	.	.	.	-0.40	0.27
Ile	240	.	.	B	B	.	.	.	-0.61	.	.	.	-0.60	0.19
Trp	241	.	.	B	B	.	.	.	-1.47	.	.	.	-0.60	0.12
Val	242	.	.	B	B	.	.	.	-1.98	.	.	.	-0.60	0.05
Ile	243	.	.	B	B	.	.	.	-2.26	.	.	.	-0.60	0.06
Leu	244	.	.	B	B	.	.	.	-3.07	.	.	.	-0.60	0.08
Val	245	.	.	B	B	.	.	.	-3.03	.	.	.	-0.60	0.09
Val	246	.	.	B	B	.	.	.	-3.60	.	.	.	-0.60	0.09
Thr	247	.	.	B	B	.	.	.	-2.96	.	.	.	-0.60	0.08
Leu	248	.	.	B	B	.	.	.	-2.88	.	.	.	-0.60	0.17
Val	249	.	.	B	B	.	.	.	-2.88	.	*	.	-0.60	0.19
Val	250	.	.	B	B	.	.	.	-2.83	.	.	.	-0.60	0.11
Pro	251	.	.	B	B	.	.	.	-2.83	.	.	.	-0.60	0.11
Leu	252	.	.	B	B	.	.	.	-3.11	.	.	.	-0.60	0.11
Leu	253	A	.	.	B	.	.	.	-3.16	.	.	.	-0.60	0.15

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Leu	254	A	.	.	B	.	.	.	-3.11	.	.	.	-0.60	0.07
Val	255	A	.	.	B	.	.	.	-3.14	.	.	.	-0.60	0.07
Ala	256	A	.	.	B	.	.	.	-3.79	.	.	.	-0.60	0.06
Val	257	.	.	B	B	.	.	.	-3.64	.	.	.	-0.60	0.05
Leu	258	.	.	B	B	.	.	.	-3.50	.	.	.	-0.60	0.04
Ile	259	.	.	B	B	.	.	.	-3.36	.	.	.	-0.60	0.02
Val	260	.	.	B	B	.	.	.	-3.39	.	.	.	-0.60	0.02
Cys	261	.	.	B	B	.	.	.	-3.14	.	.	.	-0.60	0.01
Cys	262	.	.	B	B	.	.	.	-2.59	.	.	.	-0.60	0.02
Cys	263	.	.	B	B	.	.	.	-2.12	.	.	.	-0.60	0.03
Ile	264	.	.	B	B	.	.	.	-1.90	.	.	.	-0.60	0.06
Gly	265	.	.	.	.	T	T	.	-1.39	.	.	F	0.35	0.06
Ser	266	.	.	.	.	T	T	.	-1.07	.	.	F	0.35	0.11
Gly	267	.	.	.	.	T	T	.	-0.40	.	.	F	0.65	0.16
Cys	268	.	.	.	.	T	T	.	0.06	.	.	F	1.25	0.27
Gly	269	.	.	.	.	T	.	.	0.99	.	*	F	1.39	0.31
Gly	270	.	.	.	.	T	.	.	0.67	.	.	F	2.03	0.62
Asp	271	.	.	.	.	.	T	C	0.37	.	.	F	2.37	0.62
Pro	272	.	.	.	.	T	T	.	0.71	*	*	F	2.91	0.62
Lys	273	.	.	.	.	T	T	.	1.49	*	*	F	3.40	1.05
Cys	274	.	.	B	.	.	T	.	0.98	*	*	.	2.51	1.23
Met	275	.	.	B	B	.	.	.	0.66	*	*	.	1.62	0.59
Asp	276	.	.	B	B	.	.	.	-0.04	*	*	.	1.28	0.16
Arg	277	.	.	B	B	.	.	.	-0.12	.	*	.	0.04	0.26
Val	278	.	.	B	B	.	.	.	-0.06	.	*	.	-0.60	0.27
Cys	279	.	.	B	B	.	.	.	-0.20	.	.	.	0.30	0.32
Phe	280	.	.	B	B	.	.	.	0.06	.	*	.	-0.60	0.13
Trp	281	.	.	B	B	.	.	.	-0.76	.	.	.	-0.60	0.18
Arg	282	.	.	B	B	.	.	.	-1.68	.	.	.	-0.60	0.28
Leu	283	.	.	B	B	.	.	.	-0.71	.	.	.	-0.60	0.26
Gly	284	.	.	.	B	T	.	.	-0.39	.	*	.	-0.20	0.49
Leu	285	.	.	.	B	.	.	C	0.10	.	*	.	0.50	0.25

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Leu	286	.	.	.	B	.	.	C	0.04	.	*	.	0.20	0.46
Arg	287	.	.	.	B	.	.	C	-0.66	.	.	F	0.65	0.46
Gly	288	.	.	.	.	.	T	C	0.16	.	.	F	1.35	0.57
Pro	289	.	.	.	.	.	T	C	0.50	.	*	F	2.70	1.19
Gly	290	.	.	.	.	.	T	C	1.31	*	*	F	3.00	1.01
Ala	291	A	.	.	.	.	T	.	1.53	.	*	F	2.50	1.65
Glu	292	A	.	.	.	.	.	.	1.39	.	.	F	2.00	1.08
Asp	293	A	.	.	.	.	.	.	1.73	.	.	F	1.70	1.48
Asn	294	A	.	.	.	.	T	.	1.94	.	*	.	1.45	2.36
Ala	295	A	.	.	.	.	T	.	1.40	.	.	.	1.15	2.36
His	296	A	.	.	.	.	T	.	1.18	*	.	.	1.00	0.99
Asn	297	A	.	.	.	.	T	.	0.88	.	.	.	0.10	0.51
Glu	298	A	.	.	.	.	.	.	0.88	*	.	.	-0.10	0.67
Ile	299	A	.	.	.	.	.	.	0.29	*	*	.	-0.10	0.80
Leu	300	A	.	.	.	.	.	.	0.88	*	*	.	-0.10	0.50
Ser	301	A	.	.	.	.	.	.	0.61	*	.	F	0.65	0.48
Asn	302	A	.	.	.	.	T	.	-0.20	*	.	F	0.25	0.92
Ala	303	A	.	.	.	.	T	.	-0.50	*	.	F	0.25	0.92
Asp	304	A	.	.	.	.	T	.	0.08	*	.	F	0.85	0.92
Ser	305	.	.	.	.	.	T	C	0.19	*	.	F	1.05	0.83
Leu	306	.	.	.	B	.	.	C	-0.37	*	.	F	0.05	0.71
Ser	307	.	.	B	B	.	.	.	-0.67	*	.	F	-0.15	0.31
Thr	308	.	.	B	B	.	.	.	-0.08	*	.	.	-0.60	0.31
Phe	309	.	.	B	B	.	.	.	-0.08	*	.	.	-0.30	0.66
Val	310	A	.	.	B	.	.	.	0.22	.	.	F	-0.15	0.85
Ser	311	A	A	.	.	.	.	.	0.43	.	.	F	0.00	1.03
Glu	312	A	A	.	.	.	.	.	0.73	.	.	F	0.00	1.17
Gln	313	A	A	.	.	.	.	.	0.74	.	.	F	0.90	2.73
Gln	314	A	A	.	.	.	.	.	1.44	.	.	F	0.90	2.73
Met	315	A	A	.	.	.	.	.	2.30	.	.	F	0.90	2.73
Glu	316	A	A	.	.	.	.	.	2.39	.	.	F	0.90	2.73
Ser	317	A	A	.	.	.	.	.	1.80	.	*	F	0.90	2.44

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Gln	318	A	A	.	.	.	.	.	1.80	.	*	F	0.90	2.49
Glu	319	A	A	.	.	.	.	.	0.99	.	*	F	0.90	2.40
Pro	320	A	A	.	.	.	.	.	1.28	.	*	F	0.90	1.48
Ala	321	A	A	.	.	.	.	.	0.93	.	.	F	0.60	1.23
Asp	322	A	A	.	B	.	.	.	0.38	.	.	F	0.45	0.70
Leu	323	A	A	.	B	.	.	.	0.07	.	.	F	-0.15	0.34
Thr	324	.	A	B	B	.	.	.	-0.79	.	.	F	-0.15	0.48
Gly	325	.	A	B	B	.	.	.	-0.58	.	.	.	-0.30	0.21
Val	326	.	.	B	B	.	.	.	-0.29	.	.	.	-0.60	0.45
Thr	327	.	.	B	B	.	.	.	-0.50	.	.	.	-0.60	0.42
Val	328	.	.	B	B	.	.	.	-0.03	.	*	F	-0.17	0.65
Gln	329	.	.	B	B	.	.	.	0.28	.	*	F	0.11	0.87
Ser	330	.	.	.	.	.	T	C	0.03	.	*	F	2.04	1.05
Pro	331	.	.	.	.	.	T	C	0.89	.	*	F	2.32	1.42
Gly	332	.	.	.	.	T	T	.	0.53	.	*	F	2.80	1.42
Glu	333	A	.	.	.	.	T	.	0.58	.	*	F	1.97	0.57
Ala	334	.	.	B	.	.	.	.	-0.23	.	*	.	0.74	0.30
Gln	335	.	.	B	.	.	.	.	-0.28	.	.	.	0.46	0.25
Cys	336	.	.	B	.	.	.	.	-0.28	.	.	.	0.18	0.14
Leu	337	.	.	B	.	.	.	.	-0.52	.	*	.	-0.40	0.22
Leu	338	.	.	B	.	.	.	.	-0.52	.	*	.	-0.40	0.13
Gly	339	.	A	.	.	.	.	C	-0.52	.	*	F	0.05	0.42
Pro	340	A	A	.	.	.	.	.	-0.52	.	*	F	-0.15	0.51
Ala	341	A	A	.	.	.	.	.	-0.20	.	*	F	0.60	1.07
Glu	342	A	A	.	.	.	.	.	0.31	.	*	F	0.90	1.07
Ala	343	A	A	.	.	.	.	.	1.12	*	*	F	0.75	0.93
Glu	344	A	A	.	.	.	.	.	1.58	.	*	F	0.90	1.60
Gly	345	A	A	.	.	.	.	.	1.90	.	*	F	0.90	1.80
Ser	346	A	.	.	.	.	T	.	2.60	.	*	F	1.30	3.50
Gln	347	A	.	.	.	.	T	.	1.79	.	*	F	1.30	3.96
Arg	348	A	.	.	.	.	T	.	1.57	.	*	F	1.30	3.30
Arg	349	.	.	B	.	.	T	.	0.71	.	*	F	1.30	2.03

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Arg	350	.	.	B	B	.	.	.	0.84	.	*	F	0.75	0.87
Leu	351	.	.	B	B	.	.	.	0.56	.	*	.	0.60	0.69
Leu	352	.	.	B	B	.	.	.	0.56	.	*	.	0.30	0.35
Val	353	.	.	B	B	.	.	.	0.10	*	*	.	-0.30	0.29
Pro	354	.	.	B	.	.	T	.	-0.60	*	.	.	-0.20	0.35
Ala	355	.	.	.	.	T	T	.	-0.71	.	*	.	0.50	0.43
Asn	356	.	.	.	.	.	T	C	-0.11	.	.	F	1.65	0.96
Gly	357	.	.	.	.	.	T	C	0.39	.	.	F	1.95	0.96
Ala	358	.	.	.	.	.	.	C	1.24	.	.	F	2.20	1.37
Asp	359	.	.	.	.	.	T	C	1.14	.	.	F	3.00	1.48
Pro	360	A	.	.	.	.	T	.	0.92	*	.	F	2.50	2.16
Thr	361	A	.	.	.	.	T	.	0.32	.	.	F	1.90	1.76
Glu	362	A	.	.	.	.	T	.	-0.14	.	.	F	1.60	1.04
Thr	363	A	.	.	B	.	.	.	-0.26	.	.	F	0.15	0.56
Leu	364	A	.	.	B	.	.	.	-0.96	*	.	.	-0.60	0.33
Met	365	A	.	.	B	.	.	.	-0.74	*	.	.	-0.60	0.17
Leu	366	A	.	.	B	.	.	.	-0.39	*	.	.	-0.60	0.19
Phe	367	A	.	.	B	.	.	.	-1.09	*	.	.	-0.60	0.47
Phe	368	A	.	.	B	.	.	.	-1.37	*	.	.	-0.60	0.41
Asp	369	A	.	.	B	.	.	.	-0.56	*	.	.	-0.60	0.50
Lys	370	A	A	.	.	.	.	.	-0.84	*	.	.	-0.30	0.93
Phe	371	A	A	.	B	.	.	.	-0.89	*	.	.	-0.30	0.75
Ala	372	A	A	.	B	.	.	.	-0.40	*	.	.	-0.30	0.34
Asn	373	.	A	B	B	.	.	.	-0.40	*	.	.	-0.60	0.26
Ile	374	.	A	B	B	.	.	.	-0.40	*	.	.	-0.60	0.26
Val	375	.	A	B	B	.	.	.	-0.74	.	.	.	-0.60	0.43
Pro	376	.	A	.	B	.	.	C	-0.33	.	.	.	-0.10	0.36
Phe	377	.	.	.	.	T	T	.	0.26	.	.	.	0.20	0.54
Asp	378	.	.	.	.	T	T	.	0.26	.	.	F	0.80	1.21
Ser	379	.	.	.	.	T	T	.	0.33	.	.	F	1.40	1.35
Trp	380	A	.	.	.	.	T	.	0.59	*	*	F	0.40	1.29
Asp	381	A	A	.	.	.	.	.	0.91	*	.	F	-0.15	0.76

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Gln	382	A	A	.	.	.	.	.	1.61	*	.	.	-0.15	1.11
Leu	383	A	A	.	.	.	.	.	0.80	*	.	.	-0.15	1.84
Met	384	A	A	.	.	.	.	.	1.10	*	.	.	0.30	0.91
Arg	385	A	A	.	.	.	.	.	0.58	*	.	.	0.30	0.87
Gln	386	A	A	.	.	.	.	.	0.27	*	.	.	-0.30	0.87
Leu	387	A	A	.	.	.	.	.	0.31	*	.	.	0.45	1.27
Asp	388	A	A	.	.	.	.	.	1.12	*	.	.	0.75	1.30
Leu	389	A	A	.	.	.	.	.	1.72	*	.	F	0.60	1.21
Thr	390	A	.	.	.	.	T	.	0.72	*	.	F	1.30	2.54
Lys	391	A	.	.	.	.	T	.	0.72	.	*	F	1.30	1.07
Asn	392	A	.	.	.	.	T	.	0.68	*	*	F	1.30	2.16
Glu	393	A	.	.	.	.	T	.	-0.18	*	.	F	1.30	1.11
Ile	394	.	.	B	B	.	.	.	0.74	*	.	F	0.75	0.41
Asp	395	.	.	B	B	.	.	.	0.47	*	*	.	0.60	0.50
Val	396	.	.	B	B	.	.	.	0.08	*	*	.	0.60	0.29
Val	397	.	.	B	B	.	.	.	-0.23	.	.	.	0.51	0.41
Arg	398	.	.	B	.	.	T	.	-0.82	*	.	.	1.12	0.36
Ala	399	.	.	B	.	.	T	.	-0.28	*	.	.	0.73	0.49
Gly	400	.	.	.	.	T	T	.	-0.49	*	.	F	2.09	0.65
Thr	401	.	.	.	.	.	T	C	0.02	*	*	F	2.10	0.51
Ala	402	.	.	.	.	.	.	C	0.88	*	*	F	1.09	0.50
Gly	403	.	.	.	.	.	T	C	0.18	*	*	F	1.68	0.85
Pro	404	.	.	.	.	.	T	C	-0.04	.	.	F	1.47	0.59
Gly	405	.	.	.	.	.	T	C	0.06	.	.	F	1.26	0.48
Asp	406	A	.	.	.	.	T	.	-0.22	.	.	F	0.25	0.76
Ala	407	A	A	.	.	.	.	.	-0.23	.	.	.	-0.30	0.50
Leu	408	A	A	.	.	.	.	.	-0.70	.	.	.	-0.60	0.50
Tyr	409	A	A	.	.	.	.	.	-1.09	*	.	.	-0.60	0.25
Ala	410	A	A	.	.	.	.	.	-0.70	*	.	.	-0.60	0.24
Met	411	A	A	.	.	.	.	.	-0.99	*	.	.	-0.60	0.59
Leu	412	A	A	.	.	.	.	.	-1.26	*	.	.	-0.60	0.39
Met	413	A	A	.	.	.	.	.	-0.44	*	.	.	-0.60	0.29

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Lys	414	A	A	.	B	.	.	.	-0.16	*	.	.	-0.60	0.47
Trp	415	A	A	.	B	.	.	.	0.12	*	.	.	0.15	1.14
Val	416	A	A	.	B	.	.	.	0.38	*	*	.	0.45	1.66
Asn	417	A	.	.	.	.	T	.	1.30	*	.	F	1.75	0.82
Lys	418	A	.	.	.	.	T	.	1.90	*	.	F	2.20	1.53
Thr	419	.	.	.	.	.	T	C	1.27	*	.	F	3.00	3.32
Gly	420	.	.	.	.	.	T	C	1.26	*	.	F	2.70	2.08
Arg	421	.	.	.	.	T	.	.	1.22	*	.	F	2.40	1.40
Asn	422	.	.	.	.	.	T	C	1.19	*	.	F	1.65	0.68
Ala	423	.	.	B	.	.	T	.	0.83	.	.	.	1.00	0.93
Ser	424	.	.	B	.	.	T	.	0.33	.	.	.	0.70	0.69
Ile	425	.	.	B	.	.	T	.	-0.13	.	*	.	-0.20	0.35
His	426	.	A	B	.	.	.	.	-0.24	.	*	.	-0.60	0.29
Thr	427	.	A	B	.	.	.	.	-0.83	*	*	.	-0.60	0.36
Leu	428	A	A	.	.	.	.	.	-1.06	*	*	.	-0.60	0.52
Leu	429	A	A	.	.	.	.	.	-0.76	*	*	.	-0.60	0.31
Asp	430	A	A	.	.	.	.	.	0.24	*	*	.	-0.30	0.38
Ala	431	A	A	.	.	.	.	.	-0.32	*	*	.	0.30	0.89
Leu	432	A	A	.	.	.	.	.	-0.01	*	*	.	0.75	1.07
Glu	433	A	A	.	.	.	.	.	0.80	*	*	.	0.75	1.11
Arg	434	A	A	.	.	.	.	.	1.72	*	*	F	0.90	1.90
Met	435	A	A	.	.	.	.	.	1.69	*	*	F	0.90	4.52
Glu	436	A	A	.	.	.	.	.	1.69	*	*	F	0.90	3.55
Glu	437	A	A	.	.	.	.	.	2.54	*	.	F	0.90	1.83
Arg	438	A	A	.	.	.	.	.	2.54	*	*	F	0.90	3.70
His	439	A	A	.	.	.	.	.	2.48	*	*	F	0.90	3.70
Ala	440	A	A	.	.	.	.	.	2.19	*	*	F	0.90	4.28
Lys	441	A	A	.	.	.	.	.	2.19	*	*	F	0.90	1.53
Glu	442	A	A	.	.	.	.	.	2.19	*	.	F	0.90	1.95
Lys	443	A	A	.	.	.	.	.	1.27	*	*	F	0.90	3.22
Ile	444	A	A	.	.	.	.	.	0.49	*	*	F	0.90	1.33
Gln	445	A	A	.	.	.	.	.	0.22	*	*	F	0.75	0.63

Res	Pos.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
Asp	446	A	A	.	.	.	.	.	0.18	*	*	F	-0.15	0.23
Leu	447	A	A	.	.	.	.	.	-0.12	*	.	.	-0.30	0.56
Leu	448	A	A	.	.	.	.	.	-0.51	*	.	.	0.55	0.43
Val	449	A	A	.	.	.	.	.	0.42	*	.	F	0.95	0.26
Asp	450	A	.	.	.	.	T	.	-0.28	*	.	F	1.60	0.62
Ser	451	.	.	.	.	T	T	.	-1.17	*	.	F	2.25	0.65
Gly	452	.	.	.	.	T	T	.	-0.60	*	.	F	2.50	0.62
Lys	453	.	.	B	.	.	T	.	-0.60	.	.	F	1.25	0.58
Phe	454	.	A	B	.	.	.	.	0.26	.	.	.	0.15	0.36
Ile	455	.	A	B	.	.	.	.	0.26	.	.	.	0.20	0.62
Tyr	456	.	A	B	.	.	.	.	0.21	.	.	.	0.55	0.52
Leu	457	.	A	B	.	.	.	.	0.24	.	.	.	-0.03	0.59
Glu	458	.	A	B	.	.	.	.	-0.14	.	.	F	0.54	1.22
Asp	459	.	A	.	.	T	.	.	0.26	.	.	F	1.66	0.77
Gly	460	.	.	.	.	T	T	.	0.56	.	.	F	2.78	1.26
Thr	461	.	.	.	.	.	T	C	-0.06	*	.	F	2.70	0.73
Gly	462	.	.	.	.	.	T	C	0.46	*	.	F	2.13	0.33
Ser	463	.	.	.	.	.	T	C	-0.36	.	.	F	1.26	0.44
Ala	464	A	.	.	.	.	.	.	-0.36	.	.	.	0.14	0.25
Val	465	.	.	B	.	.	.	.	-0.40	.	.	.	0.17	0.44
Ser	466	.	.	B	.	.	.	.	-0.48	.	.	.	-0.10	0.42
Leu	467	.	.	B	.	.	.	.	-0.52	.	.	.	-0.10	0.53
Glu	468	A	.	.	.	.	.	.	-0.61	.	.	.	0.50	0.92

Preferred nucleic acid fragments of the invention encode a full-length DR4 polypeptide lacking the nucleotides encoding the amino-terminal methionine (nucleotides 19-21 in SEQ ID NO:1) as it is known that the methionine is cleaved naturally and such sequences maybe useful in genetically engineering DR4 expression vectors. Polypeptides encoded by such polynucleotides are also contemplated by the invention.

Among highly preferred fragments in this regard are those that comprise, or alternatively consist of, regions of DR4 that combine several structural features, such as several of the features set out above. Preferred nucleic acid fragments of the present invention further include nucleic acid molecules encoding a polypeptide comprising, or alternatively consisting of, one, two, three, four, five, or more epitope-bearing portions of the DR4 protein. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising, or alternatively consisting of, amino acid residues from about 35 to about 92 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 114 to about 160 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 169 to about 240 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 267 to about 298 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 330 to about 364 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 391 to about 404 in SEQ ID NO:2; and a polypeptide comprising, or alternatively consisting of, amino acid residues from about 418 to about 465 in SEQ ID NO:2. In this context "about" includes the particularly recited size, larger or smaller by several (5, 4, 3, 2, or 1) amino acid residues, at either terminus or at both termini. The inventors have determined that the above polypeptide fragments are antigenic regions of the DR4 protein. Methods for determining other such epitope-bearing portions of the DR4 protein are described in detail below. Polypeptides encoded by these nucleic acids are also encompassed by the invention.

In specific embodiments, the polynucleotides of the invention are less than 100000 kb, 50000 kb, 10000 kb, 1000 kb, 500 kb, 400 kb, 350 kb, 300 kb, 250 kb, 200 kb, 175 kb, 150 kb, 125 kb, 100 kb, 75 kb, 50 kb, 40 kb, 30 kb, 25 kb, 20 kb, 15 kb, 10 kb, 7.5 kb, or 5 kb in length.

In further embodiments, polynucleotides of the invention comprise, or alternatively consist of, at least 15, at least 30, at least 50, at least 100, or at least

250, at least 500, or at least 1000 contiguous nucleotides of DR4 coding sequence, but consist of less than or equal to 1000 kb, 500 kb, 250 kb, 200 kb, 150 kb, 100 kb, 75 kb, 50 kb, 30 kb, 25 kb, 20 kb, 15 kb, 10 kb, or 5 kb of genomic DNA that flanks the 5' or 3' coding nucleotide set forth in SEQ ID NO:1. In further embodiments, polynucleotides of the invention comprise, or alternatively consist of, at least 15, at least 30, at least 50, at least 100, or at least 250, at least 500, or at least 1000 contiguous nucleotides of DR4 coding sequence, but do not comprise all or a portion of any DR4 intron. In another embodiment, the nucleic acid comprising, or alternatively consisting of, DR4 coding sequence does not contain coding sequences of a genomic flanking gene (*i.e.*, 5' or 3' to the DR4 gene in the genome). In other embodiments, the polynucleotides of the invention do not contain the coding sequence of more than 1000, 500, 250, 100, 50, 25, 20, 15, 10, 5, 4, 3, 2, or 1 genomic flanking gene(s).

In addition, the invention provides nucleic acid molecules having nucleotide sequences related to extensive portions of SEQ ID NO:1 as follows: HTOIY07R (SEQ ID NO:6) and HTXEY80R (SEQ ID NO:7) both shown in Figure 4.

Further, the invention includes a polynucleotide comprising, or alternatively consisting of, any portion of at least about 30 nucleotides, preferably at least about 50 nucleotides, of SEQ ID NO:1 from residue 365 to 1,422. In this context "about" includes the particularly recited size, larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini.

In another embodiment, the invention provides an isolated nucleic acid molecule comprising, or alternatively consisting of, a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the sequence complementary to the coding and/or noncoding (*i.e.*, transcribed, untranslated) sequence depicted in SEQ ID NO:1, the cDNA contained in ATCC Deposit No. 97853, and the sequence encoding a DR4 domain or a polynucleotide fragment as described herein. By "stringent

hybridization conditions" is intended overnight incubation at 42°C in a solution comprising, or alternatively consisting of: 50% formamide, 5x SSC (750 mM NaCl, 75mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C. Polypeptides encoded by these nucleic acids are also encompassed by the invention.

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. In this context "about" includes the particularly recited size, larger or smaller by several (5, 4, 3, 2, or 1) nucleotides, at either terminus or at both termini. By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (*e.g.*, the deposited cDNA or the nucleotide sequence as shown in SEQ ID NO:1. These have uses, which include, but are not limited to, as diagnostic probes and primers as discussed above and in more detail below.

Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3 terminal poly(A) tract of the DR4 cDNA shown in SEQ ID NO:1), or to a complementary stretch of T (or U) resides, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (*e.g.*, practically any double-stranded cDNA).

As indicated, nucleic acid molecules of the present invention which encode the DR4 polypeptide may include, but are not limited to the coding sequence for the mature polypeptide, by itself; the coding sequence for the mature polypeptide and additional sequences, such as those encoding a leader or secretary sequence,

such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al., Proc. Natl. Acad. Sci. USA 86*:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag corresponds to an epitope derived of influenza hemagglutinin protein, which has been described by Wilson *et al., Cell 37*:767 (1984), for instance.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode for fragments, analogs or derivatives of the DR4 polypeptide. Variants may occur naturally, such as an allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions which may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and functional activities of the

DR4 receptor or portions thereof. Also especially preferred in this regard are conservative substitutions.

Further embodiments of the invention include isolated nucleic acid molecules that are at least 80% identical, and more preferably at least 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical, to (a) a nucleotide sequence encoding the full-length DR4 polypeptide having the complete amino acid sequence in SEQ ID NO:2, including the predicted leader sequence; (b) nucleotide sequence encoding the full-length DR4 polypeptide having the complete amino acid sequence in SEQ ID NO:2, including the predicted leader sequence but lacking the amino terminal methionine; (c) a nucleotide sequence encoding the mature DR4 polypeptide (full-length polypeptide with the leader removed) having the amino acid sequence at positions about 24 to about 468 in SEQ ID NO:2; (d) a nucleotide sequence encoding the full-length DR4 polypeptide having the complete amino acid sequence including the leader encoded by the cDNA contained in ATCC Deposit No. 97853; (e) a nucleotide sequence encoding the full-length DR4 polypeptide having the complete amino acid sequence including the leader but lacking the amino terminal methionine encoded by the cDNA contained in ATCC Deposit No. 97853; (f) a nucleotide sequence encoding the mature DR4 polypeptide having the amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 97853; (g) a nucleotide sequence that encodes the DR4 extracellular domain having the amino acid sequence at positions about 24 to about 238 in SEQ ID NO:2, or the DR4 extracellular domain encoded by the cDNA contained in ATCC Deposit No. 97853; (h) a nucleotide sequence that encodes the DR4 transmembrane domain having the amino acid sequence at positions about 239 to about 264 in SEQ ID NO:2, or the DR4 transmembrane domain encoded by the cDNA contained in ATCC Deposit No. 97853; (i) a nucleotide sequence that encodes the DR4 intracellular domain having the amino acid sequence at positions about 265 to about 468 in SEQ ID NO:2, or the DR4 intracellular domain encoded by the cDNA contained in ATCC Deposit No. 97853; (j) a nucleotide sequence that encodes the DR4 death domain having the

amino acid sequence at positions about 379 to about 422 in SEQ ID NO:2, or the DR4 death domain encoded by the cDNA contained in ATCC Deposit No. 97853; (k) a nucleotide sequence that encodes the DR4 cysteine rich domain having the amino acid sequence at positions about 131 to about 229 in SEQ ID NO:2, or the DR4 cysteine rich domain encoded by the cDNA contained in ATCC Deposit No. 97853; (l) a nucleotide sequence that encodes the DR4 receptor extracellular and intracellular domains with all or part of the transmembrane domain deleted; (m) a nucleotide sequence that encodes a fragment of the polypeptide of (c) having DR4 functional activity (*e.g.*, antigenic or biological activity); or (n) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i), (j) , (k), (l), or (m) above. In this context "about" includes the particularly recited size, larger or smaller by several (5, 4, 3, 2, or 1) amino acid residues, at either terminus or at both termini. Polypeptides encoded by these nucleic acids are also encompassed by the invention.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a DR4 polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five mismatches per each 100 nucleotides of the reference nucleotide sequence encoding the DR4 polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mismatches of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. The reference (query) sequence may be the entire DR4 nucleotide sequence shown in SEQ ID NO:1 or any fragment (*e.g.*, a polynucleotide

encoding the amino acid sequence of a DR4 N- and/or C-terminal deletion described herein) as described herein.

As a practical matter, whether any particular nucleic acid molecule is at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NO:1 or to the nucleotide sequences of the deposited cDNA can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics 2*:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

In a specific embodiment, the identity between a reference (query) sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag *et al.* (Comp. App. Biosci. 6:237-245 (1990)). Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent

identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. A determination of whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of this embodiment. Only bases outside the 5' and 3' bases of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score. For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are made for the purposes of this embodiment.

The present application is directed to nucleic acid molecules at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in SEQ ID NO:1 or to the nucleic acid sequence of the deposited

cDNAs, irrespective of whether they encode a polypeptide having DR4 functional activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having DR4 functional activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having DR4 functional activity include, *inter alia,* (1) isolating the DR4 gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (*e.g.*, "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the DR4 gene, as described in Verma *et al., Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and (3) Northern Blot analysis for detecting DR4 mRNA expression in specific tissues.

Preferred, however, are nucleic acid molecules having sequences at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in SEQ ID NO:1 or to the nucleic acid sequence of the deposited cDNAs which do, in fact, encode a polypeptide having DR4 protein functional activity. By "a polypeptide having DR4 protein functional activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of the DR4 protein of the invention (either the full-length protein (*i.e.* complete) or, preferably, the mature protein), as measured in a particular functional and/or biological assay. For example, DR4 polypeptide functional activity can be measured by the ability of a polypeptide sequence described herein to form multimers (*e.g.*, homodimers and homotrimers) with complete DR4, and to bind a DR4 ligand (*e.g.*, TRAIL). These functional assays can be routinely performed using techniques described herein and otherwise known in the art.

For example, DR4 protein functional activity (*e.g.*, biological activity) can routinely be measured using the cell death assays performed essentially as previously described (A.M. Chinnaiyan, *et al., Cell 81*, 505-12 (1995); M.P. Boldin, *et al., J Biol Chem 270*, 7795-8 (1995); F.C. Kischkel, *et al., EMBO 14*, 5579-5588 (1995); A.M. Chinnaiyan, *et al., J Biol Chem 271*, 4961-4965 (1996))

or as set forth in Example 5, below. In MCF7 cells, plasmids encoding full-length DR4 or a candidate death domain containing receptors are co-transfected with the pLantern reporter construct encoding green fluorescent protein. Nuclei of cells transfected with DR4 will exhibit apoptotic morphology as assessed by DAPI staining. Similar to TNFR-1 and Fas/APO-1 (M. Muzio, *et al.*, *Cell 85*, 817-827 (1996); M. P. Boldin, *et al.*, *Cell 85*, 803-815 (1996); M. Tewari, *et al.*, *J Biol Chem 270*, 3255-60 (1995)), DR4-induced apoptosis is blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to, for example, the nucleic acid sequence of the deposited cDNA or the nucleic acid sequence shown in SEQ ID NO:1 will encode "a polypeptide having DR4 protein functional activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having DR4 protein functional activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (*e.g.*, replacing one aliphatic amino acid with a second aliphatic amino acid), as further described below.

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J.U. *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247:*1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

Polynucleotide Assays

This invention is also related to the use of the DR4 polynucleotides to detect complementary polynucleotides such as, for example, as a diagnostic reagent. Detection of a mutated form of DR4 associated with a dysfunction will provide a diagnostic tool that can add or define a diagnosis of a disease or susceptibility to a disease which results from under-expression over-expression or altered expression of DR4 or a soluble form thereof, such as, for example, tumors or autoimmune disease.

Individuals carrying mutations in the DR4 gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR prior to analysis. (Saiki *et al.*, *Nature 324*:163-166 (1986)). RNA or cDNA may also be used in the same ways. As an example, PCR primers complementary to the nucleic acid encoding DR4 can be used to identify and analyze DR4 expression and mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled DR4 RNA or alternatively, radiolabeled DR4 antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between a reference gene and genes having mutations also may be revealed by direct DNA sequencing. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of such methods can be greatly enhanced by appropriate use of PCR or another amplification method. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by

conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, *e.g.*, Myers *et al., Science 230*:1242 (1985)).

Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and Sl protection or the chemical cleavage method (*e.g.*, Cotton *et al., Proc. Natl. Acad. Sci. USA 85*: 4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (*e.g.*, restriction fragment length polymorphisms ("RFLP") and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations also can be detected by *in situ* analysis.

Vectors and Host Cells

The present invention also relates to vectors which include DNA molecules of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells can be genetically engineered to incorporate nucleic acid molecules and express polypeptides of the present invention. The polynucleotides may be introduced alone or with other polynucleotides. Such other

polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention.

In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single or double-stranded phage vector, a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells.

Preferred among vectors, in certain respects, are those for expression of polynucleotides and polypeptides of the present invention. Generally, such vectors comprise *cis*-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

A great variety of expression vectors can be used to express a polypeptide of the invention. Such vectors include chromosomal, episomal and virus-derived vectors *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s)), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include the phage lambda PL promoter, the *E. coli lac*, *trp* and *tac* promoters, the SV40 early

and late promoters and promoters of retroviral LTRs, to name just a few of the well-known promoters. In general, expression constructs will contain sites for transcription, initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Preferred markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing *E. coli* and other bacteria.

The vector containing the appropriate DNA sequence as described elsewhere herein, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Hosts for of a great variety of expression constructs are well known, and those of skill will be enabled by the present disclosure readily to select a host for expressing a polypeptides in accordance with this aspect of the present invention.

Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript

vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors are listed solely by way of illustration of the many commercially available and well known vectors available to those of skill in the art.

Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

The present invention also relates to host cells containing the above-described vector constructs described herein, and additionally encompasses host cells containing nucleotide sequences of the invention that are operably associated with one or more heterologous control regions (*e.g.*, promoter and/or enhancer) using techniques known of in the art. The host cell can be a higher eukaryotic cell, such as a mammalian cell (*e.g.*, a human derived cell), or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. The host strain may be chosen which modulates the expression of the inserted gene sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus expression of the genetically engineered polypeptide may be controlled. Furthermore, different host cells have characteristics and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, phosphorylation, cleavage) of proteins. Appropriate cell lines can be chosen to ensure the desired modifications and processing of the foreign protein expressed.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods.

Such methods are described in many standard laboratory manuals, such as Davis *et al., Basic Methods in Molecular Biology* (1986).

In addition to encompassing host cells containing the vector constructs discussed herein, the invention also encompasses primary, secondary, and immortalized host cells of vertebrate origin, particularly mammalian origin, that have been engineered to delete or replace endogenous genetic material (*e.g.*, DR4 coding sequence), and/or to include genetic material (*e.g.*, heterologous polynucleotide sequences) that is operably associated with DR4 polynucleotides of the invention, and which activates, alters, and/or amplifies endogenous DR4 polynucleotides. For example, techniques known in the art may be used to operably associate heterologous control regions (*e.g.*, promoter and/or enhancer) and endogenous DR4 polynucleotide sequences via homologous recombination (see, *e.g.*, US Patent Number 5,641,670, issued June 24, 1997; International Publication Number WO 96/29411, published September 26, 1996; International Publication Number WO 94/12650, published August 4, 1994; Koller *et al.*, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); and Zijlstra *et al.*, Nature 342:435-438 (1989), the disclosures of each of which are incorporated by reference in their entireties).

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Such a fusion protein can be made by ligating polynucleotides of the invention and the desired nucleic acid sequence encoding the desired amino acid sequence to each other, by methods known in the art, in the proper reading frame, and expressing the fusion protein product by methods known in the art. Alternatively, such a fusion protein can be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, region also may be added to the polypeptide to facilitate purification. Such

regions may be removed prior to final preparation of the polypeptide. For example, in one embodiment, polynucleotides encoding DR4 polypeptides of the invention may be fused to the pelB pectate lyase signal sequence to increase the efficiency to expression and purification of such polypeptides in Gram-negative bacteria. See, US Patent Nos. 5,576,195 and 5,846,818, the contents of which are herein incorporated by reference in their entireties.

The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as, hIL5-receptor has been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al.*, *Journal of Molecular Recognition*, Vol. 8:52-58 (1995) and K. Johanson *et al.*, *The Journal of Biological Chemistry*, Vol. 270, No. 16:9459-9471 (1995).

As mentioned, DR4 polypeptides may be modified by either natural processes, such as posttranslational processing, or by chemical modification techniques which are well known in the art. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given DR4 polypeptide. Also, a given DR4 polypeptide may contain many

types of modifications. DR4 polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic DR4 polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. (See, for instance, PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993); POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, pgs. 1-12 (1983); Seifter *et al.*, Meth Enzymol 182:626-646 (1990); Rattan *et al.*, Ann NY Acad Sci 663:48-62 (1992).)

The DR4 can be recovered and purified from chemical synthesis and recombinant cell cultures by standard methods which include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

DR4 polynucleotides and polypeptides may be used in accordance with the present invention for a variety of applications, particularly those that make use of the chemical and biological properties of DR4. Among these are applications in

the treatment and prevention of tumors, parasitic infections, bacterial infections, viral infections, restenosis, and graft vs. host disease; to induce resistance to parasites, bacteria and viruses; to induce proliferation of T-cells, endothelial cells and certain hematopoietic cells; to regulate anti-viral responses; and to treat and prevent certain autoimmune diseases after stimulation of DR4 by an agonist. Additional applications relate to diagnosis, treatment, and prevention of disorders of cells, tissues and organisms. These aspects of the invention are discussed further below.

DR4 Proteins and Fragments

The invention further provides for DR4 proteins containing polypeptide sequences encoded by the polynucleotides of the invention.

The DR4 proteins of the invention may be in monomers or multimers (*i.e.*, dimers, trimers, tetramers, and higher multimers). Accordingly, the present invention relates to monomers and multimers of the DR4 proteins of the invention, their preparation, and compositions (preferably, pharmaceutical compositions) containing them. In specific embodiments, the polypeptides of the invention are monomers, dimers, trimers or tetramers. In additional embodiments, the multimers of the invention are at least dimers, at least trimers, or at least tetramers.

Multimers encompassed by the invention may be homomers or heteromers. As used herein, the term homomer, refers to a multimer containing only DR4 proteins of the invention (including DR4 fragments, variants, and fusion proteins, as described herein). These homomers may contain DR4 proteins having identical or different polypeptide sequences. In a specific embodiment, a homomer of the invention is a multimer containing only DR4 proteins having an identical polypeptide sequence. In another specific embodiment, a homomer of the invention is a multimer containing DR4 proteins having different polypeptide sequences. In specific embodiments, the multimer of the invention is a homodimer

(*e.g.*, containing DR4 proteins having identical or different polypeptide sequences) or a homotrimer (*e.g.*, containing DR4 proteins having identical or different polypeptide sequences). In additional embodiments, the homomeric multimer of the invention is at least a homodimer, at least a homotrimer, or at least a homotetramer.

As used herein, the term heteromer refers to a multimer containing heterologous proteins (*i.e.*, proteins containing only polypeptide sequences that do not correspond to a polypeptide sequences encoded by the DR4 gene) in addition to the DR4 proteins of the invention. In a specific embodiment, the multimer of the invention is a heterodimer, a heterotrimer, or a heterotetramer. In additional embodiments, the homomeric multimer of the invention is at least a homodimer, at least a homotrimer, or at least a homotetramer.

Multimers of the invention may be the result of hydrophobic, hydrophilic, ionic and/or covalent associations and/or may be indirectly linked, by for example, liposome formation. Thus, in one embodiment, multimers of the invention, such as, for example, homodimers or homotrimers, are formed when proteins of the invention contact one another in solution. In another embodiment, heteromultimers of the invention, such as, for example, heterotrimers or heterotetramers, are formed when proteins of the invention contact antibodies to the polypeptides of the invention (including antibodies to the heterologous polypeptide sequence in a fusion protein of the invention) in solution. In other embodiments, multimers of the invention are formed by covalent associations with and/or between the DR4 proteins of the invention. Such covalent associations may involve one or more amino acid residues contained in the polypeptide sequence of the protein (*e.g.*, the polypeptide sequence recited in SEQ ID NO: 2 or the polypeptide encoded by the deposited cDNA). In one instance, the covalent associations are cross-linking between cysteine residues located within the polypeptide sequences of the proteins which interact in the native (*i.e.*, naturally occurring) polypeptide. In another instance, the covalent associations are the consequence of chemical or recombinant manipulation. Alternatively, such

covalent associations may involve one or more amino acid residues contained in the heterologous polypeptide sequence in a DR4 fusion protein. In one example, covalent associations are between the heterologous sequence contained in a fusion protein of the invention (see, *e.g.*, US Patent Number 5,478,925). In a specific example, the covalent associations are between the heterologous sequence contained in a DR4-Fc fusion protein of the invention (as described herein). In another specific example, covalent associations of fusion proteins of the invention are between heterologous polypeptide sequences from another TNF family ligand/receptor member that is capable of forming covalently associated multimers, such as for example, oseteoprotegerin (see, *e.g.*, International Publication No. WO 98/49305, the contents of which are herein incorporated by reference in its entirety).

The multimers of the invention may be generated using chemical techniques known in the art. For example, proteins desired to be contained in the multimers of the invention may be chemically cross-linked using linker molecules and linker molecule length optimization techniques known in the art (see, *e.g.*, US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, multimers of the invention may be generated using techniques known in the art to form one or more inter-molecule cross-links between the cysteine residues located within the polypeptide sequence of the proteins desired to be contained in the multimer (see, *e.g.*, US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Further, proteins of the invention may be routinely modified by the addition of cysteine or biotin to the C-terminus or N-terminus of the polypeptide sequence of the protein and techniques known in the art may be applied to generate multimers containing one or more of these modified proteins (see, *e.g.*, US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). Additionally, techniques known in the art may be applied to generate liposomes containing the protein components desired to be contained in the multimer of the invention (see, *e.g.*, US

Patent Number 5,478,925, which is herein incorporated by reference in its entirety).

Alternatively, multimers of the invention may be generated using genetic engineering techniques known in the art.

In one embodiment, proteins contained in multimers of the invention are produced recombinantly using fusion protein technology described herein or otherwise known in the art (see, *e.g.*, US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In a specific embodiment, polynucleotides coding for a homodimer of the invention are generated by ligating a polynucleotide sequence encoding a polypeptide of the invention to a sequence encoding a linker polypeptide and then further to a synthetic polynucleotide encoding the translated product of the polypeptide in the reverse orientation from the original C-terminus to the N-terminus (lacking the leader sequence) (see, *e.g.*, US Patent Number 5,478,925, which is herein incorporated by reference in its entirety). In another embodiment, recombinant techniques described herein or otherwise known in the art are applied to generate recombinant polypeptides of the invention which contain a transmembrane domain and which can be incorporated by membrane reconstitution techniques into liposomes (see, *e.g.*, US Patent Number 5,478,925, which is herein incorporated by reference in its entirety).

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. Accordingly, in one embodiment, the invention further provides an isolated DR4 polypeptide having the amino acid sequence encoded by the deposited cDNA, or the amino acid sequence shown in SEQ ID NO:2 or a peptide or polypeptide portion (*i.e.*, fragment) comprising a portion of the above polypeptides.

Polypeptide fragments of the present invention include polypeptides comprising, or alternatively consisting of, an amino acid sequence contained in SEQ ID NO:2, encoded by the cDNA contained in the deposited plasmid, or encoded by nucleic acids which hybridize (*e.g.*, under stringent hybridization

conditions) to the nucleotide sequence contained in the deposited plasmid, or shown in SEQ ID NO:1 or the complementary strand thereto. Protein fragments may be "free-standing," or comprised within a larger polypeptide of which the fragment forms a part or region, most preferably as a single continuous region. Representative examples of polypeptide fragments of the invention, include, for example, fragments that comprise, or alternatively consisting of, from about amino acid residues: 1 to 23, 24 to 43, 44 to 63, 64 to 83, 84 to 103, 104 to 123, 124 to 143, 144 to 163, 164 to 183, 184 to 203, 204 to 223, 224 to 238, 239 to 264, 265 to 284, 285 to 304, 305 to 324, 325 to 345, 346 to 366, 367 to 387, 388 to 418, 419 to 439, and/or 440 to the end of the coding region of SEQ ID NO:2. Additional representative examples of polypeptide fragments of the invention, include, for example, fragments that comprise, or alternatively consisting of, from about amino acid residues: 1-60, 11-70, 21-80, 31-90, 41-100, 51-110, 61-120, 71-130, 81-140, 91-150, 101-160, 111-170, 121-180, 131-190, 141-200, 151-210, 161-220, 171-230, 181-240, 191-250, 201-260, 211-270, 221-280, 231-290, 241-300, 251-310, 261-320, 271-330, 281-340, 291-350, 301-360, 311-370, 321-380, 331-390, 341-400, 351-410, 361-420, 371-430, 381-440, 391-450, and/or 401-468 of SEQ ID NO:2, as well as isolated polynucleotides which encode these polypeptides. In this context "about" includes the particularly recited value, larger or smaller by several (5, 4, 3, 2, or 1) amino acids, at either extreme or at both extremes. Moreover, polypeptide fragments can be at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 175 or 200 amino acids in length. Polynucleotides encoding these polypeptides are also encompassed by the invention.

Preferred polypeptide fragments of the present invention include a member selected from the group: a polypeptide comprising, or alternatively consisting of, the DR4 receptor extracellular domain (predicted to constitute amino acid residues from about 24 to about 238 in SEQ ID NO:2); a polypeptide comprising, or alternatively consisting of, the DR4 cysteine rich domain (predicted to constitute amino acid residues from about 131 to about 229 in SEQ ID NO:2); a

polypeptide comprising, or alternatively consisting of, the DR4 receptor transmembrane domain (predicted to constitute amino acid residues from about 239 to about 264 in SEQ ID NO:2); a polypeptide comprising, or alternatively consisting of, a fragment of the predicted mature DR4 polypeptide, wherein the fragment has a DR4 functional activity (*e.g.*, antigenic activity or biological activity); a polypeptide comprising, or alternatively consisting of, the DR4 receptor intracellular domain (predicted to constitute amino acid residues from about 265 to about 468 in SEQ ID NO:2); a polypeptide comprising, or alternatively consisting of, the DR4 receptor extracellular and intracellular domains with all or part of the transmembrane domain deleted; a polypeptide comprising, or alternatively consisting of, the DR4 receptor death domain (predicted to constitute amino acid residues from about 379 to about 422 in SEQ ID NO:2); and a polypeptide comprising, or alternatively consisting of, one, two, three, four or more, epitope bearing portions of the DR4 receptor protein. In additional embodiments, the polypeptide fragments of the invention comprise, or alternatively consist of, any combination of 1, 2, 3, 4, 5, 6, 7, or all 8 of the above members. As above, with the leader sequence, the amino acid residues constituting the DR4 receptor extracellular, transmembrane and intracellular domains have been predicted by computer analysis. Thus, as one of ordinary skill would appreciate, the amino acid residues constituting these domains may vary slightly (*e.g.*, by about 1 to about 15 amino acid residues) depending on the criteria used to define each domain. Polynucleotides encoding these polypeptides are also encompassed by the invention.

As discussed above, it is believed that one or both of the extracellular cysteine rich motifs of DR4 is important for interactions between DR4 and its ligands (*e.g.*, TRAIL). Accordingly, in preferred embodiments, polypeptide fragments of the invention comprise, or alternatively consist of, amino acid residues 131 to 183, and/or 184 to 229 of SEQ ID NO:2. In a specific embodiment the polypeptides of the invention comprise, or alternatively consist of, both of the extracellular cysteine rich motifs disclosed in SEQ ID NO:2.

Polynucleotides encoding these polypeptide fragments are also encompassed by the invention.

Among the especially preferred polypeptide fragments of the invention are fragments comprising, or alternatively consisting of, structural or functional attributes of DR4. Such fragments include amino acid residues that comprise, or alternatively consisting of, one, two, three, four or more of the following functional domains: alpha-helix and alpha-helix forming regions ("alpha-regions"), beta-sheet and beta-sheet-forming regions ("beta-regions"), turn and turn-forming regions ("turn-regions"), coil and coil-forming regions ("coil-regions"), hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, surface forming regions, and high antigenic index regions (*i.e.*, containing four or more contiguous amino acids having an antigenic index of greater than or equal to 1.5, as identified using the default parameters of the Jameson-Wolf program) of complete (*i.e.*, full-length) DR4. Certain preferred regions are those set out in Figure 3 and Table I and include, but are not limited to, regions of the aforementioned types identified by analysis of the amino acid sequence depicted in SEQ ID NO:2, such preferred regions include; Garnier-Robson predicted alpha-regions, beta-regions, turn-regions, and coil-regions; Chou-Fasman predicted alpha-regions, beta-regions, and turn-regions; Kyte-Doolittle predicted hydrophilic regions; Eisenberg alpha and beta amphipathic regions; Emini surface-forming regions; and Jameson-Wolf high antigenic index regions, as predicted using the default parameters of these computer programs. Polynucleotides encoding these polypeptides are also encompassed by the invention.

The present invention encompasses polypeptides comprising, or alternatively consisting of, an epitope of the polypeptide having an amino acid sequence of SEQ ID NO:2, or an epitope of the polypeptide sequence encoded by a polynucleotide sequence contained in the cDNA assigned ATCC Accession No. 97853, encoded by a polynucleotide that hybridizes to the complement of the sequence of SEQ ID NO:1, or contained in the cDNA assigned ATCC Accession

No. 97853 under stringent hybridization conditions or lower stringency hybridization conditions as defined *supra*. The present invention further encompasses polynucleotide sequences encoding an epitope of a polypeptide sequence of the invention (such as, for example, the sequence disclosed in SEQ ID NO:1), polynucleotide sequences of the complementary strand of a polynucleotide sequence encoding an epitope of the invention, and polynucleotide sequences which hybridize to the complementary strand under stringent hybridization conditions or lower stringency hybridization conditions defined *supra*.

The term "epitopes," as used herein, refers to portions of a polypeptide having antigenic or immunogenic activity in an animal, preferably a mammal, and most preferably in a human. In a preferred embodiment, the present invention encompasses a polypeptide comprising an epitope, as well as the polynucleotide encoding this polypeptide. An "immunogenic epitope," as used herein, is defined as a portion of a protein that elicits an antibody response in an animal, as determined by any method known in the art, for example, by the methods for generating antibodies described *infra*. (*See*, for example, Geysen *et al.*, *Proc. Natl. Acad. Sci. USA 81*:3998-4002 (1983)). The term "antigenic epitope," as used herein, is defined as a portion of a protein to which an antibody can immunospecifically bind its antigen as determined by any method well known in the art, for example, by the immunoassays described herein. Immunospecific binding excludes non-specific binding but does not necessarily exclude cross-reactivity with other antigens. Antigenic epitopes need not necessarily be immunogenic.

Fragments that function as epitopes may be produced by any conventional means. (*See*, *e.g.*, Houghten, *Proc. Natl. Acad. Sci. USA 82*:5131-5135 (1985), further described in U.S. Patent No. 4,631,211).

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind

specifically to a polypeptide of the invention. See, for instance, Wilson *et al.*, *Cell* *37*:767-778 (1984) at 777.

In the present invention, antigenic epitopes preferably contain a sequence of at least 4, at least 5, at least 6, at least 7, more preferably at least 8, at least 9, at least 10, at least 15, at least 20, at least 25, and, most preferably, between about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. Preferred polypeptides comprising immunogenic or antigenic epitopes are at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acid residues in length. Antigenic epitopes are useful, for example, to raise antibodies, including monoclonal antibodies, that specifically bind the epitope. Antigenic epitopes can be used as the target molecules in immunoassays. (See, for instance, Wilson *et al.*, Cell 37:767-778 (1984); Sutcliffe *et al.*, *Science* 219:660-666 (1983)). Polynucleotides encoding these polypeptides are also encompassed by the invention.

Similarly, immunogenic epitopes can be used, for example, to induce antibodies according to methods well known in the art. (*See*, for instance, Sutcliffe *et al.*, *supra*; Wilson *et al.*, *supra*; Chow *et al.*, Proc. Natl. Acad. Sci. USA 82:910-914; and Bittle *et al.*, J. Gen. Virol. 66:2347-2354 (1985). A preferred immunogenic epitope includes the secreted protein. The polypeptides comprising one or more immunogenic epitopes may be presented for eliciting an antibody response together with a carrier protein, such as an albumin, to an animal system (such as, for example, rabbit or mouse), or, if the polypeptide is of sufficient length (at least about 25 amino acids), the polypeptide may be presented without a carrier. However, immunogenic epitopes comprising as few as 8 to 10 amino acids have been shown to be sufficient to raise antibodies capable of binding to, at the very least, linear epitopes in a denatured polypeptide (*e.g.*, in Western blotting).

Epitope-bearing polypeptides of the present invention may be used to induce antibodies according to methods well known in the art including, but not limited to, *in vivo* immunization, *in vitro* immunization, and phage display

methods. *See, e.g.*, Sutcliffe *et al.*, *supra*; Wilson *et al.*, *supra*, and Bittle *et al.*, *J. Gen. Virol.*, *66*:2347-2354 (1985). If *in vivo* immunization is used, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling the peptide to a macromolecular carrier, such as keyhole limpet hemacyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine residues may be coupled to a carrier using a linker such as maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), while other peptides may be coupled to carriers using a more general linking agent such as glutaraldehyde. Animals such as, for example, rabbits, rats, and mice are immunized with either free or carrier-coupled peptides, for instance, by intraperitoneal and/or intradermal injection of emulsions containing about 100 micrograms of peptide or carrier protein and Freund's adjuvant or any other adjuvant known for stimulating an immune response. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody that can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

As to the selection of peptides or polypeptides bearing an antigenic epitope (*i.e.*, that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R.A. (1983) Antibodies that react with predetermined sites on proteins. *Science 219:*660-666. Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (*i.e.*, immunogenic epitopes) nor to the amino or carboxyl terminals.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate DR4-specific antibodies include: a polypeptide comprising, or alternatively consisting of, amino acid residues from about 35 to about 92 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 114 to about 160 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 169 to about 240 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 267 to about 298 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 330 to about 364 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 391 to about 404 in SEQ ID NO:2; and a polypeptide comprising, or alternatively consisting of, amino acid residues from about 418 to about 465 in SEQ ID NO:2. In this context "about" includes the particularly recited range, larger or smaller by several (5, 4, 3, 2, or 1) amino acid residues, at either terminus or at both termini. As indicated above, the inventors have determined that the above polypeptide fragments are antigenic regions of the DR4 protein. Polynucleotides encoding these polypeptides are also encompassed by the invention.

As one of skill in the art will appreciate, and as discussed above, the polypeptides of the present invention comprising an immunogenic or antigenic epitope can be fused to other polypeptide sequences. For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, or any combination thereof and portions thereof) resulting in chimeric polypeptides. Such fusion proteins may facilitate purification and may increase half-life *in vivo*. This has been shown for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. *See, e.g.*, EP 394,827; Traunecker *et al.*, *Nature, 331*:84-86 (1988). IgG Fusion proteins that have a disulfide-linked dimeric structure due to the IgG portion disulfide bonds have also

been found to be more efficient in binding and neutralizing other molecules than monomeric polypeptides or fragments thereof alone. *See, e.g.*, Fountoulakis *et al.*, *J. Biochem.*, *270*:3958-3964 (1995). Nucleic acids encoding the above epitopes can also be recombined with a gene of interest as an epitope tag (*e.g.*, the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht *et al.* allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88:8972-897). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix-binding domain for the fusion protein. Extracts from cells infected with the recombinant vaccinia virus are loaded onto Ni^{2+} nitriloacetic acid-agarose column and histidine-tagged proteins can be selectively eluted with imidazole-containing buffers.

Additional fusion proteins of the invention may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to modulate the activities of polypeptides of the invention, such methods can be used to generate polypeptides with altered activity, as well as agonists and antagonists of the polypeptides. See, generally, U.S. Patent Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Patten *et al.*, Curr. Opinion Biotechnol. 8:724-33 (1997); Harayama, Trends Biotechnol. 16(2):76-82 (1998); Hansson *et al.*, J. Mol. Biol. 287:265-76 (1999); and Lorenzo and Blasco, Biotechniques 24(2):308-13 (1998) (each of these patents and publications are hereby incorporated by reference in its entirety). In one embodiment, alteration of polynucleotides corresponding to SEQ ID NO:1 and the polypeptides encoded by these polynucleotides may be achieved by DNA shuffling. DNA shuffling involves the assembly of two or more DNA segments by homologous or site-specific recombination to generate variation in the

polynucleotide sequence. In another embodiment, polynucleotides of the invention, or the encoded polypeptides, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of a polynucleotide coding a polypeptide of the invention may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

As one of skill in the art will appreciate, DR4 polypeptides of the present invention and the epitope-bearing fragments thereof described herein (*e.g.*, corresponding to a portion of the extracellular domain such as, for example, amino acid residues 1 to 240 of SEQ ID NO:2) can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life *in vivo.* This has been shown, *e.g.*, for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker *et al., Nature 331*:84-86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric DR4 protein or protein fragment alone (Fountoulakis *et al., J Biochem 270*:3958-3964 (1995)). The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means. Houghten, R.A., "General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids," *Proc. Natl. Acad. Sci. USA 82*:5131-5135 (1985). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986).

To improve or alter the characteristics of DR4 polypeptides, protein engineering may be employed. Recombinant DNA technology known to those

skilled in the art can be used to create novel mutant proteins or "muteins" including single or multiple amino acid substitutions, deletions, additions or fusion proteins. Such modified polypeptides can show, *e.g.*, enhanced activity or increased stability. In addition, they may be purified in higher yields and show better solubility than the corresponding natural polypeptide, at least under certain purification and storage conditions.

For instance, for many proteins, including the extracellular domain of a membrane associated protein or the mature form(s) of a secreted protein, it is known in the art that one or more amino acids may be deleted from the N-terminus or C-terminus without substantial loss of biological function. For instance, Ron *et al., J. Biol. Chem., 268*:2984-2988 (1993) reported modified KGF proteins that had heparin binding activity even if 3, 8, or 27 amino-terminal amino acid residues were missing. In the present case, since the protein of the invention is a member of the death domain containing receptor (DDCR) polypeptide family, deletions of N-terminal amino acids up to the cysteine residue at position 132 in SEQ ID NO:2 may retain some biological activity such as the ability to induce apoptosis. Polypeptides having further N-terminal deletions including the cysteine residue at position 132 (C-132) in SEQ ID NO:2 would not be expected to retain such biological activities because this residue is conserved among family members, see Figure 2, and may be required for forming a disulfide bridge to provide structural stability which is needed for receptor binding.

However, even if deletion of one or more amino acids from the N-terminus of a protein results in modification or loss of one or more biological functions of the protein, other functional activities (*e.g.*, biological activities, ability to multimerize, ability to bind DR4 ligand (*e.g.*, TRAIL)) may still be retained. For example, the ability of shortened DR4 muteins to induce and/or bind to antibodies which recognize the complete or mature forms of the DR4 polypeptides of the invention (preferably antibodies that bind specifically to DR4) generally will be retained when less than the majority of the residues of the complete or mature polypeptide are removed from the N-terminus. Whether a particular polypeptide

lacking N-terminal residues of a complete polypeptide retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art. It is not unlikely that an DR4 mutein with a large number of deleted N-terminal amino acid residues may retain some biological or immunogenic activities. In fact, peptides composed of as few as six DR4 amino acid residues may often evoke an immune response.

Accordingly, the present invention further provides polypeptides having one or more residues deleted from the amino terminus of the DR4 amino acid sequence shown in SEQ ID NO:2, up to the serine residue at position number 463 and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues n^1-468 of SEQ ID NO:2, where n^1 is an integer from 2 to 463 corresponding to the position of the amino acid residue in SEQ ID NO:2.

More in particular, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues of A-2 to E-468; P-3 to E-468; P-4 to E-468; P-5 to E-468; A-6 to E-468; R-7 to E-468; V-8 to E-468; H-9 to E-468; L-10 to E-468; G-11 to E-468; A-12 to E-468; F-13 to E-468; L-14 to E-468; A-15 to E-468; V-16 to E-468; T-17 to E-468; P-18 to E-468; N-19 to E-468; P-20 to E-468; G-21 to E-468; S-22 to E-468; A-23 to E-468; A-24 to E-468; S-25 to E-468; G-26 to E-468; T-27 to E-468; E-28 to E-468; A-29 to E-468; A-30 to E-468; A-31 to E-468; A-32 to E-468; T-33 to E-468; P-34 to E-468; S-35 to E-468; K-36 to E-468; V-37 to E-468; W-38 to E-468; G-39 to E-468; S-40 to E-468; S-41 to E-468; A-42 to E-468; G-43 to E-468; R-44 to E-468; I-45 to E-468; E-46 to E-468; P-47 to E-468; R-48 to E-468; G-49 to E-468; G-50 to E-468; G-51 to E-468; R-52 to E-468; G-53 to E-468; A-54 to E-468; L-55 to E-468; P-56 to E-468; T-57 to E-468; S-58 to E-468; M-59 to E-468; G-60 to E-468; Q-61 to E-468; H-62 to E-468; G-63 to E-468; P-64 to E-468; S-65 to E-468; A-66 to E-468; R-67 to E-468; A-68 to E-468; R-69 to E-468; A-70 to E-468; G-71 to E-468; R-72 to E-

468; A-73 to E-468; P-74 to E-468; G-75 to E-468; P-76 to E-468; R-77 to E-468; P-78 to E-468; A-79 to E-468; R-80 to E-468; E-81 to E-468; A-82 to E-468; S-83 to E-468; P-84 to E-468; R-85 to E-468; L-86 to E-468; R-87 to E-468; V-88 to E-468; H-89 to E-468; K-90 to E-468; T-91 to E-468; F-92 to E-468; K-93 to E-468; F-94 to E-468; V-95 to E-468; V-96 to E-468; V-97 to E-468; G-98 to E-468; V-99 to E-468; L-100 to E-468; L-101 to E-468; Q-102 to E-468; V-103 to E-468; V-104 to E-468; P-105 to E-468; S-106 to E-468; S-107 to E-468; A-108 to E-468; A-109 to E-468; T-110 to E-468; I-111 to E-468; K-112 to E-468; L-113 to E-468; H-114 to E-468; D-115 to E-468; Q-116 to E-468; S-117 to E-468; I-118 to E-468; G-119 to E-468; T-120 to E-468; Q-121 to E-468; Q-122 to E-468; W-123 to E-468; E-124 to E-468; H-125 to E-468; S-126 to E-468; P-127 to E-468; L-128 to E-468; G-129 to E-468; E-130 to E-468; L-131 to E-468; C-132 to E-468; P-133 to E-468; P-134 to E-468; G-135 to E-468; S-136 to E-468; H-137 to E-468; R-138 to E-468; S-139 to E-468; E-140 to E-468; R-141 to E-468; P-142 to E-468; G-143 to E-468; A-144 to E-468; C-145 to E-468; N-146 to E-468; R-147 to E-468; C-148 to E-468; T-149 to E-468; E-150 to E-468; G-151 to E-468; V-152 to E-468; G-153 to E-468; Y-154 to E-468; T-155 to E-468; N-156 to E-468; A-157 to E-468; S-158 to E-468; N-159 to E-468; N-160 to E-468; L-161 to E-468; F-162 to E-468; A-163 to E-468; C-164 to E-468; L-165 to E-468; P-166 to E-468; C-167 to E-468; T-168 to E-468; A-169 to E-468; C-170 to E-468; K-171 to E-468; S-172 to E-468; D-173 to E-468; E-174 to E-468; E-175 to E-468; E-176 to E-468; R-177 to E-468; S-178 to E-468; P-179 to E-468; C-180 to E-468; T-181 to E-468; T-182 to E-468; T-183 to E-468; R-184 to E-468; N-185 to E-468; T-186 to E-468; A-187 to E-468; C-188 to E-468; Q-189 to E-468; C-190 to E-468; K-191 to E-468; P-192 to E-468; G-193 to E-468; T-194 to E-468; F-195 to E-468; R-196 to E-468; N-197 to E-468; D-198 to E-468; N-199 to E-468; S-200 to E-468; A-201 to E-468; E-202 to E-468; M-203 to E-468; C-204 to E-468; R-205 to E-468; K-206 to E-468; C-207 to E-468; S-208 to E-468; T-209 to E-468; G-210 to E-468; C-211 to E-468; P-212 to E-468; R-213 to E-468; G-214 to E-

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468; N-356 to E-468; G-357 to E-468; A-358 to E-468; D-359 to E-468; P-360 to E-468; T-361 to E-468; E-362 to E-468; T-363 to E-468; L-364 to E-468; M-365 to E-468; L-366 to E-468; F-367 to E-468; F-368 to E-468; D-369 to E-468; K-370 to E-468; F-371 to E-468; A-372 to E-468; N-373 to E-468; I-374 to E-468; V-375 to E-468; P-376 to E-468; F-377 to E-468; D-378 to E-468; S-379 to E-468; W-380 to E-468; D-381 to E-468; Q-382 to E-468; L-383 to E-468; M-384 to E-468; R-385 to E-468; Q-386 to E-468; L-387 to E-468; D-388 to E-468; L-389 to E-468; T-390 to E-468; K-391 to E-468; N-392 to E-468; E-393 to E-468; I-394 to E-468; D-395 to E-468; V-396 to E-468; V-397 to E-468; R-398 to E-468; A-399 to E-468; G-400 to E-468; T-401 to E-468; A-402 to E-468; G-403 to E-468; P-404 to E-468; G-405 to E-468; D-406 to E-468; A-407 to E-468; L-408 to E-468; Y-409 to E-468; A-410 to E-468; M-411 to E-468; L-412 to E-468; M-413 to E-468; K-414 to E-468; W-415 to E-468; V-416 to E-468; N-417 to E-468; K-418 to E-468; T-419 to E-468; G-420 to E-468; R-421 to E-468; N-422 to E-468; A-423 to E-468; S-424 to E-468; I-425 to E-468; H-426 to E-468; T-427 to E-468; L-428 to E-468; L-429 to E-468; D-430 to E-468; A-431 to E-468; L-432 to E-468; E-433 to E-468; R-434 to E-468; M-435 to E-468; E-436 to E-468; E-437 to E-468; R-438 to E-468; H-439 to E-468; A-440 to E-468; K-441 to E-468; E-442 to E-468; K-443 to E-468; I-444 to E-468; Q-445 to E-468; D-446 to E-468; L-447 to E-468; L-448 to E-468; V-449 to E-468; D-450 to E-468; S-451 to E-468; G-452 to E-468; K-453 to E-468; F-454 to E-468; I-455 to E-468; Y-456 to E-468; L-457 to E-468; E-458 to E-468; D-459 to E-468; G-460 to E-468; T-461 to E-468; G-462 to E-468; and S-463 to E-468 of the DR4 sequence shown in SEQ ID NO:2.

The present invention is also directed to nucleic acid molecules comprising, or alternatively consisting of, a polynucleotide sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the polynucleotide sequences encoding the polypeptides described above. The invention is further directed to nucleic acid molecules comprising, or alternatively consisting of, polynucleotide sequences which encode polypeptides that are at least 80%, 85%,

90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the polypeptides described above. The present invention also encompasses the above polynucleotide sequences fused to a heterologous polynucleotide sequence. Polypeptides encoded by these polynucleotides are also encompassed by the invention.

In another embodiment, N-terminal deletions of the DR4 polypeptide can be described by the general formula n^2 to 238 where n^2 is a number from 2 to 238 corresponding to the amino acid sequence identified in SEQ ID NO:2. In specific embodiments, N-terminal deletions of the DR4 receptors of the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues: A-2 to H-238; P-3 to H-238; P-4 to H-238; P-5 to H-238; A-6 to H-238; R-7 to H-238; V-8 to H-238; H-9 to H-238; L-10 to H-238; G-11 to H-238; A-12 to H-238; F-13 to H-238; L-14 to H-238; A-15 to H-238; V-16 to H-238; T-17 to H-238; P-18 to H-238; N-19 to H-238; P-20 to H-238; G-21 to H-238; S-22 to H-238; A-23 to H-238; A-24 to H-238; S-25 to H-238; G-26 to H-238; T-27 to H-238; E-28 to H-238; A-29 to H-238; A-30 to H-238; A-31 to H-238; A-32 to H-238; T-33 to H-238; P-34 to H-238; S-35 to H-238; K-36 to H-238; V-37 to H-238; W-38 to H-238; G-39 to H-238; S-40 to H-238; S-41 to H-238; A-42 to H-238; G-43 to H-238; R-44 to H-238; I-45 to H-238; E-46 to H-238; P-47 to H-238; R-48 to H-238; G-49 to H-238; G-50 to H-238; G-51 to H-238; R-52 to H-238; G-53 to H-238; A-54 to H-238; L-55 to H-238; P-56 to H-238; T-57 to H-238; S-58 to H-238; M-59 to H-238; G-60 to H-238; Q-61 to H-238; H-62 to H-238; G-63 to H-238; P-64 to H-238; S-65 to H-238; A-66 to H-238; R-67 to H-238; A-68 to H-238; R-69 to H-238; A-70 to H-238; G-71 to H-238; R-72 to H-238; A-73 to H-238; P-74 to H-238; G-75 to H-238; P-76 to H-238; R-77 to H-238; P-78 to H-238; A-79 to H-238; R-80 to H-238; E-81 to H-238; A-82 to H-238; S-83 to H-238; P-84 to H-238; R-85 to H-238; L-86 to H-238; R-87 to H-238; V-88 to H-238; H-89 to H-238; K-90 to H-238; T-91 to H-238; F-92 to H-238; K-93 to H-238; F-94 to H-238; V-95 to H-238; V-96 to H-238; V-97 to H-238; G-98 to H-238; V-99 to H-238; L-100 to H-238; L-101 to H-238; Q-102 to H-238; V-103 to H-238; V-104 to H-238;

P-105 to H-238; S-106 to H-238; S-107 to H-238; A-108 to H-238; A-109 to H-238; T-110 to H-238; I-111 to H-238; K-112 to H-238; L-113 to H-238; H-114 to H-238; D-115 to H-238; Q-116 to H-238; S-117 to H-238; I-118 to H-238; G-119 to H-238; T-120 to H-238; Q-121 to H-238; Q-122 to H-238; W-123 to H-238; E-124 to H-238; H-125 to H-238; S-126 to H-238; P-127 to H-238; L-128 to H-238; G-129 to H-238; E-130 to H-238; L-131 to H-238; C-132 to H-238; P-133 to H-238; P-134 to H-238; G-135 to H-238; S-136 to H-238; H-137 to H-238; R-138 to H-238; S-139 to H-238; E-140 to H-238; R-141 to H-238; P-142 to H-238; G-143 to H-238; A-144 to H-238; C-145 to H-238; N-146 to H-238; R-147 to H-238; C-148 to H-238; T-149 to H-238; E-150 to H-238; G-151 to H-238; V-152 to H-238; G-153 to H-238; Y-154 to H-238; T-155 to H-238; N-156 to H-238; A-157 to H-238; S-158 to H-238; N-159 to H-238; N-160 to H-238; L-161 to H-238; F-162 to H-238; A-163 to H-238; C-164 to H-238; L-165 to H-238; P-166 to H-238; C-167 to H-238; T-168 to H-238; A-169 to H-238; C-170 to H-238; K-171 to H-238; S-172 to H-238; D-173 to H-238; E-174 to H-238; E-175 to H-238; E-176 to H-238; R-177 to H-238; S-178 to H-238; P-179 to H-238; C-180 to H-238; T-181 to H-238; T-182 to H-238; T-183 to H-238; R-184 to H-238; N-185 to H-238; T-186 to H-238; A-187 to H-238; C-188 to H-238; Q-189 to H-238; C-190 to H-238; K-191 to H-238; P-192 to H-238; G-193 to H-238; T-194 to H-238; F-195 to H-238; R-196 to H-238; N-197 to H-238; D-198 to H-238; N-199 to H-238; S-200 to H-238; A-201 to H-238; E-202 to H-238; M-203 to H-238; C-204 to H-238; R-205 to H-238; K-206 to H-238; C-207 to H-238; S-208 to H-238; T-209 to H-238; G-210 to H-238; C-211 to H-238; P-212 to H-238; R-213 to H-238; G-214 to H-238; M-215 to H-238; V-216 to H-238; K-217 to H-238; V-218 to H-238; K-219 to H-238; D-220 to H-238; C-221 to H-238; T-222 to H-238; P-223 to H-238; W-224 to H-238; S-225 to H-238; D-226 to H-238; I-227 to H-238; E-228 to H-238; C-229 to H-238; V-230 to H-238; H-231 to H-238; K-232 to H-238; and E-233 to H-238 of the DR4 extracellular domain sequence shown in SEQ ID NO:2.

The present invention is also directed to nucleic acid molecules comprising, or alternatively consisting of, a polynucleotide sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the polynucleotide sequences encoding the polypeptides described above. The invention is further directed to nucleic acid molecules comprising, or alternatively consisting of, polynucleotide sequences which encode polypeptides that are at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the polypeptides described above. The present invention also encompasses the above polynucleotide sequences fused to a heterologous polynucleotide sequence. Polypeptides encoded by these polynucleotides are also encompassed by the invention.

Similarly, many examples of functional C-terminal deletion muteins are known. For instance, interferon gamma shows up to ten times higher activities by deleting 8-10 amino acid residues from the carboxy terminus of the protein (Döbeli *et al., J. Biotechnology* 7:199-216 (1988). In the present case, since the protein of the invention is a member of the DDCR polypeptide family, deletions of C-terminal amino acids up to the cysteine at position 221 (C-221) of SEQ ID NO:2 may retain some biological activity such as receptor binding. Polypeptides having further C-terminal deletions including C-221 of SEQ ID NO:2 would not be expected to retain such biological activities because this residue is conserved among DDCR family members and is required for forming a disulfide bridge to provide structural stability which is needed for receptor-ligand binding.

As mentioned above, even if deletion of one or more amino acids from the C-terminus of a protein results in modification of loss of one or more biological functions of the protein, other functional activities (*e.g.*, biological activities, ability to multimerize, ability to bind DR4 ligand (*e.g.*, TRAIL)) may still be retained. For example the ability of the shortened DR4 mutein to induce and/or bind to antibodies which recognize the complete or mature forms of the polypeptide generally will be retained when less than the majority of the residues of the complete or mature polypeptide are removed from the C-terminus. Whether a particular polypeptide lacking C-terminal residues of a complete

polypeptide retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art. It is not unlikely that a DR4 mutein with a large number of deleted C-terminal amino acid residues may retain some biological or immunogenic activities. In fact, peptides composed of as few as six DR4 amino acid residues may often evoke an immune response.

Accordingly, the present invention further provides polypeptides having one or more residues deleted from the carboxy terminus of the amino acid sequence of the DR4 polypeptide shown in SEQ ID NO:2, up to the alanine residue at position number 30, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues 24-m^1 of SEQ ID NO:2, where m^1 is an integer from 30 to 467 corresponding to the position of the amino acid residue in SEQ ID NO:2.

More in particular, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues A-24 to L-467; A-24 to S-466; A-24 to V-465; A-24 to A-464; A-24 to S-463; A-24 to G-462; A-24 to T-461; A-24 to G-460; A-24 to D-459; A-24 to E-458; A-24 to L-457; A-24 to Y-456; A-24 to I-455; A-24 to F-454; A-24 to K-453; A-24 to G-452; A-24 to S-451; A-24 to D-450; A-24 to V-449; A-24 to L-448; A-24 to L-447; A-24 to D-446; A-24 to Q-445; A-24 to I-444; A-24 to K-443; A-24 to E-442; A-24 to K-441; A-24 to A-440; A-24 to H-439; A-24 to R-438; A-24 to E-437; A-24 to E-436; A-24 to M-435; A-24 to R-434; A-24 to E-433; A-24 to L-432; A-24 to A-431; A-24 to D-430; A-24 to L-429; A-24 to L-428; A-24 to T-427; A-24 to H-426; A-24 to I-425; A-24 to S-424; A-24 to A-423; A-24 to N-422; A-24 to R-421; A-24 to G-420; A-24 to T-419; A-24 to K-418; A-24 to N-417; A-24 to V-416; A-24 to W-415; A-24 to K-414; A-24 to M-413; A-24 to L-412; A-24 to M-411; A-24 to A-410; A-24 to Y-409; A-24 to L-408; A-24 to A-407; A-24 to D-406; A-24 to G-405; A-24 to P-404; A-24 to G-403; A-24 to A-402; A-24 to T-401; A-24 to G-400; A-24 to A-399; A-24 to R-

398; A-24 to V-397; A-24 to V-396; A-24 to D-395; A-24 to I-394; A-24 to E-393; A-24 to N-392; A-24 to K-391; A-24 to T-390; A-24 to L-389; A-24 to D-388; A-24 to L-387; A-24 to Q-386; A-24 to R-385; A-24 to M-384; A-24 to L-383; A-24 to Q-382; A-24 to D-381; A-24 to W-380; A-24 to S-379; A-24 to D-378; A-24 to F-377; A-24 to P-376; A-24 to V-375; A-24 to I-374; A-24 to N-373; A-24 to A-372; A-24 to F-371; A-24 to K-370; A-24 to D-369; A-24 to F-368; A-24 to F-367; A-24 to L-366; A-24 to M-365; A-24 to L-364; A-24 to T-363; A-24 to E-362; A-24 to T-361; A-24 to P-360; A-24 to D-359; A-24 to A-358; A-24 to G-357; A-24 to N-356; A-24 to A-355; A-24 to P-354; A-24 to V-353; A-24 to L-352; A-24 to L-351; A-24 to R-350; A-24 to R-349; A-24 to R-348; A-24 to Q-347; A-24 to S-346; A-24 to G-345; A-24 to E-344; A-24 to A-343; A-24 to E-342; A-24 to A-341; A-24 to P-340; A-24 to G-339; A-24 to L-338; A-24 to L-337; A-24 to C-336; A-24 to Q-335; A-24 to A-334; A-24 to E-333; A-24 to G-332; A-24 to P-331; A-24 to S-330; A-24 to Q-329; A-24 to V-328; A-24 to T-327; A-24 to V-326; A-24 to G-325; A-24 to T-324; A-24 to L-323; A-24 to D-322; A-24 to A-321; A-24 to P-320; A-24 to E-319; A-24 to Q-318; A-24 to S-317; A-24 to E-316; A-24 to M-315; A-24 to Q-314; A-24 to Q-313; A-24 to E-312; A-24 to S-311; A-24 to V-310; A-24 to F-309; A-24 to T-308; A-24 to S-307; A-24 to L-306; A-24 to S-305; A-24 to D-304; A-24 to A-303; A-24 to N-302; A-24 to S-301; A-24 to L-300; A-24 to I-299; A-24 to E-298; A-24 to N-297; A-24 to H-296; A-24 to A-295; A-24 to N-294; A-24 to D-293; A-24 to E-292; A-24 to A-291; A-24 to G-290; A-24 to P-289; A-24 to G-288; A-24 to R-287; A-24 to L-286; A-24 to L-285; A-24 to G-284; A-24 to L-283; A-24 to R-282; A-24 to W-281; A-24 to F-280; A-24 to C-279; A-24 to V-278; A-24 to R-277; A-24 to D-276; A-24 to M-275; A-24 to C-274; A-24 to K-273; A-24 to P-272; A-24 to D-271; A-24 to G-270; A-24 to G-269; A-24 to C-268; A-24 to G-267; A-24 to S-266; A-24 to G-265; A-24 to I-264; A-24 to C-263; A-24 to C-262; A-24 to C-261; A-24 to V-260; A-24 to I-259; A-24 to L-258; A-24 to V-257; A-24 to A-256; A-24 to V-255; A-24 to L-254; A-24 to L-253; A-24 to L-252; A-24 to P-251; A-24 to V-250; A-24 to V-249; A-24 to L-

248; A-24 to T-247; A-24 to V-246; A-24 to V-245; A-24 to L-244; A-24 to I-243; A-24 to V-242; A-24 to W-241; A-24 to I-240; A-24 to N-239; A-24 to H-238; A-24 to G-237; A-24 to N-236; A-24 to G-235; A-24 to S-234; A-24 to E-233; A-24 to K-232; A-24 to H-231; A-24 to V-230; A-24 to C-229; A-24 to E-228; A-24 to I-227; A-24 to D-226; A-24 to S-225; A-24 to W-224; A-24 to P-223; A-24 to T-222; A-24 to C-221; A-24 to D-220; A-24 to K-219; A-24 to V-218; A-24 to K-217; A-24 to V-216; A-24 to M-215; A-24 to G-214; A-24 to R-213; A-24 to P-212; A-24 to C-211; A-24 to G-210; A-24 to T-209; A-24 to S-208; A-24 to C-207; A-24 to K-206; A-24 to R-205; A-24 to C-204; A-24 to M-203; A-24 to E-202; A-24 to A-201; A-24 to S-200; A-24 to N-199; A-24 to D-198; A-24 to N-197; A-24 to R-196; A-24 to F-195; A-24 to T-194; A-24 to G-193; A-24 to P-192; A-24 to K-191; A-24 to C-190; A-24 to Q-189; A-24 to C-188; A-24 to A-187; A-24 to T-186; A-24 to N-185; A-24 to R-184; A-24 to T-183; A-24 to T-182; A-24 to T-181; A-24 to C-180; A-24 to P-179; A-24 to S-178; A-24 to R-177; A-24 to E-176; A-24 to E-175; A-24 to E-174; A-24 to D-173; A-24 to S-172; A-24 to K-171; A-24 to C-170; A-24 to A-169; A-24 to T-168; A-24 to C-167; A-24 to P-166; A-24 to L-165; A-24 to C-164; A-24 to A-163; A-24 to F-162; A-24 to L-161; A-24 to N-160; A-24 to N-159; A-24 to S-158; A-24 to A-157; A-24 to N-156; A-24 to T-155; A-24 to Y-154; A-24 to G-153; A-24 to V-152; A-24 to G-151; A-24 to E-150; A-24 to T-149; A-24 to C-148; A-24 to R-147; A-24 to N-146; A-24 to C-145; A-24 to A-144; A-24 to G-143; A-24 to P-142; A-24 to R-141; A-24 to E-140; A-24 to S-139; A-24 to R-138; A-24 to H-137; A-24 to S-136; A-24 to G-135; A-24 to P-134; A-24 to P-133; A-24 to C-132; A-24 to L-131; A-24 to E-130; A-24 to G-129; A-24 to L-128; A-24 to P-127; A-24 to S-126; A-24 to H-125; A-24 to E-124; A-24 to W-123; A-24 to Q-122; A-24 to Q-121; A-24 to T-120; A-24 to G-119; A-24 to I-118; A-24 to S-117; A-24 to Q-116; A-24 to D-115; A-24 to H-114; A-24 to L-113; A-24 to K-112; A-24 to I-111; A-24 to T-110; A-24 to A-109; A-24 to A-108; A-24 to S-107; A-24 to S-106; A-24 to P-105; A-24 to V-104; A-24 to V-103; A-24 to Q-102; A-24 to L-101; A-24 to L-100; A-24 to V-99; A-24 to G-

98; A-24 to V-97; A-24 to V-96; A-24 to V-95; A-24 to F-94; A-24 to K-93; A-24 to F-92; A-24 to T-91; A-24 to K-90; A-24 to H-89; A-24 to V-88; A-24 to R-87; A-24 to L-86; A-24 to R-85; A-24 to P-84; A-24 to S-83; A-24 to A-82; A-24 to E-81; A-24 to R-80; A-24 to A-79; A-24 to P-78; A-24 to R-77; A-24 to P-76; A-24 to G-75; A-24 to P-74; A-24 to A-73; A-24 to R-72; A-24 to G-71; A-24 to A-70; A-24 to R-69; A-24 to A-68; A-24 to R-67; A-24 to A-66; A-24 to S-65; A-24 to P-64; A-24 to G-63; A-24 to H-62; A-24 to Q-61; A-24 to G-60; A-24 to M-59; A-24 to S-58; A-24 to T-57; A-24 to P-56; A-24 to L-55; A-24 to A-54; A-24 to G-53; A-24 to R-52; A-24 to G-51; A-24 to G-50; A-24 to G-49; A-24 to R-48; A-24 to P-47; A-24 to E-46; A-24 to I-45; A-24 to R-44; A-24 to G-43; A-24 to A-42; A-24 to S-41; A-24 to S-40; A-24 to G-39; A-24 to W-38; A-24 to V-37; A-24 to K-36; A-24 to S-35; A-24 to P-34; A-24 to T-33; A-24 to A-32; A-24 to A-31; and A-24 to A-30 of the DR4 sequence shown in SEQ ID NO:2.

The present invention is also directed to nucleic acid molecules comprising, or alternatively consisting of, a polynucleotide sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the polynucleotide sequences encoding the polypeptides described above. The invention is further directed to nucleic acid molecules comprising, or alternatively consisting of, polynucleotide sequences which encode polypeptides that are at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the polypeptides described above. The present invention also encompasses the above polynucleotide sequences fused to a heterologous polynucleotide sequence. Polypeptides encoded by these polynucleotides are also encompassed by the invention.

In another embodiment, C-terminal deletions of the DR4 polypeptide can be described by the general formula 24-m^2 where m^2 is a number from 30 to 238 corresponding to the amino acid sequence identified in SEQ ID NO:2. In specific embodiments, the invention provides polynucleotides encoding polypeptides comprising, or alternatively consisting of, the amino acid sequence of residues: A-24 to G-237; A-24 to N-236; A-24 to G-235; A-24 to S-234; A-24 to E-233; A-

24 to K-232; A-24 to H-231; A-24 to V-230; A-24 to C-229; A-24 to E-228; A-24 to I-227; A-24 to D-226; A-24 to S-225; A-24 to W-224; A-24 to P-223; A-24 to T-222; A-24 to C-221; A-24 to D-220; A-24 to K-219; A-24 to V-218; A-24 to K-217; A-24 to V-216; A-24 to M-215; A-24 to G-214; A-24 to R-213; A-24 to P-212; A-24 to C-211; A-24 to G-210; A-24 to T-209; A-24 to S-208; A-24 to C-207; A-24 to K-206; A-24 to R-205; A-24 to C-204; A-24 to M-203; A-24 to E-202; A-24 to A-201; A-24 to S-200; A-24 to N-199; A-24 to D-198; A-24 to N-197; A-24 to R-196; A-24 to F-195; A-24 to T-194; A-24 to G-193; A-24 to P-192; A-24 to K-191; A-24 to C-190; A-24 to Q-189; A-24 to C-188; A-24 to A-187; A-24 to T-186; A-24 to N-185; A-24 to R-184; A-24 to T-183; A-24 to T-182; A-24 to T-181; A-24 to C-180; A-24 to P-179; A-24 to S-178; A-24 to R-177; A-24 to E-176; A-24 to E-175; A-24 to E-174; A-24 to D-173; A-24 to S-172; A-24 to K-171; A-24 to C-170; A-24 to A-169; A-24 to T-168; A-24 to C-167; A-24 to P-166; A-24 to L-165; A-24 to C-164; A-24 to A-163; A-24 to F-162; A-24 to L-161; A-24 to N-160; A-24 to N-159; A-24 to S-158; A-24 to A-157; A-24 to N-156; A-24 to T-155; A-24 to Y-154; A-24 to G-153; A-24 to V-152; A-24 to G-151; A-24 to E-150; A-24 to T-149; A-24 to C-148; A-24 to R-147; A-24 to N-146; A-24 to C-145; A-24 to A-144; A-24 to G-143; A-24 to P-142; A-24 to R-141; A-24 to E-140; A-24 to S-139; A-24 to R-138; A-24 to H-137; A-24 to S-136; A-24 to G-135; A-24 to P-134; A-24 to P-133; A-24 to C-132; A-24 to L-131; A-24 to E-130; A-24 to G-129; A-24 to L-128; A-24 to P-127; A-24 to S-126; A-24 to H-125; A-24 to E-124; A-24 to W-123; A-24 to Q-122; A-24 to Q-121; A-24 to T-120; A-24 to G-119; A-24 to I-118; A-24 to S-117; A-24 to Q-116; A-24 to D-115; A-24 to H-114; A-24 to L-113; A-24 to K-112; A-24 to I-111; A-24 to T-110; A-24 to A-109; A-24 to A-108; A-24 to S-107; A-24 to S-106; A-24 to P-105; A-24 to V-104; A-24 to V-103; A-24 to Q-102; A-24 to L-101; A-24 to L-100; A-24 to V-99; A-24 to G-98; A-24 to V-97; A-24 to V-96; A-24 to V-95; A-24 to F-94; A-24 to K-93; A-24 to F-92; A-24 to T-91; A-24 to K-90; A-24 to H-89; A-24 to V-88; A-24 to R-87; A-24 to L-86; A-24 to R-85; A-24 to P-84; A-24 to S-83; A-24 to A-82; A-24 to E-81;

A-24 to R-80; A-24 to A-79; A-24 to P-78; A-24 to R-77; A-24 to P-76; A-24 to G-75; A-24 to P-74; A-24 to A-73; A-24 to R-72; A-24 to G-71; A-24 to A-70; A-24 to R-69; A-24 to A-68; A-24 to R-67; A-24 to A-66; A-24 to S-65; A-24 to P-64; A-24 to G-63; A-24 to H-62; A-24 to Q-61; A-24 to G-60; A-24 to M-59; A-24 to S-58; A-24 to T-57; A-24 to P-56; A-24 to L-55; A-24 to A-54; A-24 to G-53; A-24 to R-52; A-24 to G-51; A-24 to G-50; A-24 to G-49; A-24 to R-48; A-24 to P-47; A-24 to E-46; A-24 to I-45; A-24 to R-44; A-24 to G-43; A-24 to A-42; A-24 to S-41; A-24 to S-40; A-24 to G-39; A-24 to W-38; A-24 to V-37; A-24 to K-36; A-24 to S-35; A-24 to P-34; A-24 to T-33; A-24 to A-32; A-24 to A-31; and A-24 to A-30 of the DR4 extracellular domain sequence shown in SEQ ID NO:2.

The present invention is also directed to nucleic acid molecules comprising, or alternatively consisting of, a polynucleotide sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the polynucleotide sequences encoding the polypeptides described above. The invention is further directed to nucleic acid molecules comprising, or alternatively consisting of, polynucleotide sequences which encode polypeptides that are at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the polypeptides described above. The present invention also encompasses the above polynucleotide sequences fused to a heterologous polynucleotide sequence. Polypeptides encoded by these polynucleotides are also encompassed by the invention.

The present invention further provides polypeptides having one or more residues from the carboxy terminus of the amino acid sequence of the DR4 shown in SEQ ID NO:2, up to C-221 of SEQ ID NO:2, and polynucleotides encoding such polypeptides. In particular, the present invention provides polypeptides having the amino acid sequence of residues 1-m^2 of the amino acid sequence in SEQ ID NO:2, where m^2 is any integer in the range of 221-468 and residue C-221 is the position of the first residue from the C-terminus of the complete DR4 polypeptide (shown in SEQ ID NO:2) believed to be required for receptor binding

activity of the DR4 protein. Polynucleotides encoding these polypeptides also are provided.

The invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini of an DR4 polypeptide, which may be described generally as having residues n^1- m^1 and/or n^2- m^2 of SEQ ID NO:2, where n^1, n^2, m^1, and m^2 are integers as described above.

Also included are a nucleotide sequence encoding a polypeptide consisting of a portion of the complete DR4 amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 97853, where this portion excludes from 1 to about 108 amino acids from the amino terminus of the complete amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 97853, or from 1 to about 247 amino acids from the carboxy terminus, or any combination of the above amino terminal and carboxy terminal deletions, of the complete amino acid sequence encoded by the cDNA contained in ATCC Deposit No. 97853. Polynucleotides encoding all of the above deletion mutant polypeptide forms also are provided.

Preferred amongst the N- and C-terminal deletion mutants are those comprising only a portion of the extracellular domain; *i.e.*, within residues 24-238, since any portion therein is expected to be soluble.

It will be recognized in the art that some amino acid sequence of DR4 can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity. Such areas will usually comprise residues which make up the ligand binding site or the death domain, or which form tertiary structures which affect these domains.

Thus, the invention further includes variations of the DR4 protein which show substantial DR4 protein activity or which include regions of DR4 such as the protein fragments discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above,

guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U. *et al., Science 247:*1306-1310 (1990).

Thus, the fragment, derivative, or analog of the polypeptide of SEQ ID NO:2, or that encoded by the deposited cDNA, may be (i) one in which at least one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue(s), and more preferably at least one but less than ten conserved amino acid residues) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein. Polynucleotides encoding these fragments, derivatives or analogs are also encompassed by the invention.

Of particular interest are substitutions of charged amino acids with another charged amino acid and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the DR4 protein. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al., Clin Exp. Immunol. 2*:331-340 (1967); Robbins *et al., Diabetes 36*:838-845 (1987); Cleland *et al. Crit. Rev. Therapeutic Drug Carrier Systems 10*:307-377 (1993)).

The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Ostade *et al., Nature 361*:266-268 (1993) describes certain mutations resulting in selective binding of TNF-alpha to only one of the

two known types of TNF receptors. Thus, the DR4 receptor of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table II).

TABLE II. Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

In specific embodiments, the number of substitutions, additions or deletions in the amino acid sequence of SEQ ID NO:2 and/or any of the polypeptide fragments described herein (*e.g.*, the extracellular domain or intracellular domain) is 75, 70, 60, 50, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 or 30-20, 20-15, 20-10, 15-10, 10-1, 5-10, 1-5, 1-3 or 1-2.

Amino acids in the DR4 protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science*

244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro*, or *in vitro* proliferative activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al., J. Mol. Biol. 224*:899-904 (1992) and de Vos *et al. Science 255*:306-312 (1992)).

Additionally, protein engineering may be employed to improve or alter the characteristics of DR4 polypeptides. Recombinant DNA technology known to those skilled in the art can be used to create novel mutant proteins or muteins including single or multiple amino acid substitutions, deletions, additions or fusion proteins. Such modified polypeptides can show, *e.g.*, enhanced activity or increased stability. In addition, they may be purified in higher yields and show better solubility than the corresponding natural polypeptide, at least under certain purification and storage conditions.

Non-naturally occurring variants may be produced using art-known mutagenesis techniques, which include, but are not limited to oligonucleotide mediated mutagenesis, alanine scanning, PCR mutagenesis, site directed mutagenesis (see *e.g., Carter et al., Nucl. Acids Res. 13*:4331 (1986); and Zoller *et al., Nucl. Acids Res. 10*:6487 (1982)), cassette mutagenesis (see *e.g.*, Wells *et al., Gene 34*:315 (1985)), restriction selection mutagenesis (see *e.g.*, Wells *et al., Philos. Trans. R. Soc. London SerA 317*:415 (1986)).

Thus, the invention also encompasses DR4 derivatives and analogs that have one or more amino acid residues deleted, added, or substituted to generate DR4 polypeptides that are better suited for expression, scale up, etc., in the host cells chosen. For example, cysteine residues can be deleted or substituted with another amino acid residue in order to eliminate disulfide bridges; N-linked glycosylation sites can be altered or eliminated to achieve, for example, expression of a homogeneous product that is more easily recovered and purified from yeast hosts which are known to hyperglycosylate N-linked sites. To this end, a variety

of amino acid substitutions at one or both of the first or third amino acid positions on any one or more of the glycosylation recognitions sequences in the DR4 polypeptides of the invention, and/or an amino acid deletion at the second position of any one or more such recognition sequences will prevent glycosylation of the DR4 at the modified tripeptide sequence (see, *e.g.*, Miyajimo *et al.*, EMBO J 5(6):1193-1197).

The polypeptides of the present invention also include a polypeptide comprising, or alternatively consisting of, the polypeptide encoded by the deposited cDNA (the deposit having ATCC Accession No. 97853) including the leader; a polypeptide comprising, or alternatively consisting of, the mature polypeptide encoded by the deposited the cDNA minus the leader (*i.e.*, the mature protein); a polypeptide comprising, or alternatively consisting of, the polypeptide of SEQ ID NO:2 including the leader; a polypeptide comprising, or alternatively consisting of, the polypeptide of SEQ ID NO:2 minus the amino terminal methionine; a polypeptide comprising, or alternatively consisting of, the polypeptide of SEQ ID NO:2 minus the leader; a polypeptide comprising, or alternatively consisting of, the DR4 extracellular domain; a polypeptide comprising, or alternatively consisting of, the DR4 cysteine rich domain; a polypeptide comprising, or alternatively consisting of, the DR4 transmembrane domain; a polypeptide comprising, or alternatively consisting of, the DR4 intracellular domain; a polypeptide comprising, or alternatively consisting of, the DR4 death domain; a polypeptide comprising, or alternatively consisting of, soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain; as well as polypeptides which are at least 80% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to the polypeptides described above (*e.g.*, the polypeptide encoded by the deposited cDNA, the polypeptide of SEQ ID NO:2, and portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids. Polynucleotides encoding these polypeptides are also encompassed by the invention.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a DR4 polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the DR4 polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2 or to the amino acid sequence encoded by the deposited cDNA can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

In a specific embodiment, the identity between a reference (query) sequence (a sequence of the present invention) and a subject sequence, also

referred to as a global sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag *et al.* (Comp. App. Biosci. 6:237-245 (1990)). Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. A determination of whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of this embodiment. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence. For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and

C-termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are made for the purposes of this embodiment.

The present application is also directed to proteins containing polypeptides at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to the DR4 polypeptide sequence set forth herein as n^1-m^1, and/or n^2-m^2. In preferred embodiments, the application is directed to proteins containing polypeptides at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to polypeptides having the amino acid sequence of the specific DR4 N- and C-terminal deletions recited herein. Polynucleotides encoding these polypeptides are also encompassed by the invention.

In certain preferred embodiments, DR4 proteins of the invention comprise fusion proteins as described above wherein the DR4 polypeptides are those described as n^1-m^1, and/or n^2-m^2 herein. In preferred embodiments, the application is directed to nucleic acid molecules at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequences encoding polypeptides having the amino acid sequence of the specific N- and C-terminal deletions recited herein. Polynucleotides encoding these polypeptides are also encompassed by the invention.

The present inventors have discovered that the DR4 polypeptide is a 468 residue protein exhibiting three main structural domains. First, the ligand binding

domain (extracellular domain) was identified within residues from about 24 to about 238 in SEQ ID NO:2. Second, the transmembrane domain was identified within residues from about 239 to about 264 in SEQ ID NO:2. Third, the intracellular domain was identified within residues from about 265 to about 468 in SEQ ID NO:2. Importantly, the intracellular domain includes a death domain at residues from about 379 to about 422 in SEQ ID NO:2. Further preferred fragments of the polypeptide shown in SEQ ID NO:2 include the mature protein from residues about 24 to about 468 and soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain. In this context "about" includes the particularly recited range, larger or smaller by several (5, 4, 3, 2, or 1) amino acid residues, at either terminus or at both termini.

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide described herein. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen *et al., Proc. Natl. Acad. Sci. USA 81*:3998-4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (*i.e.*, that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R.A. (1983) Antibodies that react with predetermined sites on proteins. *Science 219*:660-666. Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (*i.e.*, immunogenic epitopes) nor to the amino or carboxyl terminals.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate DR4-specific antibodies include: a polypeptide comprising, or alternatively consisting of, amino acid residues from about 35 to about 92 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 114 to about 160 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 169 to about 240 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 267 to about 298 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 330 to about 364 in SEQ ID NO:2; a polypeptide comprising, or alternatively consisting of, amino acid residues from about 391 to about 404 in SEQ ID NO:2; and a polypeptide comprising, or alternatively consisting of, amino acid residues from about 418 to about 465 in SEQ ID NO:2. In this context "about" includes the particularly recited range, larger or smaller by several (5, 4, 3, 2, or 1) amino acids, at either terminus or at both termini. As indicated above, the inventors have determined that the above polypeptide fragments are antigenic regions of the DR4 protein. Polynucleotides encoding these polypeptides are also encompassed by the invention.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means. Houghten, R.A., "General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids," *Proc. Natl. Acad. Sci. USA 82*:5131-5135 (1985). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986). As one of skill in the art will appreciate, DR4 polypeptides of the present invention and the epitope-bearing fragments thereof described herein (*e.g.*, corresponding to a portion of the extracellular domain such as, for example, amino acid residues 1 to 240 of SEQ ID NO:2) can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased

half-life *in vivo*. This has been shown, *e.g.*, for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker *et al.*, *Nature 331*:84- 86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric DR4 protein or protein fragment alone (Fountoulakis *et al.*, *J Biochem 270*:3958-3964 (1995)).

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

In addition, proteins of the invention can be chemically synthesized using techniques known in the art (*e.g.*, see Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y., and Hunkapiller, M. *et al.*, *Nature 310*:105-111 (1984)). For example, a peptide corresponding to a fragment of the DR4 polypeptides of the invention can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the DR4 polypeptide sequence. Non-classical amino acids include, but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid, alpha-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, alpha-Abu, alpha-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, alpha-alanine, fluoro-amino acids, designer

amino acids such as alpha-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

Non-naturally occurring variants may be produced using art-known mutagenesis techniques, which include, but are not limited to oligonucleotide mediated mutagenesis, alanine scanning, PCR mutagenesis, site directed mutagenesis (*see, e.g.*, Carter *et al., Nucl. Acids Res. 13*:4331 (1986); and Zoller *et al., Nucl. Acids Res. 10*:6487 (1982)), cassette mutagenesis (*see, e.g.*, Wells *et al., Gene 34*:315 (1985)), restriction selection mutagenesis (*see, e.g.*, Wells *et al., Philos. Trans. R. Soc. London SerA 317*:415 (1986)).

The invention additionally, encompasses DR4 polypeptides which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to, specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin; etc.

Additional post-translational modifications encompassed by the invention include, for example, *e.g.*, N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of procaryotic host cell expression. The polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

Also provided by the invention are chemically modified derivatives of DR4 which may provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (see U. S. Patent

No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the preferred molecular weight is between about 1 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (*e.g.*, the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog). For example, the polyethylene glycol may have an average molecular weight of about 200, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10,000, 10,500, 11,000, 11,500, 12,000, 12,500, 13,000, 13,500, 14,000, 14,500, 15,000, 15,500, 16,000, 16,500, 17,000, 17,500, 18,000, 18,500, 19,000, 19,500, 20,000, 25,000, 30,000, 35,000, 40,000, 50,000, 55,000, 60,000, 65,000, 70,000, 75,000, 80,000, 85,000, 90,000, 95,000, or 100,000 kDa.

As noted above, the polyethylene glycol may have a branched structure. Branched polyethylene glycols are described, for example, in U.S. Patent No. 5,643,575; Morpurgo *et al., Appl. Biochem. Biotechnol. 56*:59-72 (1996); Vorobjev *et al., Nucleosides Nucleotides 18*:2745-2750 (1999); and Caliceti *et al., Bioconjug. Chem. 10*:638-646 (1999), the disclosures of each of which are incorporated herein by reference.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the protein with consideration of effects on functional or antigenic

domains of the protein. There are a number of attachment methods available to those skilled in the art, *e.g.*, EP 0 401 384, herein incorporated by reference (coupling PEG to G-CSF), see also Malik *et al.*, *Exp. Hematol. 20*:1028-1035 (1992) (reporting pegylation of GM-CSF using tresyl chloride). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues, glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group.

As suggested above, polyethylene glycol may be attached to proteins via linkage to any of a number of amino acid residues. For example, polyethylene glycol can be linked to a proteins via covalent bonds to lysine, histidine, aspartic acid, glutamic acid, or cysteine residues. One or more reaction chemistries may be employed to attach polyethylene glycol to specific amino acid residues (*e.g.*, lysine, histidine, aspartic acid, glutamic acid, or cysteine) of the protein or to more than one type of amino acid residue (*e.g.*, lysine, histidine, aspartic acid, glutamic acid, cysteine and combinations thereof) of the protein.

One may specifically desire proteins chemically modified at the N-terminus. Using polyethylene glycol as an illustration of the present composition, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (or peptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (*i.e.*, separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of

pegylated protein molecules. Selective proteins chemically modified at the N-terminus modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved.

As indicated above, pegylation of the proteins of the invention may be accomplished by any number of means. For example, polyethylene glycol may be attached to the protein either directly or by an intervening linker. Linkerless systems for attaching polyethylene glycol to proteins are described in Delgado *et al., Crit. Rev. Thera. Drug Carrier Sys. 9*:249-304 (1992); Francis *et al., Intern. J. of Hematol. 68*:1-18 (1998); U.S. Patent No. 4,002,531; U.S. Patent No. 5,349,052; WO 95/06058; and WO 98/32466, the disclosures of each of which are incorporated herein by reference.

One system for attaching polyethylene glycol directly to amino acid residues of proteins without an intervening linker employs tresylated MPEG, which is produced by the modification of monmethoxy polyethylene glycol (MPEG) using tresylchloride ($ClSO_2CH_2CF_3$). Upon reaction of protein with tresylated MPEG, polyethylene glycol is directly attached to amine groups of the protein. Thus, the invention includes protein-polyethylene glycol conjugates produced by reacting proteins of the invention with a polyethylene glycol molecule having a 2,2,2-trifluoreothane sulphonyl group.

Polyethylene glycol can also be attached to proteins using a number of different intervening linkers. For example, U.S. Patent No. 5,612,460, the entire disclosure of which is incorporated herein by reference, discloses urethane linkers for connecting polyethylene glycol to proteins. Protein-polyethylene glycol conjugates wherein the polyethylene glycol is attached to the protein by a linker can also be produced by reaction of proteins with compounds such as MPEG-succinimidylsuccinate, MPEG activated with 1,1'-carbonyldiimidazole, MPEG-2,4,5-trichloropenylcarbonate, MPEG-p-nitrophenolcarbonate, and various

MPEG-succinate derivatives. A number additional polyethylene glycol derivatives and reaction chemistries for attaching polyethylene glycol to proteins are described in WO 98/32466, the entire disclosure of which is incorporated herein by reference. Pegylated protein products produced using the reaction chemistries set out herein are included within the scope of the invention.

The number of polyethylene glycol moieties attached to each protein of the invention (*i.e.*, the degree of substitution) may also vary. For example, the pegylated proteins of the invention may be linked, on average, to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 17, 20, or more polyethylene glycol molecules. Similarly, the average degree of substitution within ranges such as 1-3, 2-4, 3-5, 4-6, 5-7, 6-8, 7-9, 8-10, 9-11, 10-12, 11-13, 12-14, 13-15, 14-16, 15-17, 16-18, 17-19, or 18-20 polyethylene glycol moieties per protein molecule. Methods for determining the degree of substitution are discussed, for example, in Delgado *et al., Crit. Rev. Thera. Drug Carrier Sys. 9*:249-304 (1992).

Antibodies

The present invention further relates to antibodies and T-cell antigen receptors (TCR) which immunospecifically bind a polypeptide, preferably an epitope, of the present invention (as determined by immunoassays well known in the art for assaying specific antibody-antigen binding). Antibodies of the invention include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that immunospecifically binds an antigen. The immunoglobulin molecules of the invention can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*,

IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

Most preferably the antibodies are human antigen-binding antibody fragments of the present invention and include, but are not limited to, Fab, Fab' and F(ab')2, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V_L or V_H domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, and CH3 domains. The antibodies of the invention may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, donkey, ship rabbit, goat, guinea pig, camel, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulin and that do not express endogenous immunoglobulins, as described *infra* and, for example in, U.S. Patent No. 5,939,598 by Kucherlapati *et al.*

The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of a polypeptide of the present invention or may be specific for both a polypeptide of the present invention as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material. *See, e.g.*, PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt *et al.*, J. Immunol. 147:60-69 (1991); U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny *et al.*, J. Immunol. 148:1547-1553 (1992).

Antibodies of the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide of the present invention that they

recognize or specifically bind. The epitope(s) or polypeptide portion(s) may be specified as described herein, *e.g.*, by N-terminal and C-terminal positions, by size in contiguous amino acid residues, or listed in the Tables and Figures. Antibodies that specifically bind any epitope or polypeptide of the present invention may also be excluded. Therefore, the present invention includes antibodies that specifically bind polypeptides of the present invention, and allows for the exclusion of the same.

Antibodies of the present invention may also be described or specified in terms of their cross-reactivity. Antibodies that do not bind any other analog, ortholog, or homolog of a polypeptide of the present invention are included. Antibodies that bind polypeptides with at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, and at least 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. Antibodies that do not bind polypeptides with less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, and less than 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. Further included in the present invention are antibodies that bind polypeptides encoded by polynucleotides which hybridize to a polynucleotide of the present invention under stringent hybridization conditions (as described herein). Antibodies of the present invention may also be described or specified in terms of their binding affinity to a polypeptide of the invention. Preferred binding affinities include those with a dissociation constant or Kd less than $5X10^{-2}M$, $10^{-2}M$, $5X10^{-3}M$, $10^{-3}M$, $5X10^{-4}M$, $10^{-4}M$, $5X10^{-5}M$, $10^{-5}M$, $5X10^{-6}M$, $10^{-6}M$, $5X10^{-7}M$, $10^{-7}M$, $5X10^{-8}M$, $10^{-8}M$, $5X10^{-9}M$, $10^{-9}M$, $5X10^{-10}M$, $10^{-10}M$, $5X10^{-11}M$, $10^{-11}M$, $5X10^{-12}M$, $10^{-12}M$, $5X10^{-13}M$, $10^{-13}M$, $5X10^{-14}M$, $10^{-14}M$, $5X10^{-15}M$, and $10^{-15}M$.

The invention also provides antibodies that competitively inhibit binding of an antibody to an epitope of the invention as determined by any method known

in the art for determining competitive binding, for example, the immunoassays described herein. In preferred embodiments, the antibody competitively inhibits binding to the epitope by at least 90%, at least 80%, at least 70%, at least 60%, or at least 50%.

Antibodies of the present invention may act as agonists or antagonists of the polypeptides of the present invention. For example, the present invention includes antibodies which disrupt the receptor/ligand interactions with the polypeptides of the invention either partially or fully. The invention features both receptor-specific antibodies and ligand-specific antibodies. The invention also features receptor-specific antibodies which do not prevent ligand binding but prevent receptor activation. Receptor activation (*i.e.*, signaling) may be determined by techniques described herein or otherwise known in the art. For example, receptor activation can be determined by detecting the phosphorylation (*e.g.*, tyrosine or serine/threonine) of the receptor or its substrate by immunoprecipitation followed by western blot analysis (for example, as described *supra*). In specific embodiments, antibodies are provided that inhibit ligand or receptor activity by at least 90%, at least 80%, at least 70%, at least 60%, or at least 50% of the activity in absence of the antibody.

The invention also features receptor-specific antibodies which both prevent ligand binding and receptor activation as well as antibodies that recognize the receptor-ligand complex, and, preferably, do not specifically recognize the unbound receptor or the unbound ligand. Likewise, included in the invention are neutralizing antibodies which bind the ligand and prevent binding of the ligand to the receptor, as well as antibodies which bind the ligand, thereby preventing receptor activation, but do not prevent the ligand from binding the receptor. Further included in the invention are antibodies which activate the receptor. These antibodies may act as receptor agonists, *i.e.*, potentiate or activate either all or a subset of the biological activities of the ligand-mediated receptor activation. The antibodies may be specified as agonists, antagonists or inverse agonists for biological activities comprising the specific biological activities of the

peptides of the invention disclosed herein. Thus, the invention further relates to antibodies which act as agonists or antagonists of the polypeptides of the present invention. The above antibody agonists can be made using methods known in the art. *See, e.g.*, PCT publication WO 96/40281; U.S. Patent No. 5,811,097; Deng *et al.*, *Blood 92(6)*:1981-1988 (1998); Chen *et al.*, *Cancer Res. 58(16)*:3668-3678 (1998); Harrop *et al.*, *J. Immunol. 161(4)*:1786-1794 (1998); Zhu *et al.*, *Cancer Res. 58(15)*:3209-3214 (1998); Yoon *et al.*, *J. Immunol. 160(7)*:3170-3179 (1998); Prat *et al.*, *J. Cell. Sci. 111(Pt2)*:237-247 (1998); Pitard *et al.*, *J. Immunol. Methods 205(2)*:177-190 (1997); Liautard *et al.*, *Cytokine 9(4)*:233-241 (1997); Carlson *et al.*, *J. Biol. Chem. 272(17)*:11295-11301 (1997); Taryman *et al.*, *Neuron 14(4)*:755-762 (1995); Muller *et al.*, *Structure 6(9)*:1153-1167 (1998); Bartunek *et al.*, *Cytokine 8(1)*:14-20 (1996) (which are all incorporated by reference herein in their entireties).

Antibodies of the present invention may be used, for example, but not limited to, to purify, detect, and target the polypeptides of the present invention, including both *in vitro* and *in vivo* diagnostic and therapeutic methods. For example, the antibodies have use in immunoassays for qualitatively and quantitatively measuring levels of the polypeptides of the present invention in biological samples. *See, e.g.*, Harlow *et al.*, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988) (incorporated by reference herein in its entirety).

As discussed in more detail below, the antibodies of the present invention may be used either alone or in combination with other compositions. The antibodies may further be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to polypeptides or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as heterologous polypeptides, drugs, or toxins. *See, e.g.*, PCT publications

WO 92/08495; WO 91/14438; WO 89/12624; U.S. Patent No. 5,314,995; and EP 396,387.

The antibodies of the invention include derivatives that are modified, *i.e*, by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotypic response. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

The antibodies of the present invention may be generated by any suitable method known in the art. Polyclonal antibodies to an antigen of interest can be produced by various procedures well known in the art. For example, a polypeptide of the invention can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Such adjuvants are also well known in the art.

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and

taught, for example, in Harlow *et al.*, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling *et al.*, in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced. Thus, the term "monoclonal antibody" is not limited to antibodies produced through hybridoma technology. Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma and recombinant and phage display technology.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well-known in the art and are discussed in detail in Example 21. Briefly, mice can be immunized with a polypeptide of the invention or a cell expressing such peptide. Once an immune response is detected, *e.g.*, antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well-known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a polypeptide of the invention. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

Accordingly, the present invention provides methods of generating monoclonal antibodies as well as antibodies produced by the method comprising culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with an antigen of the invention with myeloma cells and then

screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a polypeptide of the invention.

Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, Fab and F(ab')2 fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments). F(ab')2 fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

For example, the antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular, such phage can be utilized to display antigen-binding domains expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, *e.g.*, using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman *et al., J. Immunol. Methods 182*:41-50 (1995); Ames *et al., J. Immunol. Methods 184*:177-186 (1995); Kettleborough *et al., Eur. J. Immunol. 24*:952-958 (1994); Persic *et al., Gene 187*:9-18 (1997); Burton *et al., Advances in Immunology 57*:191-280 (1994); PCT application No. PCT/GB91/01134; PCT publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, *e.g.*, as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax *et al.*, *BioTechniques 12(6)*:864-869 (1992); and Sawai *et al.*, *AJRI 34*:26-34 (1995); and Better *et al.*, *Science 240*:1041-1043 (1988) (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston *et al.*, *Methods in Enzymology 203*:46-88 (1991); Shu *et al.*, *PNAS 90*:7995-7999 (1993); and Skerra *et al.*, *Science 240*:1038-1040 (1988). For some uses, including *in vivo* use of antibodies in humans and *in vitro* detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. *See, e.g.*, Morrison, *Science 229*:1202 (1985); Oi *et al.*, *BioTechniques 4*:214 (1986); Gillies *et al.*, (1989) J. *Immunol. Methods 125*:191-202; U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816397, which are incorporated herein by reference in their entireties. Humanized antibodies are antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and framework regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve,

antigen binding. These framework substitutions are identified by methods well known in the art, *e.g.*, by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (*See, e.g.*, Queen *et al.*, U.S. Patent No. 5,585,089; Riechmann *et al.*, *Nature 332*:323 (1988), which are incorporated herein by reference in their entireties.) Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, Molecular Immunology 28(4/5):489-498 (1991); Studnicka *et al.*, Protein Engineering 7(6):805-814 (1994); Roguska. *et al.*, PNAS 91:969-973 (1994)), and chain shuffling (U.S. Patent No. 5,565,332).

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. *See also*, U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety.

Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous

deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring that express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B-cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, Int. Rev. Immunol. 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, *see, e.g.*, PCT publications WO 98/24893; WO 96/34096; WO 96/33735; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, CA) and GenPharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers *et al.*, *Bio/technology 12*:899-903 (1988)).

Further, antibodies to the polypeptides of the invention can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" polypeptides of the invention using techniques well known to those skilled in the art. (*See, e.g.*, Greenspan & Bona, *FASEB J. 7(5)*:437-444 (1989) and Nissinoff, *J. Immunol.*

147(8):2429-2438 (1991)). For example, antibodies which bind to and competitively inhibit polypeptide multimerization and/or binding of a polypeptide of the invention to a ligand can be used to generate anti-idiotypes that "mimic" the polypeptide multimerization and/or binding domain and, as a consequence, bind to and neutralize polypeptide and/or its ligand. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize polypeptide ligand. For example, such anti-idiotypic antibodies can be used to bind a polypeptide of the invention and/or to bind its ligands/receptors, and thereby block its biological activity.

A. Polynucleotides Encoding Antibodies.

The invention further provides polynucleotides comprising a nucleotide sequence encoding an antibody of the invention and fragments thereof. The invention also encompasses polynucleotides that hybridize under stringent or lower stringency hybridization conditions, *e.g.*, as defined *supra*, to polynucleotides that encode an antibody, preferably, that specifically binds to a polypeptide of the invention, preferably, an antibody that binds to a polypeptide having the amino acid sequence of SEQ ID NO:2.

The polynucleotides may be obtained, and the nucleotide sequence of the polynucleotides determined, by any method known in the art. For example, if the nucleotide sequence of the antibody is known, a polynucleotide encoding the antibody may be assembled from chemically synthesized oligonucleotides (*e.g.*, as described in Kutmeier *et al., BioTechniques 17*:242 (1994)), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the antibody, annealing and ligation of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, a polynucleotide encoding an antibody may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid encoding a particular antibody is not available, but the sequence of the antibody

molecule is known, a nucleic acid encoding the immunoglobulin may be obtained from a suitable source (*e.g.*, an antibody cDNA library, or a cDNA library generated from, or nucleic acid, preferably poly A+ RNA, isolated from, any tissue or cells expressing the antibody, such as hybridoma cells selected to express an antibody of the invention) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, *e.g.*, a cDNA clone from a cDNA library that encodes the antibody. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method well known in the art.

Once the nucleotide sequence and corresponding amino acid sequence of the antibody is determined, the nucleotide sequence of the antibody may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, *e.g.*, recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for example, the techniques described in Sambrook *et al.*, 1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY and Ausubel *et al.*, eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their entireties), to generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

In a specific embodiment, the amino acid sequence of the heavy and/or light chain variable domains may be inspected to identify the sequences of the complementarity determining regions (CDRs) by methods that are well know in the art, *e.g.*, by comparison to known amino acid sequences of other heavy and light chain variable regions to determine the regions of sequence hypervariability. Using routine recombinant DNA techniques, one or more of the CDRs may be inserted within framework regions, *e.g.*, into human framework regions to humanize a non-human antibody, as described *supra*. The framework regions may be naturally occurring or consensus framework regions, and preferably human

framework regions (*see, e.g.*, Chothia *et al.*, *J. Mol. Biol. 278*:457-479 (1998) for a listing of human framework regions). Preferably, the polynucleotide generated by the combination of the framework regions and CDRs encodes an antibody that specifically binds a polypeptide of the invention. Preferably, as discussed *supra*, one or more amino acid substitutions may be made within the framework regions, and, preferably, the amino acid substitutions improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present invention and within the skill of the art.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, 1984, *Proc. Natl. Acad. Sci. 81*:851-855; Neuberger *et al.*, 1984, *Nature 312*:604-608; Takeda *et al.*, 1985, *Nature 314*:452-454) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described *supra*, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region, *e.g.*, humanized antibodies.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,694,778; Bird, 1988, *Science 242*:423-42; Huston *et al.*, 1988, *Proc. Natl. Acad. Sci. USA 85*:5879-5883; and Ward *et al.*, 1989, *Nature 334*:544-554) can be adapted to produce single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in *E. coli* may also be used (Skerra *et al.*, 1988, *Science 242*:1038- 1041).

B. Methods of Producing Antibodies

The antibodies of the invention can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

Recombinant expression of an antibody of the invention, or fragment, derivative or analog thereof, *e.g.*, a heavy or light chain of an antibody of the invention, requires construction of an expression vector containing a polynucleotide that encodes the antibody. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an antibody, or portion thereof (preferably containing the heavy or light chain variable domain), of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding an antibody molecule of the invention, or a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (*see, e.g.*, PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an antibody of the invention. Thus, the invention includes host cells

containing a polynucleotide encoding an antibody of the invention, or a heavy or light chain thereof, operably linked to a heterologous promoter. In preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

A variety of host-expression vector systems may be utilized to express the antibody molecules of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express an antibody molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (*e.g., E. coli, B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (*e.g., Saccharomyces, Pichia*) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing antibody coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial cells such as *Escherichia coli*, and more preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecule, are used for the expression of a recombinant antibody molecule. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system

for antibodies (Foecking *et al.*, 1986, *Gene 45*:101; Cockett *et al.*, 1990, *Bio/Technology 8*:2).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, *EMBO J. 2*:1791), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, *Nucleic Acids Res. 13*:3101-3109; Van Heeke & Schuster, 1989, *J. Biol. Chem. 24*:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite

leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non- essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody molecule in infected hosts. (*e.g.*, see Logan & Shenk, 1984, *Proc. Natl. Acad. Sci. USA 81*:355-359). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner *et al.*, 1987, *Methods in Enzymol. 153*:51-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, breast cancer cell lines such as, for example, BT483, Hs578T, HTB2, BT20 and T47D, and normal mammary gland cell line such as, for example, CRL7030 and Hs578Bst.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the antibody molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the antibody molecule. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibody molecule.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, *Cell 11*:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 192, *Proc. Natl. Acad. Sci. USA 48*:202), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:817) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler *et al.*, 1980, Natl. Acad. Sci. USA 77:357; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIB TECH 11(5):155-215); and hygro, which confers resistance to hygromycin (Santerre *et*

al., 1984, Gene 30:147). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), 1993, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY; Kriegler, 1990, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli *et al.* (eds), 1994, *Current Protocols in Human Genetics*, John Wiley & Sons, NY.; Colberre-Garapin *et al.*, 1981, *J. Mol. Biol. 150*:1, which are incorporated by reference herein in their entireties.

The expression levels of an antibody molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, *The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning*, Vol. 3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse *et al.*, 1983, *Mol. Cell. Biol. 3*:257).

The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, 1986, *Nature 322*:52; Kohler, 1980, *Proc. Natl. Acad. Sci. USA 77*:2197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once an antibody molecule of the invention has been recombinantly expressed, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (*e.g.*, ion exchange, affinity, particularly by affinity for the specific antigen after Protein A,

and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

C. Antibody Conjugates

The present invention encompasses antibodies recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to a polypeptide (or portion thereof, preferably at least 10, 20 or 50 amino acids of the polypeptide) of the present invention to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences. The antibodies may be specific for antigens other than polypeptides (or portion thereof, preferably at least 10, 20 or 50 amino acids of the polypeptide) of the present invention. For example, antibodies may be used to target the polypeptides of the present invention to particular cell types, either *in vitro* or *in vivo*, by fusing or conjugating the polypeptides of the present invention to antibodies specific for particular cell surface receptors. Antibodies fused or conjugated to the polypeptides of the present invention may also be used in *in vitro* immunoassays and purification methods using methods known in the art. *See e.g.*, Harbor *et al.*, *supra*, and PCT publication WO 93/21232; EP 439,095; Naramura *et al.*, *Immunol. Lett. 39*:91-99 (1994); U.S. Patent 5,474,981; Gillies *et al.*, *PNAS 89*:1428-1432 (1992); Fell *et al.*, *J. Immunol. 146*:2446-2452 (1991), which are incorporated by reference in their entireties.

The present invention further includes compositions comprising the polypeptides of the present invention fused or conjugated to antibody domains other than the variable regions. For example, the polypeptides of the present invention may be fused or conjugated to an antibody Fc region, or portion thereof. The antibody portion fused to a polypeptide of the present invention may comprise the constant region, hinge region, CH1 domain, CH2 domain, and CH3 domain or any combination of whole domains or portions thereof. The polypeptides may also be fused or conjugated to the above antibody portions to

form multimers. For example, Fc portions fused to the polypeptides of the present invention can form dimers through disulfide bonding between the Fc portions. Higher multimeric forms can be made by fusing the polypeptides to portions of IgA and IgM. Methods for fusing or conjugating the polypeptides of the present invention to antibody portions are known in the art. *See, e.g.*, U.S. Patent Nos. 5,336,603; 5,622,929; 5,359,046; 5,349,053; 5,447,851; 5,112,946; EP 307,434; EP 367,166; PCT publications WO 96/04388; WO 91/06570; Ashkenazi *et al.*, Proc. Natl. Acad. Sci. USA 88:10535-10539 (1991); Zheng *et al.*, J. Immunol. 154:5590-5600 (1995); and Vil *et al.*, Proc. Natl. Acad. Sci. USA 89:11337-11341(1992) (said references incorporated by reference in their entireties).

As discussed, *supra*, the polypeptides of the present invention may be fused or conjugated to the above antibody portions to increase the *in vivo* half life of the polypeptides or for use in immunoassays using methods known in the art. Further, the polypeptides of the present invention may be fused or conjugated to the above antibody portions to facilitate purification. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EP 394,827; Traunecker *et al.*, Nature 331:84-86 (1988). The polypeptides of the present invention fused or conjugated to an antibody having disulfide- linked dimeric structures (due to the IgG) may also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. (Fountoulakis *et al.*, J. Biochem. 270:3958-3964 (1995)). In many cases, the Fc part in a fusion protein is beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. (EP A 232,262). Alternatively, deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired. For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5 receptor, have been fused with Fc portions for the purpose of high-throughput screening assays to identify

antagonists of hIL-5. (*See*, D. Bennett *et al.*, *J. Molecular Recognition 8*:52-58 (1995); K. Johanson *et al.*, *J. Biol. Chem. 270*:9459-9471 (1995).

Moreover, the antibodies or fragments thereof of the present invention can be fused to marker sequences, such as a peptide to facilitates their purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA 86*:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson *et al.*, *Cell 37*:767 (1984)) and the "flag" tag.

The present invention further encompasses antibodies or fragments thereof conjugated to a diagnostic or therapeutic agent. The antibodies can be used diagnostically to, for example, monitor the development or progression of a tumor as part of a clinical testing procedure to, *e.g.*, determine the efficacy of a given treatment and/or prevention regimens. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. *See, for example*, U.S. Patent No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples

of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{111}In or ^{99}Tc.

Further, an antibody or fragment thereof may be conjugated to a therapeutic moiety such as a cytotoxin, *e.g.*, a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and *cis*-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological response, the therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, a-interferon, ß-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, a thrombotic agent or an anti-angiogenic agent, *e.g.*, angiostatin or endostatin; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"),

granulocyte macrophase colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Antibodies may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

Techniques for conjugating such therapeutic moiety to antibodies are well known, *see, e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in *Controlled Drug Delivery* (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev. 62*:119-58 (1982).

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980, which is incorporated herein by reference in its entirety.

An antibody, with or without a therapeutic moiety conjugated to it, administered alone or in combination with cytotoxic factor(s) and/or cytokine(s) can be used as a therapeutic.

D. Assays For Antibody Binding

The antibodies of the invention may be assayed for immunospecific binding by any method known in the art. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (*see, e.g.*, Ausubel *et al.*, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasylol) supplemented with protein phosphatase and/or protease inhibitors (*e.g.*, EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody of interest to the cell lysate, incubating for a period of time (*e.g.*, 1-4 hours) at 4° C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 4° C, washing the beads in lysis buffer and resuspending the beads in SDS/sample buffer. The ability of the antibody of interest to immunoprecipitate a particular antigen can be assessed by, *e.g.*, western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to an antigen and decrease the background (*e.g.*, pre-clearing the cell lysate with sepharose beads). For further discussion regarding immunoprecipitation protocols *see, e.g.*, Ausubel

et al., eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.16.1.

Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (*e.g.*, 8%-20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (*e.g.*, PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (*e.g.*, PBS-Tween 20), blocking the membrane with primary antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, blocking the membrane with a secondary antibody (which recognizes the primary antibody, *e.g.*, an anti-human antibody) conjugated to an enzymatic substrate (*e.g.*, horseradish peroxidase or alkaline phosphatase) or radioactive molecule (*e.g.*, ^{32}P or ^{125}I) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the antigen. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding western blot protocols *see, e.g.*, Ausubel *et al.*, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.8.1.

ELISAs comprise preparing antigen, coating the well of a 96 well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (*e.g.*, horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of the antigen of interest to the coated well. One of skill in the art would be

knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs *see, e.g.*, Ausubel *et al.*, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1.

The binding affinity of an antibody to an antigen and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (*e.g.*, ^{3}H or ^{125}I) with the antibody of interest in the presence of increasing amounts of unlabeled antigen, and the detection of the antibody bound to the labeled antigen. The affinity of the antibody of interest for a particular antigen and the binding off-rates can be determined from the data by scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, the antigen is incubated with antibody of interest is conjugated to a labeled compound (*e.g.*, ^{3}H or ^{125}I) in the presence of increasing amounts of an unlabeled second antibody.

E. Antibody Based Therapies

The present invention is further directed to antibody-based therapies which involve administering antibodies of the invention to an animal, preferably a mammal, and most preferably a human, patient for treating and/or preventing one or more of the disorders or conditions described herein. Therapeutic compounds of the invention include, but are not limited to, antibodies of the invention (including fragments, analogs and derivatives thereof as described herein) and nucleic acids encoding antibodies of the invention (including fragments, analogs and derivatives thereof as described herein).

While not intending to be bound to theory, DR4 receptors are believed to induce programmed cell death by a process which involves the association/cross-linking of death domains between different receptor molecules.

Further, DR4 ligands (*e.g.*, TRAIL) which induce DR4 mediated programmed cell death are believed to function by causing the association/cross-linking of DR4 death domains. Thus, agents (*e.g.*, antibodies) which prevent association/cross-linking of DR4 death domains will prevent DR4 mediated programmed cell death, and agents (*e.g.*, antibodies) which facilitate the association/cross-linking of DR4 death domains will induce DR4 mediated programmed cell death.

As noted above, DR4 receptors have been shown to bind TRAIL. DR4 receptors are also known to be present in a number of tissues and on the surfaces of a number of cell types. These tissues and cell types include amniotic cells, heart, liver cancer cells, kidney, leukocytes, activated T-cells, K562 cells (an erythroleukemia cell line) plus PMA, W138 cells (a human lung fibroblast cell line), Th2 cells (lymphocytes), human tonsils, and CD34 depleted buffy coat cells of cord blood. Further, as explained in more detail below, TRAIL has been shown to induce apoptosis and to inhibit the growth of tumor cells *in vivo*. Additionally, TRAIL activities are believed to be modulated, at least in part, through interaction with DR4 and DR5 receptors.

TRAIL is a member of the TNF family of cytokines which has been shown to induce apoptotic cell death in a number of tumor cell lines and appears to mediate its apoptosis inducing effects through interaction with DR4 and DR5 receptors. These death domain containing receptors are believed to form membrane-bound self-activating signaling complexes which initiate apoptosis through cleavage of caspases.

In addition to DR4 and DR5 receptors, TRAIL also binds to several receptors proposed to be "decoy" receptors, DcR2 (a receptor with a truncated death domain), DcR1 (a GPI-anchored receptor), and OPG (a secreted protein which binds to another member of the TNF family, RANKL).

Further, recent studies have shown that the rank-order of affinities of TRAIL for the recombinant soluble forms of its receptors is strongly temperature

dependent. In particular, at 37° C, DR5 has the highest affinity for TRAIL and OPG having the lowest affinity.

The DR4 and DR5 receptor genes, as well as genes encoding two decoy receptors, have been shown to be located on human chromosome 8p21-22. Further, this region of the human genome is frequently disrupted in head and neck cancers.

It has recently been found that the FaDu nasopharyngeal cancer cell line contains an abnormal chromosome 8p21-22 region. (Ozoren *et al., Int. J. Oncol.16*:917-925 (2000).) In particular, a homozygous deletion involving DR4, but not DR5, has been found in these cells. (Ozoren *et al., Int. J. Oncol.16*:917-925 (2000).) The homozygous loss within the DR4 receptor gene in these FaDu cells encompasses the DR4 receptor death domain. This disruption of the DR4 receptor death domain is associated with resistance to TRAIL-mediated cytotoxicity. Further, re-introduction of a wild-type DR4 receptor gene has been shown to both lead to apoptosis and restoration of TRAIL sensitivity of FaDu cells. (Ozoren *et al., Int. J. Oncol. 16*:917-925 (2000).) These data indicate that the DR4 receptor gene may be inactivated in human cancers and DR4 receptor gene disruption may contribute to resistance to TRAIL therapy. It is expected that similar results would be found in cells having analogous deletions in the DR5 gene.

It has also been shown that overexpression of the cytoplasmic domain of the DR4 receptor in human breast, lung, and colon cancer cell lines leads to p53-independent apoptotic cell death which involves the cleavage of caspases. (Xu *et al., Biochem. Biophys. Res. Commun. 269*:179-190 (2000).) Further, DR4 cytoplasmic domain overexpression has also been shown to result in cleavage of both poly(ADP-ribose) polymerase (PARP) and a DNA fragmentation factor (*i.e.*, ICAD-DFF45). (Xu *et al., Biochem. Biophys. Res. Commun. 269*:179-190 (2000).) In addition, despite similar levels of DR4 cytoplasmic domain protein as compared to cancer cells tested, normal lung fibroblasts have been shown to be resistant to DR4 cytoplasmic domain overexpression and show no evidence of

caspase-cleavage. (Xu *et al., Biochem. Biophys. Res. Commun. 269*:179-190 (2000).) Again, similar results are expected with cells that overexpress the cytoplasmic domain of DR5. Thus, the cytoplasmic domains of the DR4 and DR5 receptors are useful as agents for inducing apoptosis, for example, in cancer cells.

Further, overexpression of the cyclin-dependent kinase inhibitor p21(WAF1/CIP1), as well as the N-terminal 91 amino acids of this protein, has cell cycle-inhibitory activity and inhibits DR4 cytoplasmic domain-dependent caspase cleavage. Thus, DR4 receptors are also involved in the regulation of cell cycle progression. As above, similar results are expected with the DR5 receptor. Thus, the DR4 and DR5 receptors, as well as agonists and antagonists of these receptors, are useful for regulating cell cycle progression.

Antibodies which bind to DR4 receptors are useful for treating and/or preventing diseases and conditions associated with increased or decreased DR4-induced apoptotic cells death. Further, these antibodies vary in the effect they have on DR4 receptors. These effects differ based on the specific portions of the DR4 receptor to which the antibodies bind, the three-dimensional conformation of the antibody molecules themselves, and/or the manner in which they interact with the DR4 receptor. Thus, antibodies which bind to the extracellular domain of a DR4 receptor can either stimulate or inhibit DR4 activities (*e.g.*, the induction of apoptosis). Antibodies which stimulate DR4 receptor activities (*e.g.*, by facilitating the association between DR4 receptor death domains) are DR4 agonists, and antibodies which inhibit DR4 receptor activities (*e.g.*, by blocking the binding of TRAIL and/or preventing the association between DR4 receptor death domains) are DR4 antagonists.

Antibodies of the invention which function as agonists and antagonists of DR4 receptors include antigen-binding antibody fragments such as Fab and $F(ab')_2$ fragments, Fd, single-chain Fvs (scFv), disulfide-linked Fvs (sdFv) and fragments comprising either a V_L or V_H domain, as well as polyclonal, monoclonal and humanized antibodies. Divalent antibodies are preferred as agonists. Each of

these antigen-binding antibody fragments and antibodies are described in more detail elsewhere herein.

In view of the above, antibodies of the invention, as well as other agonists, are useful for stimulating DR4 death domain activity to promote apoptosis in cells which express DR4 receptors (*e.g.*, cancer cells). Antibodies of this type are useful for prevention and/or treating diseases and conditions associated with increased cell survival and/or insensitivity to apoptosis-inducing agents (*e.g.*, TRAIL), such as solid tissue cancers (*e.g.*, skin cancer, head and neck tumors, breast tumors, endothelioma, lung cancer, osteoblastoma, osteoclastoma, and Kaposi's sarcoma) and leukemias.

Antagonists of the invention (*e.g.*, anti-DR4 antibodies) function by preventing DR4 mediated apoptosis and are useful for preventing and/or treating diseases associated with increased apoptotic cell death. Examples of such diseases include diabetes mellitus, AIDS, neurodegenerative disorders, myelodysplastic syndromes, ischemic injury, toxin-induced liver disease, septic shock, cachexia and anorexia.

As noted above, DR4 receptors are present on the surfaces of T-cells. Thus, agonists of the invention (*e.g.*, anti-DR4 receptor antibodies) are also useful for inhibiting T-cell mediated immune responses, as well as preventing and/or treating diseases and conditions associated with increased T-cell proliferation. Diseases and conditions associated with T-cell mediated immune responses and increased T-cell proliferation include graft-v-host responses and diseases, osteoarthritis, psoriasis, septicemia, inflammatory bowel disease, inflammation in general, autoimmune diseases, and T-cell leukemias.

When an agonist of the invention is administered to an individual for the treatment and/or prevention of a disease or condition associated with increased T-cell populations or increased cell proliferation (*e.g.*, cancer), the antagonist may be co-administered with another agent which induces apoptosis (*e.g.*, TRAIL) or otherwise inhibits cell proliferation (*e.g.*, an anti-cancer drug). Combination

therapies of this nature, as well as other combination therapies, are discussed below in more detail.

Further, antagonists of the invention (*e.g.*, anti-DR4 receptor antibodies) are also useful for enhancing T-cell mediated immune responses, as well as preventing and/or treating diseases and conditions associated with decreased T-cell proliferation. Antibodies of the invention which block the binding of DR4 receptor ligands to DR4 receptors or interfere with DR4 receptor conformational changes associated with membrane signal transduction can inhibit DR4 mediated T-cell apoptosis. The inhibition of DR4 mediated apoptosis can, for examples, either result in an increase in the expansion rate of *in vivo* T-cell populations or prevent a decrease in the size of such populations. Thus, antagonists of the invention can be used to prevent and/or treat diseases or conditions associated with decreased or decreases in T-cell populations. Examples of such diseases and conditions included acquired immune deficiency syndrome (AIDS) and related afflictions (*e.g.*, AIDS related complexes), T-cell immunodeficiencies, radiation sickness, and T-cell depletion due to radiation and/or chemotherapy.

When an antagonist of the invention is administered to an individual for the treatment and/or prevention of a disease or condition associated with decreased T-cell populations, the antagonist may be co-administered with an agent which activates and/or induces lymphocyte proliferation (*e.g.*, a cytokine). Combination therapies of this nature, as well as other combination therapies, are discussed below in more detail.

Anti-DR4 antibodies are thus useful for treating and/or preventing malignancies, abnormalities, diseases and/or conditions involving tissues and cell types which express DR4 receptors. Further, malignancies, abnormalities, diseases and/or conditions which can be treated and/or prevented by the induction of programmed cell death in cells which express DR4 receptors can be treated and/or prevented using DR4 receptor agonists of the invention. Similarly, malignancies, abnormalities, diseases and/or conditions which can be treated and/or prevented by inhibiting programmed cell death in cells which express DR4

receptors can be treated and/or prevented using DR4 receptor antagonists of the invention.

A number of additional malignancies, abnormalities, diseases and/or conditions which can be treated using the agonists and antagonists of the invention are set out elsewhere herein, for example, in the section below entitled "Therapeutics".

The antibodies of the present invention may be used therapeutically in a number of ways. For example, antibodies which bind polynucleotides or polypeptides of the present invention can be administered to an individual (*e.g.*, a human) either locally or systemically. Further, these antibodies can be administered alone, in combination with another therapeutic agent, or associated with or bound to a toxin.

Anti-DR4 antibodies may be utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines, tumor necrosis factors or TNF-related molecules (*e.g.*, TNF-α, TNF-β, TNF-γ, TNF-γ-α, TNF-γ-β, and TRAIL), or hematopoietic growth factors (*e.g.*, IL-2, IL-3 and IL-7). For example, agonistic anti-DR4 antibodies may be administered in conjunction with TRAIL when one seeks to induce DR4 mediated cell death in cells which express DR4 receptors of the invention. Combination therapies of this nature, as well as other combination therapies, are discussed below in more detail.

The antibodies of the invention may be administered alone or in combination with other types of treatments (*e.g.*, radiation therapy, chemotherapy, hormonal therapy, immunotherapy and anti-tumor agents). Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, human antibodies, fragments derivatives, analogs, or nucleic acids, are administered to a human patient for therapy or prophylaxis.

It is preferred to use high affinity and/or potent *in vivo* inhibiting and/or neutralizing antibodies against polypeptides or polynucleotides of the present invention, fragments or regions thereof, for both immunoassays directed to and

therapy of disorders related to polynucleotides or polypeptides, including fragments thereof, of the present invention. Such antibodies, fragments, or regions, will preferably have an affinity for polynucleotides or polypeptides, including fragments thereof. Preferred binding affinities include those with a dissociation constant or Kd less than $5X10^{-6}M$, $10^{-6}M$, $5X10^{-7}M$, $10^{-7}M$, $5X10^{-8}M$, $10^{-8}M$, $5X10^{-9}M$, $10^{-9}M$, $5X10^{-10}M$, $10^{-10}M$, $5X10^{-11}M$, $10^{-11}M$, $5X10^{-12}M$, $10^{-12}M$, $5X10^{-13}M$, $10^{-13}M$, $5X10^{-14}M$, $10^{-14}M$, $5X10^{-15}M$, and $10^{-15}M$.

Polypeptide Assays

The present invention also relates to diagnostic assays such as quantitative and diagnostic assays for detecting levels of DR4 protein, or the soluble form thereof, in cells and tissues, including determination of normal and abnormal levels. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of DR4, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors, for example. Assay techniques that can be used to determine levels of a protein, such as a DR4 protein of the present invention, or a soluble form thereof, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays.

Assaying DR4 protein levels in a biological sample can occur using any art-known method. By "biological sample" is intended any biological sample obtained from an individual, cell line, tissue culture, or other source containing DR4 receptor protein or mRNA. Preferred for assaying DR4 protein levels in a biological sample are antibody-based techniques. For example, DR4 protein expression in tissues can be studied with classical immunohistological methods. (Jalkanen, M., *et al., J. Cell. Biol. 101:*976-985 (1985); Jalkanen, M.; *et al., J. Cell. Biol. 105*:3087-3096 (1987)). Other antibody-based methods useful for

detecting DR4 protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA).

Suitable labels are known in the art and include enzyme labels, such as glucose oxidase, radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulphur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium (^{99m}Tc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Transgenics and "Knock-outs"

The proteins of the invention can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are used to express polypeptides of the invention in humans, as part of a gene therapy protocol.

Any technique known in the art may be used to introduce the transgene (*i.e.*, nucleic acids of the invention) into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Paterson *et al.*, Appl. Microbiol. Biotechnol. 40:691-698 (1994); Carver *et al.*, Biotechnology (NY) 11:1263-1270 (1993); Wright *et al.*, Biotechnology (NY) 9:830-834 (1991); and Hoppe *et al.*, US Patent Number 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines (Van der Putten *et al.*, Proc. Natl. Acad. Sci., USA 82:6148-6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells (Thompson *et al.*, Cell 56:313-321 (1989)); electroporation of cells or embryos (Lo, Mol Cell. Biol. 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun (see, *e.g.*, Ulmer *et al.*, Science 259:1745 (1993); introducing nucleic acid constructs into embryonic pleuripotent stem cells and transferring the stem cells back into the blastocyst; and sperm-mediated gene transfer (Lavitrano *et al.*, Cell

57:717-723 (1989); etc. For a review of such techniques, *see* Gordon, "Transgenic Animals," *Intl. Rev. Cytol. 115*:171-229 (1989), which is incorporated by reference herein in its entirety. Further, the contents of each of the documents recited in this paragraph is herein incorporated by reference in its entirety. *See also*, U.S. Patent No. 5,464,764 (Capecchi *et al.*, Positive-Negative Selection Methods and Vectors); U.S. Patent No. 5,631,153 (Capecchi *et al.*, Cells and Non-Human Organisms Containing Predetermined Genomic Modifications and Positive-Negative Selection Methods and Vectors for Making Same); U.S. Patent No. 4,736,866 (Leder *et al.*, Transgenic Non-Human Animals); and U.S. Patent No. 4,873,191 (Wagner *et al.*, Genetic Transformation of Zygotes); each of which is hereby incorporated by reference in its entirety.

Any technique known in the art may be used to produce transgenic clones containing polynucleotides of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to quiescence (Campell *et al.*, Nature 380:64-66 (1996); Wilmut *et al.*, *Nature 385*:810-813 (1997)), each of which is herein incorporated by reference in its entirety).

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, *i.e.*, mosaic animals or chimeric animals. The transgene may be integrated as a single transgene or as multiple copies such as in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko *et al.* (*Proc. Natl. Acad. Sci. USA 89*:6232-6236 (1992)). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the polynucleotide transgene be integrated into the chromosomal site of the endogenous gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for

the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu *et al.* (*Science 265*:103-106 (1994)). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. The contents of each of the documents recited in this paragraph is herein incorporated by reference in its entirety.

Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of transgenic gene-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the transgene product.

Once the founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; and

breeding to place the transgene on a distinct background that is appropriate for an experimental model of interest.

Transgenic and "knock-out" animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of DR4 polypeptides, studying conditions and/or disorders associated with aberrant DR4 expression, and in screening for compounds effective in ameliorating such conditions and/or disorders.

In further embodiments of the invention, cells that are genetically engineered to express the proteins of the invention, or alternatively, that are genetically engineered not to express the proteins of the invention (*e.g.*, knockouts) are administered to a patient *in vivo*. Such cells may be obtained from the patient (*i.e.*, animal, including human) or an MHC compatible donor and can include, but are not limited to fibroblasts, bone marrow cells, blood cells (*e.g.*, lymphocytes), adipocytes, muscle cells, endothelial cells, etc. The cells are genetically engineered *in vitro* using recombinant DNA techniques to introduce the coding sequence of polypeptides of the invention into the cells, or alternatively, to disrupt the coding sequence and/or endogenous regulatory sequence associated with the polypeptides of the invention, *e.g.*, by transduction (using viral vectors, and preferably vectors that integrate the transgene into the cell genome) or transfection procedures, including, but not limited to, the use of plasmids, cosmids, YACs, naked DNA, electroporation, liposomes, etc. The coding sequence of the polypeptides of the invention can be placed under the control of a strong constitutive or inducible promoter or promoter/enhancer to achieve expression, and preferably secretion, of the polypeptides of the invention. The engineered cells which express and preferably secrete the polypeptides of the invention can be introduced into the patient systemically, *e.g.*, in the circulation, or intraperitoneally. Alternatively, the cells can be incorporated into a matrix and implanted in the body, *e.g.*, genetically engineered fibroblasts can be implanted as part of a skin graft; genetically engineered endothelial cells can be implanted as part of a lymphatic or vascular graft. (See, for example, Anderson et al. US

Patent Number 5,399,349; and Mulligan & Wilson, US Patent Number 5,460,959, each of which is incorporated by reference herein in its entirety).

When the cells to be administered are non-autologous or non-MHC compatible cells, they can be administered using well known techniques which prevent the development of a host immune response against the introduced cells. For example, the cells may be introduced in an encapsulated form that, while allowing for an exchange of components with the immediate extracellular environment, does not allow the introduced cells to be recognized by the host immune system.

Therapeutics

The Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes (Goeddel, D.V. *et al.*, "Tumor Necrosis Factors: Gene Structure and Biological Activities," *Symp. Quant. Biol. 51*:597-609 (1986), Cold Spring Harbor; Beutler, B., and Cerami, A., *Annu. Rev. Biochem. 57*:505-518 (1988); Old, L.J., *Sci. Am. 258*:59-75 (1988); Fiers, W., *FEBS Lett. 285*:199-224 (1991)). The TNF-family ligands induce such various cellular responses by binding to TNF-family receptors, including the DR4 receptors of the present invention.

DR4 polynucleotides and polypeptides of the invention may be used in developing treatments and/or prevention methods for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of DR4. DR4 polypeptides may be administered to a patient (*e.g.*, mammal, preferably human) afflicted with such a disorder. Alternatively, a gene therapy approach may be applied to treat and/or prevent such disorders. Disclosure herein of DR4 nucleotide sequences permits the detection of defective DR4 genes, and the replacement thereof with normal DR4 -encoding genes. Defective genes may be

detected in *in vitro* diagnostic assays, and by comparison of the DR4 nucleotide sequence disclosed herein with that of a DR4 gene derived from a patient suspected of harboring a defect in this gene.

In another embodiment, the polypeptides of the present invention are used as a research tool for studying the biological effects that result from inhibiting TRAIL/DR4 interactions on different cell types. DR4 polypeptides also may be employed in *in vitro* assays for detecting TRAIL or DR4 or the interactions thereof.

In another embodiment, a purified DR4 polypeptide or DR4 antagonist of the invention is used to inhibit binding of TRAIL to endogenous cell surface TRAIL receptors. Certain ligands of the TNF family (of which TRAIL is a member) have been reported to bind to more than one distinct cell surface receptor protein. TRAIL likewise is believed to bind multiple cell surface proteins. By binding TRAIL, soluble DR4 polypeptides and/or DR4 antagonists of the present invention may be employed to inhibit the binding of TRAIL not only to cell surface DR4, but also to TRAIL receptor proteins that are distinct from DR4. Thus, in another embodiment, a DR4 polypeptide and/or antagonist is used to inhibit a biological activity of TRAIL, in *in vitro* or *in vivo* procedures. By inhibiting binding of TRAIL to cell surface receptors, a DR4 polypeptide and antagonist also inhibits biological effects that result from the binding of TRAIL to endogenous receptors. Various forms of DR4 polypeptides may be employed, including, for example, the above-described DR4 fragments, derivatives, and variants that are capable of binding TRAIL. In a preferred embodiment, a soluble DR4 is employed to inhibit a biological activity of TRAIL, *e.g.*, to inhibit TRAIL-mediated apoptosis of cells susceptible to such apoptosis.

In a further embodiment, DR4 compositions (*e.g.*, DR4 polynucleotides, polypeptides, agonists and/or antagonists) of the invention are administered to a mammal (*e.g.*, a human) to treat and/or prevent a TRAIL-mediated disorder. Such TRAIL-mediated disorders include conditions caused (directly or indirectly) or exacerbated by TRAIL.

Cells which express the DR4 polypeptide and are believed to have a potent cellular response to DR4 ligands include amniotic cells, heart, liver cancer, kidney, peripheral blood leukocytes, activated T-cells, tissue corresponding to Th2 cells, human tonsils, and CD34 depleted buffy coat (cord blood). By "a cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphologic change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis-programmed cell death-is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes (Ameisen, J.C., *AIDS 8*:1197-1213 (1994); Krammer, P.H. *et al., Curr. Opin. Immunol. 6*:279-289 (1994)).

Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, including, but not limited to colon cancer, cardiac tumors, pancreatic cancer, melanoma, retinoblastoma, glioblastoma, lung cancer, intestinal cancer, testicular cancer, stomach cancer, neuroblastoma, myxoma, myoma, lymphoma, endothelioma, osteoblastoma, osteoclastoma, osteosarcoma, chondrosarcoma, adenoma, breast cancer, prostrate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), information graft v. host disease, acute graft rejection, and chronic graft rejection. In preferred embodiments, DR4 polynucleotides, polypeptides, and/or antagonists of the invention are used to inhibit growth, progression, and/or metastasis of cancers, in particular those listed above or in the paragraph that follows.

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

Diseases associated with increased apoptosis include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration); and brain tumor or prior associated disease); autoimmune disorders (such as, multiple sclerosis, Sjogren's syndrome, Hashimoto's thyroiditis, biliary cirrhosis, Behcet's disease, Crohn's disease, polymyositis, systemic lupus erythematosus and immune-related glomerulonephritis and rheumatoid arthritis) myelodysplastic

syndromes (such as aplastic anemia), graft v. host disease, ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), liver injury (*e.g.*, hepatitis related liver injury, ischemia/reperfusion injury, cholestosis (bile duct injury) and liver cancer); toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia. In preferred embodiments, DR4 polynucleotides, polypeptides and/or agonists are used to treat the diseases and disorders listed above.

Many of the pathologies associated with HIV are mediated by apoptosis, including HIV-induced nephropathy and HIV encephalitis. Thus, in additional preferred embodiments, DR4 polynucleotides, polypeptides, and/or DR4 agonists of the invention are used to treat and/or prevent AIDS and pathologies associated with AIDS. Another embodiment of the present invention is directed to the use of DR4 to reduce TRAIL-mediated death of T-cells in HIV-infected patients.

The state of immunodeficiency that defines AIDS is secondary to a decrease in the number and function of $CD4^+$ T-lymphocytes. Recent reports estimate the daily loss of $CD4^+$ T-cells to be between 3.5×10^7 and 2×10^9 cells (Wei X., *et al., Nature 373*:117-122 (1995)). One cause of $CD4^+$ T-cell depletion in the setting of HIV infection is believed to be HIV-induced apoptosis (see, for example, Meyaard *et al., Science 257*:217-219, (1992); Groux *et al., J Exp. Med., 175*:331, (1992); and Oyaizu *et al.*, in *Cell Activation and Apoptosis in HIV Infection,* Andrieu and Lu, Eds., Plenum Press, New York, 1995, pp. 101-114). Indeed, HIV-induced apoptotic cell death has been demonstrated not only *in vitro* but also, more importantly, in infected individuals (Ameisen, J.C., *AIDS 8*:1197-1213 (1994) ; Finkel, T.H., and Banda, N.K., *Curr. Opin. Immunol. 6*:605-615(1995); Muro-Cacho, C.A. *et al., J. Immunol. 154*:5555-5566 (1995)). Furthermore, apoptosis and $CD4^+$ T-lymphocyte depletion is tightly correlated in different animal models of AIDS (Brunner, T., *et al., Nature 373*:441-444 (1995); Gougeon, M.L., *et al., AIDS Res. Hum. Retroviruses 9*:553-563 (1993)) and, apoptosis is not observed in those animal models in which viral replication does not result in AIDS (Gougeon, M.L. *et al., AIDS Res. Hum. Retroviruses 9*:553-

563 (1993)). Further data indicates that uninfected but primed or activated T lymphocytes from HIV-infected individuals undergo apoptosis after encountering the TNF-family ligand FasL. Using monocytic cell lines that result in death following HIV infection, it has been demonstrated that infection of U937 cells with HIV results in the *de novo* expression of FasL and that FasL mediates HIV-induced apoptosis (Badley, A.D. *et al., J. Virol. 70*:199-206 (1996)). Further the TNF-family ligand was detectable in uninfected macrophages and its expression was upregulated following HIV infection resulting in selective killing of uninfected CD4 T-lymphocytes (Badley, A.D *et al., J. Virol. 70*:199-206 (1996)). Further, additional studies have implicated Fas-mediated apoptosis in the loss of T-cells in HIV individuals (*Katsikis et al., J. Exp. Med. 181*:2029-2036 (1995)). It is also possible that T-cell apoptosis occurs through multiple mechanisms. Further, at least some of the T-cell death seen in HIV patients may be mediated by TRAIL.

Thus, by the invention, a method for treating and/or preventing HIV^+ individuals is provided which involves administering DR4, DR4 polypeptides, polynucleotides, antagonists, and/or agonists of the present invention to reduce selective killing of $CD4^+$ T-lymphocytes. While not wanting to be bound by theory, activated human T-cells are believed to be induced to undergo programmed cell death (apoptosis) upon triggering through the CD3/T-cell receptor complex, a process termed activated-induced cell death (AICD). AICD of $CD4^+$ T-cells isolated from HIV-Infected asymptomatic individuals has been reported (Groux *et al., supra)*. Thus, AICD may play a role in the depletion of $CD4^+$T-cells and the progression to AIDS in HIV-infected individuals. Thus, the present invention provides a method of inhibiting TRAIL-mediated T-cell death in HIV patients, comprising administering a DR4 polypeptide of the invention (preferably, a soluble DR4 polypeptide) and/or DR4 antagonist of the invention to the patients. Modes of administration and dosages are discussed in detail below. In one embodiment, the patient is asymptomatic when treatment with DR4 commences. If desired, prior to treatment, peripheral blood T-cells may be extracted from an HIV patient, and tested for susceptibility to TRAIL-mediated

cell death by procedures known in the art. In one embodiment, a patient's blood or plasma is contacted with DR4 polypeptides of the invention *ex vivo*. The DR4 polypeptides of the invention may be bound to a suitable chromatography matrix by procedures known in the art. The patient's blood or plasma flows through a chromatography column containing DR4 bound to the matrix, before being returned to the patient. The immobilized DR4 polypeptide binds TRAIL, thus removing TRAIL protein from the patient's blood.

In additional embodiments a DR4 polypeptide and/or antagonist of the invention is administered in combination with other inhibitors of T-cell apoptosis. For example, as discussed above, Fas-mediated apoptosis also has been implicated in loss of T-cells in HIV individuals (Katsikis *et al.*, J. Exp. *Med.* 181:2029-2036, 1995). Thus, a patient susceptible to both Fas ligand mediated and TRAIL mediated T-cell death may be treated with both an agent that blocks TRAIL/TRAIL receptor interactions and an agent that blocks Fas-ligand/Fas interactions. Suitable agents for blocking binding of Fas-ligand to Fas include, but are not limited to, soluble Fas polypeptides; multimeric forms of soluble Fas polypeptides (*e.g.*, dimers of sFas/Fc); anti-Fas antibodies that bind Fas without transducing the biological signal that results in apoptosis; anti-Fas-ligand antibodies that block binding of Fas-ligand to Fas; and muteins of Fas-ligand that bind Fas but do not transduce the biological signal that results in apoptosis. Preferably, the antibodies employed according to this method are monoclonal antibodies. Examples of suitable agents for blocking Fas-ligand/Fas interactions, including blocking anti-Fas monoclonal antibodies, are described in International application publication number WO 95/10540, hereby incorporated by reference.

Suitable agents, which also block binding of TRAIL to a TRAIL receptor that may be administered with the polynucleotides and/or polypeptides of the present invention include, but are not limited to, soluble TRAIL receptor polypeptides (*e.g.*, a soluble form of OPG, TR5 (International application publication number WO 98/30693); DR5 (International application publication number WO 98/41629); and TR10 (International application publication number

WO 98/54202)); multimeric forms of soluble TRAIL receptor polypeptides; and TRAIL receptor antibodies that bind the TRAIL receptor without transducing the biological signal that results in apoptosis, anti-TRAIL antibodies that block binding of TRAIL to one or more TRAIL receptors, and muteins of TRAIL that bind TRAIL receptors but do not transduce the biological signal that results in apoptosis. Preferably, the antibodies employed according to this method are monoclonal antibodies.

In rejection of an allograft, the immune system of the recipient animal has not previously been primed to respond because the immune system for the most part is only primed by environmental antigens. Tissues from other members of the same species have not been presented in the same way that, for example, viruses and bacteria have been presented. In the case of allograft rejection, immunosuppressive regimens are designed to prevent the immune system from reaching the effector stage. However, the immune profile of xenograft rejection may resemble disease recurrence more that allograft rejection. In the case of disease recurrence, the immune system has already been activated, as evidenced by destruction of the native islet cells. Therefore, in disease recurrence the immune system is already at the effector stage. DR4 polynucleotides, polypeptides and/or agonists of the present invention are able to suppress the immune response to both allografts and xenografts because lymphocytes activated and differentiated into effector cells will express the DR4 polypeptide, and thereby are susceptible to compounds which enhance apoptosis. Thus, the present invention further provides a method for creating immune privileged tissues. Antagonist of the invention can further be used in the treatment and/or prevention of Inflammatory Bowel-Disease.

DR4 antagonists or agonists of the invention may be useful for treating and/or preventing inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

In addition, due to lymphoblast expression of DR4, soluble DR4, agonist or antagonist monoclonal antibodies may be used to treat and/or prevent this form

of cancer. Further, soluble DR4 or neutralizing monoclonal antibodies may be used to treat and/or prevent various chronic and acute forms of inflammation such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

In one embodiment, DR4 polypeptides, polynucleotides, and/or antagonists of the invention may be used to treat and/or prevent cardiovascular disorders, including peripheral artery disease, such as limb ischemia.

Cardiovascular disorders include cardiovascular abnormalities, such as arterio-arterial fistula, arteriovenous fistula, cerebral arteriovenous malformations, congenital heart defects, pulmonary atresia, and Scimitar Syndrome. Congenital heart defects include aortic coarctation, cor triatriatum, coronary vessel anomalies, crisscross heart, dextrocardia, patent ductus arteriosus, Ebstein's anomaly, Eisenmenger complex, hypoplastic left heart syndrome, levocardia, tetralogy of fallot, transposition of great vessels, double outlet right ventricle, tricuspid atresia, persistent truncus arteriosus, and heart septal defects, such as aortopulmonary septal defect, endocardial cushion defects, Lutembacher's Syndrome, trilogy of Fallot, ventricular heart septal defects.

Cardiovascular disorders also include heart disease, such as arrhythmias, carcinoid heart disease, high cardiac output, low cardiac output, cardiac tamponade, endocarditis (including bacterial), heart aneurysm, cardiac arrest, congestive heart failure, congestive cardiomyopathy, paroxysmal dyspnea, cardiac edema, heart hypertrophy, congestive cardiomyopathy, left ventricular hypertrophy, right ventricular hypertrophy, post-infarction heart rupture, ventricular septal rupture, heart valve diseases, myocardial diseases, myocardial ischemia, pericardial effusion, pericarditis (including constrictive and tuberculous), pneumopericardium, postpericardiotomy syndrome, pulmonary heart disease, rheumatic heart disease, ventricular dysfunction, hyperemia, cardiovascular pregnancy complications, Scimitar Syndrome, cardiovascular syphilis, and cardiovascular tuberculosis.

Arrhythmias include sinus arrhythmia, atrial fibrillation, atrial flutter, bradycardia, extrasystole, Adams-Stokes Syndrome, bundle-branch block, sinoatrial block, long QT syndrome, parasystole, Lown-Ganong-Levine Syndrome, Mahaim-type pre-excitation syndrome, Wolff-Parkinson-White syndrome, sick sinus syndrome, tachycardias, and ventricular fibrillation. Tachycardias include paroxysmal tachycardia, supraventricular tachycardia, accelerated idioventricular rhythm, atrioventricular nodal reentry tachycardia, ectopic atrial tachycardia, ectopic junctional tachycardia, sinoatrial nodal reentry tachycardia, sinus tachycardia, Torsades de Pointes, and ventricular tachycardia.

Heart valve disease include aortic valve insufficiency, aortic valve stenosis, hear murmurs, aortic valve prolapse, mitral valve prolapse, tricuspid valve prolapse, mitral valve insufficiency, mitral valve stenosis, pulmonary atresia, pulmonary valve insufficiency, pulmonary valve stenosis, tricuspid atresia, tricuspid valve insufficiency, and tricuspid valve stenosis.

Myocardial diseases include alcoholic cardiomyopathy, congestive cardiomyopathy, hypertrophic cardiomyopathy, aortic subvalvular stenosis, pulmonary subvalvular stenosis, restrictive cardiomyopathy, Chagas cardiomyopathy, endocardial fibroelastosis, endomyocardial fibrosis, Kearns Syndrome, myocardial reperfusion injury, and myocarditis.

Myocardial ischemias include coronary disease, such as angina pectoris, coronary aneurysm, coronary arteriosclerosis, coronary thrombosis, coronary vasospasm, myocardial infarction and myocardial stunning.

Cardiovascular diseases also include vascular diseases such as aneurysms, angiodysplasia, angiomatosis, bacillary angiomatosis, Hippel-Lindau Disease, Klippel-Trenaunay-Weber Syndrome, Sturge-Weber Syndrome, angioneurotic edema, aortic diseases, Takayasu's Arteritis, aortitis, Leriche's Syndrome, arterial occlusive diseases, arteritis, enarteritis, polyarteritis nodosa, cerebrovascular disorders, diabetic angiopathies, diabetic retinopathy, embolisms, thrombosis, erythromelalgia, hemorrhoids, hepatic veno-occlusive disease, hypertension, hypotension, ischemia, peripheral vascular diseases, phlebitis, pulmonary veno-

occlusive disease, Raynaud's disease, CREST syndrome, retinal vein occlusion, Scimitar syndrome, superior vena cava syndrome, telangiectasia, atacia telangiectasia, hereditary hemorrhagic telangiectasia, varicocele, varicose veins, varicose ulcer, vasculitis, and venous insufficiency.

Aneurysms include dissecting aneurysms, false aneurysms, infected aneurysms, ruptured aneurysms, aortic aneurysms, cerebral aneurysms, coronary aneurysms, heart aneurysms, and iliac aneurysms.

Arterial occlusive diseases include arteriosclerosis, intermittent claudication, carotid stenosis, fibromuscular dysplasias, mesenteric vascular occlusion, Moyamoya disease, renal artery obstruction, retinal artery occlusion, and thromboangiitis obliterans.

Cerebrovascular disorders include carotid artery diseases, cerebral amyloid angiopathy, cerebral aneurysm, cerebral anoxia, cerebral arteriosclerosis, cerebral arteriovenous malformation, cerebral artery diseases, cerebral embolism and thrombosis, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, cerebral hemorrhage, epidural hematoma, subdural hematoma, subaraxhnoid hemorrhage, cerebral infarction, cerebral ischemia (including transient), subclavian steal syndrome, periventricular leukomalacia, vascular headache, cluster headache, migraine, and vertebrobasilar insufficiency.

Embolisms include air embolisms, amniotic fluid embolisms, cholesterol embolisms, blue toe syndrome, fat embolisms, pulmonary embolisms, and thromoboembolisms. Thrombosis include coronary thrombosis, hepatic vein thrombosis, retinal vein occlusion, carotid artery thrombosis, sinus thrombosis, Wallenberg's syndrome, and thrombophlebitis.

Ischemia includes cerebral ischemia, ischemic colitis, compartment syndromes, anterior compartment syndrome, myocardial ischemia, reperfusion injuries, and peripheral limb ischemia. Vasculitis includes aortitis, arteritis, Behcet's Syndrome, Churg-Strauss Syndrome, mucocutaneous lymph node syndrome, thromboangiitis obliterans, hypersensitivity vasculitis, Schoenlein-Henoch purpura, allergic cutaneous vasculitis, and Wegener's granulomatosis.

In one embodiment, DR4 polynucleotides, polypeptides and/or antagonists of the invention is used to treat and/or prevent thrombotic microangiopathies. One such disorder is thrombotic thrombocytopenic purpura (TTP) (Kwaan, H.C., *Semin. Hematol. 24*:71 (1987); Thompson *et al., Blood 80*:1890 (1992)). Increasing TTP-associated mortality rates have been reported by the U.S. Centers for Disease Control (Torok *et al., Am. J. Hematol. 50*:84 (1995)). Plasma from patients afflicted with TTP (including HIV+ and HIV- patients) induces apoptosis of human endothelial cells of dermal microvascular origin, but not large vessel origin (Laurence *et al., Blood 87*:3245 (1996)). Plasma of TTP patients thus is thought to contain one or more factors that directly or indirectly induce apoptosis. As described in International patent application number WO 97/01633 (hereby incorporated by reference), TRAIL is present in the serum of TTP patients, and is likely to play a role in inducing apoptosis of microvascular endothelial cells. Another thrombotic microangiopathy is hemolytic-uremic syndrome (HUS) (Moake, J.L., *Lancet*, 343:393 (1994); Melnyk *et al., (Arch. Intern. Med., 155*:2077 (1995); Thompson *et al., supra*). Thus, in one embodiment, the invention is directed to use of DR4 to treat and/or prevent the condition that is often referred to as "adult HUS" (even though it can strike children as well). A disorder known as childhood/diarrhea-associated HUS differs in etiology from adult HUS. In another embodiment, conditions characterized by clotting of small blood vessels may be treated and/or prevented using DR4. Such conditions include, but are not limited to, those described herein. For example, cardiac problems seen in about 5-10% of pediatric AIDS patients are believed to involve clotting of small blood vessels. Breakdown of the microvasculature in the heart has been reported in multiple sclerosis patients. As a further example, treatment and/or prevention of systemic lupus erythematosus (SLE) is contemplated. In one embodiment, a patient's blood or plasma is contacted with DR4 polynucleotides and/or polypeptides of the invention *ex vivo*. The DR4 polynucleotides and/or polypeptides of the invention may be bound to a suitable chromatography matrix by procedures known in the art. According to this embodiment, the patient's

blood or plasma flows through a chromatography column containing DR4 polynucleotides and/or polypeptides of the invention bound to the matrix, before being returned to the patient. The immobilized DR4 binds TRAIL, thus removing TRAIL protein from the patient's blood. Alternatively, DR4 polynucleotides and/or polypeptides of the invention may be administered *in vivo* to a patient afflicted with a thrombotic microangiopathy. In one embodiment, a soluble form of DR4 polypeptide of the invention is administered to the patient. Thus, the present invention provides a method for treating and/or preventing a thrombotic microangiopathy, involving use of an effective amount of DR4. A DR4 polypeptide may be employed in *in vivo* or *ex vivo* procedures, to inhibit TRAIL-mediated damage to (*e.g.*, apoptosis of) microvascular endothelial cells.

DR4 polynucleotides and/or polypeptides of the invention may be employed in combination with other agents useful in treating and/or preventing a particular disorder. For example, in an *in vitro* study reported by Laurence *et al.* (*Blood* 87:3245 (1996)), some reduction of TTP plasma-mediated apoptosis of microvascular endothelial cells was achieved by using an anti-Fas blocking antibody, aurintricarboxylic acid, or normal plasma depleted of cryoprecipitate. Thus, a patient may be treated with a polynucleotide and/or polypeptide of the invention in combination with an agent that inhibits Fas-ligand-mediated apoptosis of endothelial cells, such as, for example, an agent described above. In one embodiment, DR4 polynucleotides and/or polypeptides of the invention and an anti-FAS blocking antibody are both administered to a patient afflicted with a disorder characterized by thrombotic microanglopathy, such as TTP or HUS. Examples of blocking monoclonal antibodies directed against Fas antigen (CD95) are described in International patent application publication number WO 95/10540, hereby incorporated by reference.

The naturally occurring balance between endogenous stimulators and inhibitors of angiogenesis is one in which inhibitory influences predominate (Rastinejad *et al.*, Cell 56:345-355 (1989)). In those rare instances in which neovascularization occurs under normal physiological conditions, such as wound

healing, organ regeneration, embryonic development, and female reproductive processes, angiogenesis is stringently regulated and spatially and temporally delimited. Under conditions of pathological angiogenesis such as that characterizing solid tumor growth, these regulatory controls fail. Unregulated angiogenesis becomes pathologic and sustains progression of many neoplastic and non-neoplastic diseases. A number of serious diseases are dominated by abnormal neovascularization including solid tumor growth and metastases, arthritis, some types of eye disorders, and psoriasis. See, *e.g.*, reviews by Moses *et al., Biotech. 9*:630-634 (1991); *Folkman et al., N. Engl. J. Med., 333*:1757-1763 (1995); Auerbach *et al., J. Microvasc. Res. 29*:401-411 (1985); Folkman, *Advances in Cancer Research*, eds. Klein and Weinhouse, Academic Press, New York, pp. 175-203 (1985); Patz, *Am. J. Opthalmol. 94*:715-743 (1982); and *Folkman et al., Science 221*:719-725 (1983). In a number of pathological conditions, the process of angiogenesis contributes to the disease state. For example, significant data have accumulated which suggest that the growth of solid tumors is dependent on angiogenesis. Folkman and Klagsbrun, *Science 235*:442-447 (1987).

The present invention provides for treatment and/or prevention of diseases or disorders associated with neovascularization by administration of the DR4 polynucleotides and/or polypeptides of the invention (including DR4 agonists and/or antagonists). Malignant and metastatic conditions which can be treated and/or prevented with the polynucleotides and polypeptides of the invention include, but are not limited to those malignancies, solid tumors, and cancers described herein and otherwise known in the art (for a review of such disorders, see Fishman *et al., Medicine*, 2d Ed., J. B. Lippincott Co., Philadelphia (1985)).

Additionally, ocular disorders associated with neovascularization which can be treated and/or prevented with the DR4 polynucleotides and polypeptides of the present invention (including DR4 agonists and DR4 antagonists) include, but are not limited to: neovascular glaucoma, diabetic retinopathy, retinoblastoma, retrolental fibroplasia, uveitis, retinopathy of prematurity macular degeneration, corneal graft neovascularization, as well as other eye inflammatory diseases,

ocular tumors and diseases associated with choroidal or iris neovascularization. See, *e.g.*, reviews by Waltman *et al.*, *Am. J. Ophthal.* *85*:704-710 (1978) and Gartner *et al.*, *Surv. Ophthal.* *22*:291-312 (1978).

Additionally, disorders which can be treated and/or prevented with the DR4 polynucleotides and polypeptides of the present invention (including DR4 agonists and DR4 antagonists) include, but are not limited to, hemangioma, arthritis, psoriasis, angiofibroma, atherosclerotic plaques, delayed wound healing, granulations, hemophilic joints, hypertrophic scars, nonunion fractures, Osler-Weber syndrome, pyogenic granuloma, scleroderma, trachoma, and vascular adhesions.

Polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful in the diagnosis, prognosis, treatment and/or prevention of a wide range of diseases and/or conditions. Such diseases and conditions include, but are not limited to, cancer (*e.g.*, immune cell related cancers, breast cancer, prostate cancer, ovarian cancer, follicular lymphoma, cancer associated with mutation or alteration of p53, brain tumor, bladder cancer, uterocervical cancer, colon cancer, colorectal cancer, non-small cell carcinoma of the lung, small cell carcinoma of the lung, stomach cancer, etc.), lymphoproliferative disorders (*e.g.*, lymphadenopathy), microbial (*e.g.*, viral, bacterial, etc.) infection (*e.g.*, HIV-1 infection, HIV-2 infection, herpesvirus infection (including, but not limited to, HSV-1, HSV-2, CMV, VZV, HHV-6, HHV-7, EBV), adenovirus infection, poxvirus infection, human papilloma virus infection, hepatitis infection (*e.g.*, HAV, HBV, HCV, etc.), *Helicobacter pylori* infection, invasive Staphylococcia, etc.), parasitic infection, nephritis, bone disease (*e.g.*, osteoporosis), atherosclerosis, pain, cardiovascular disorders (*e.g.*, neovascularization, hypovascularization or reduced circulation (*e.g.*, ischemic disease (*e.g.*, myocardial infarction, stroke, etc.))), AIDS, allergy, inflammation, neurodegenerative disease (*e.g.*, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, pigmentary retinitis, cerebellar degeneration, etc.), graft rejection (acute and chronic), graft vs. host disease, diseases due to

osteomyelodysplasia (*e.g.*, aplastic anemia, etc.), joint tissue destruction in rheumatism, liver disease (*e.g.*, acute and chronic hepatitis, liver injury, and cirrhosis), autoimmune disease (*e.g.*, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, immune complex glomerulonephritis, autoimmune diabetes, autoimmune thrombocytopenic purpura, Grave's disease, Hashimoto's thyroiditis, etc.), cardiomyopathy (*e.g.*, dilated cardiomyopathy), diabetes, diabetic complications (*e.g.*, diabetic nephropathy, diabetic neuropathy, diabetic retinopathy), influenza, asthma, psoriasis, glomerulonephritis, septic shock, and ulcerative colitis.

Polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful in promoting angiogenesis, wound healing (*e.g.*, wounds, burns, and bone fractures).

Polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are also useful as an adjuvant to enhance immune responsiveness to specific antigen, anti-viral immune responses.

More generally, polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful in regulating (*i.e.*, elevating or reducing) immune response. For example, polynucleotides and/or polypeptides of the invention may be useful in preparation or recovery from surgery, trauma, radiation therapy, chemotherapy, and transplantation, or may be used to boost immune response and/or recovery in the elderly and immunocompromised individuals. Alternatively, polynucleotides and/or polypeptides of the invention and/or agonists and/or antagonists thereof are useful as immunosuppressive agents, for example in the treatment and/or prevention of autoimmune disorders. In specific embodiments, polynucleotides and/or polypeptides of the invention are used to treat and/or prevent chronic inflammatory, allergic or autoimmune conditions, such as those described herein or are otherwise known in the art.

In one aspect, the present invention is directed to a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR4 polypeptide an effective amount of DR4 ligand, analog

or an agonist capable of increasing DR4 mediated signaling. Preferably, DR4 mediated signaling is increased to treat and/or prevent a disease wherein decreased apoptosis or decreased cytokine and adhesion molecule expression is exhibited. An agonist can include soluble forms of DR4 and monoclonal antibodies directed against the DR4 polypeptide.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the, DR4 polypeptide an effective amount of an antagonist capable of decreasing DR4 mediated signaling. Preferably, DR4 mediated signaling is decreased to treat and/or prevent a disease wherein increased apoptosis or NF-kB expression is exhibited. An antagonist can include soluble forms of DR4 (*e.g.*, polypeptides containing all or a portion of the DR4 extracellular domain) and monoclonal antibodies directed against the DR4 polypeptide.

By "agonist" is intended naturally occurring and synthetic compounds capable of enhancing or potentiating apoptosis (*e.g.*, stimulating DR4 activities). By "antagonist" is intended naturally occurring and synthetic compounds capable of inhibiting apoptosis (*e.g.*, inhibiting DR4 activities). Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below.

One such screening procedure involves the use of melanophores which are transfected to express the receptor of the present invention. Such a screening technique is described in PCT WO 92/01810, published February 6, 1992. Such an assay may be employed, for example, for screening for a compound which inhibits (or enhances) activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with both a TNF-family ligand and the candidate antagonist (or agonist). Inhibition or enhancement of the signal generated by the ligand indicates that the compound is an antagonist or agonist of the ligand/receptor signaling pathway.

Other screening techniques include the use of cells which express the receptor (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in *Science 246*:181-296 (October 1989). For example, compounds may be contacted with a cell which expresses the receptor polypeptide of the present invention and a second messenger response, *e.g.*, signal transduction or pH changes, may be measured to determine whether the potential compound activates or inhibits the receptor.

Another such screening technique involves introducing RNA encoding the receptor into *Xenopus* oocytes to transiently express the receptor. The receptor oocytes may then be contacted with the receptor ligand and a compound to be screened, followed by detection of inhibition or activation of a calcium signal in the case of screening for compounds which are thought to inhibit activation of the receptor.

Another screening technique involves expressing in cells a construct wherein the receptor is linked to a phospholipase C or D. Such cells include endothelial cells, smooth muscle cells, embryonic kidney cells, etc. The screening may be accomplished as herein above described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase signal.

Another method involves screening for compounds which inhibit activation of the receptor polypeptide of the present invention antagonists by determining inhibition of binding of labeled ligand to cells which have the receptor on the surface thereof. Such a method involves transfecting a eukaryotic cell with DNA encoding the receptor such that the cell expresses the receptor on its surface and contacting the cell with a compound in the presence of a labeled form of a known ligand. The ligand can be labeled, *e.g.*, by radioactivity. The amount of labeled ligand bound to the receptors is measured, *e.g.*, by measuring radioactivity of the receptors. If the compound binds to the receptor as determined by a reduction of labeled ligand which binds to the receptors, the binding of labeled ligand to the receptor is inhibited.

Further screening assays for agonist and antagonist of the present invention are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem. 267(7)*:4304-4307(1992).

Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR4 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response to a candidate compound and/or a TNF-family ligand (*e.g.*, determining or estimating an increase or decrease in T-cell proliferation or tritiated thymidine labeling). By the invention, a cell expressing the DR4 polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Agonist according to the present invention include naturally occurring and synthetic compounds such as, for example, TNF family ligand peptide fragments, transforming growth factor, neurotransmitters (such as glutamate, dopamine, *N*-methyl-D-aspartate), tumor suppressors (p53), cytolytic T-cells and antimetabolites. Preferred agonist include chemotherapeutic drugs such as, for example, cisplatin, doxorubicin, bleomycin, cytosine arabinoside, nitrogen mustard, methotrexate and vincristine. Others include ethanol and β-amyloid peptide. (*Science 267*:1457-1458 (1995)). Further preferred agonist include polyclonal and monoclonal antibodies raised against the DR4 polypeptide, or a fragment thereof. Such agonist antibodies raised against a TNF-family receptor are disclosed in Tartaglia, L.A., *et al., Proc. Natl. Acad. Sci. USA 88*:9292-9296

(1991); and Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem. 267* (7):4304-4307 (1992). See, also, PCT Application WO 94/09137.

Antagonist according to the present invention include naturally occurring and synthetic compounds such as, for example, the CD40 ligand, neutral amino acids, zinc, estrogen, androgens, viral genes (such as Adenovirus *E1B*, Baculovirus *p35* and *IAP*, Cowpox virus *crmA*, Epstein-Barr virus *BHRF1, LMP-1,* African swine fever virus *LMW5-HL,* and Herpesvirus yl 34.5), calpain inhibitors, cysteine protease inhibitors, and tumor promoters (such as PMA, Phenobarbital, and hexachlorocyclohexanes (*e.g.*, α-, β-, or γ-hexachlorocyclohexane).

Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through triple-helix formation. Antisense techniques are discussed, for example, in Okano, *J. Neurochem. 56*:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance Lee *et al., Nucleic Acids Research 6*:3073 (1979); Cooney *et al., Science 241*:456 (1988); and Dervan *et al., Science 251*:1360 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into receptor polypeptide. The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the DR4 receptor.

In one embodiment, the DR4 antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For

example, a vector or a portion thereof, is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the DR4 antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others know in the art, used for replication and expression in vertebrate cells. Expression of the sequence encoding DR4, or fragments thereof, can be by any promoter known in the art to act in vertebrate, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, *Nature* 29:304-310 (1981), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, *Cell* 22:787-797 (1980), the herpes thymidine promoter (Wagner *et al.*, *Proc. Natl. Acad. Sci.* U.S.A. 78:1441-1445 (1981), the regulatory sequences of the metallothionein gene (Brinster *et al.*, *Nature* 296:39-42 (1982)), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a DR4 gene. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double stranded DR4 antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the larger the hybridizing nucleic acid, the more base mismatches with a DR4 RNA it may contain and still form a stable duplex (or triplex as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, *e.g.*, the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. See generally, Wagner, R., *Nature 372*:333-335 (1994). Thus, oligonucleotides complementary to either the 5'- or 3'- non- translated, non-coding regions of the DR4 shown in SEQ ID NO:1 could be used in an antisense approach to inhibit translation of endogenous DR4 mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5'-, 3'- or coding region of DR4 mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

The polynucleotides of the invention can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al., Proc. Natl. Acad. Sci. U.S.A. 86*:6553-6556 (1989); Lemaitre *et al., Proc. Natl. Acad. Sci. 84*:648-652 (1987); PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, *e.g.*, PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents. (*See, e.g.*, Krol *et al., BioTechniques 6*:958-976 (1988)) or intercalating agents. (*See, e.g.*, Zon, *Pharm. Res. 5*:539-549 (1988)). To this end, the oligonucleotide may be conjugated to

another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group including, but not limited to, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier *et al., Nucl. Acids Res. 15*:6625-6641 (1987)). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue *et al., Nucl.*

Acids Res. 15:6131-6148 (1987)), or a chimeric RNA-DNA analogue (Inoue *et al., FEBS Lett. 215*:327-330 (1987)).

Polynucleotides of the invention may be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al.* (*Nucl. Acids Res. 16*:3209 (1988)), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (*Sarin et al., Proc. Natl. Acad. Sci. U.S.A. 85*:7448-7451 (1988)), etc.

While antisense nucleotides complementary to the DR4 coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred.

Potential antagonists according to the invention also include catalytic RNA, or a ribozyme (*See, e.g.*, PCT International Publication WO 90/11364, published October 4, 1990; Sarver *et al, Science 247*:1222-1225 (1990). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy DR4 mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, *Nature 334*:585-591 (1988). There are numerous potential hammerhead ribozyme cleavage sites within the nucleotide sequence of DR4 (SEQ ID NO:2). Preferably, the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the DR4 mRNA; *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

As in the antisense approach, the ribozymes of the invention can be composed of modified oligonucleotides (*e.g.* for improved stability, targeting, etc.) and should be delivered to cells which express DR4 *in vivo*. DNA constructs

encoding the ribozyme may be introduced into the cell in the same manner as described above for the introduction of antisense encoding DNA. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive promoter, such as, for example, pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous DR4 messages and inhibit translation. Since ribozymes, unlike antisense molecules are catalytic, a lower intracellular concentration is required for efficiency.

Endogenous gene expression can also be reduced by inactivating or "knocking out" the DR4 gene and/or its promoter using targeted homologous recombination. (*e.g., see* Smithies *et al., Nature 317*:230-234 (1985); Thomas & Capecchi, *Cell 51*:503-512 (1987); Thompson *et al., Cell 5*:313-321 (1989); each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional polynucleotide of the invention (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous polynucleotide sequence (either the coding regions or regulatory regions of the gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express polypeptides of the invention *in vivo*. In another embodiment, techniques known in the art are used to generate knockouts in cells that contain, but do not express the gene of interest. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the targeted gene. Such approaches are particularly suited in research and agricultural fields where modifications to embryonic stem cells can be used to generate animal offspring with an inactive targeted gene (*e.g., see* Thomas & Capecchi (1987) and Thompson (1989), *supra*). However this approach can be routinely adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors that will be apparent to those of skill in the art. The contents of each of the documents recited in this paragraph is herein incorporated by reference in its entirety.

Further antagonist according to the present invention include soluble forms of DR4, *i.e.*, DR4 fragments that include the ligand binding domain from the extracellular region of the full length receptor. Such soluble forms of the receptor, which may be naturally occurring or synthetic, antagonize DR4 mediated signaling by competing with the cell surface DR4 for binding to TNF-family ligands. Thus, soluble forms of the receptor that include the ligand binding domain are novel cytokines capable of inhibiting apoptosis induced by TNF-family ligands. These are preferably expressed as dimers or trimers, since these have been shown to be superior to monomeric forms of soluble receptor as antagonists, *e.g.*, IgGFc-TNF receptor family fusions. Other such cytokines are known in the art and include Fas B (a soluble form of the mouse Fas receptor) that acts physiologically to limit apoptosis induced by Fas ligand (Hughes, D.P. and Crispe, I.N., *J. Exp. Med. 182*:1395-1401 (1995)).

The experiments set forth in Example 5 demonstrates that DR4 is a death domain-containing molecule capable of triggering apoptosis which is important in the regulation of the immune system. In addition, the experiments set forth below demonstrate that DR4-induced apoptosis was blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk. Thus, inhibitors of ICE-like proteases, FADD-DN and FLICE-DN/MACHa1C360S could also be used as antagonists for DR4 activity.

As discussed above, the term "antibody" (Ab) or "monoclonal antibody" (mAb) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and $F(ab')_2$ fragments) which are capable of binding an antigen. Fab and F $(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al., J. Nucl. Med. 24*:316-325 (1983)).

Antibodies according to the present invention may be prepared by any of a variety of methods using DR4 immunogens of the present invention. As indicated, such DR4 immunogens include the full length (complete) DR4

polypeptide (which may or may not include the leader sequence) and DR4 polypeptide fragments such as the ligand binding domain, the transmembrane domain, the intracellular domain and the death domain.

Proteins and other compounds which bind the DR4 domains are also candidate agonist and antagonist according to the present invention. Such binding compounds can be "captured" using the yeast two-hybrid system (Fields and Song, *Nature 340*:245-246 (1989)). A modified version of the yeast two-hybrid system has been described by Roger Brent and his colleagues (Gyuris, J. *et al.*, *Cell 75*:791-803 (1993); Zervos, A.S. *et al.*, *Cell 72*:223-232 (1993)). Preferably, the yeast two-hybrid system is used according to the present invention to capture compounds which bind to either the DR4 ligand binding domain or to the DR4 intracellular domain. Such compounds are good candidate agonist and antagonist of the present invention.

By a "TNF-family ligand" is intended naturally occurring, recombinant, and synthetic ligands that are capable of binding to a member of the TNF receptor family and inducing and/or blocking the ligand/receptor signaling pathway. Members of the TNF ligand family include, but are not limited to, DR4 ligands, TRAIL, TNF-α, TNF-β-α, lymphotoxin-α (LT-α, also known as TNF-β), LT-β (found in complex heterotrimer LT-α2-β), FasL, VEGI (International Publication No. WO 96/14328), AIM-I (International Publication No. WO 97/33899), AIM-II (International Publication No. WO 97/34911), APRIL (J. Exp. Med. 188(6):1185-1190), endokine-alpha (International Publication No. WO 98/07880), neutrokine-alpha (International Publication No. WO 98/18921), CD40L, CD27L, CD30L, 4-lBBL, OX40L and nerve growth factor (NGF).

Gene Therapy

In a specific embodiment, nucleic acids comprising sequences encoding antibodies or functional derivatives thereof, are administered to treat, inhibit and/or prevent a disease or disorder associated with aberrant expression and/or

activity of a polypeptide of the invention, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded protein that mediates a therapeutic effect.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel *et al.*, 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the compound comprises nucleic acid sequences encoding an antibody, said nucleic acid sequences being part of expression vectors that express the antibody or fragments or chimeric proteins or heavy or light chains thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the antibody coding region, said promoter being inducible or constitutive, and, optionally, tissue- specific. In another particular embodiment, nucleic acid molecules are used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody nucleic acids (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438). In specific embodiments, the expressed antibody molecule is a single chain antibody; alternatively, the nucleic acid sequences include sequences encoding both the heavy and light chains, or fragments thereof, of the antibody.

Delivery of the nucleic acids into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid- carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered in vivo, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing them as part of an appropriate nucleic acid expression vector and administering it so that they become intracellular, *e.g.*, by infection using defective or attenuated retrovirals or other viral vectors (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, 1987, *J. Biol. Chem. 262*:4429-4432) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (*see, e.g.*, PCT Publications WO 92/06180 dated April 16, 1992 (Wu *et al.*); WO 92/22635 dated December 23, 1992 (Wilson *et al.*); WO92/20316 dated November 26, 1992 (Findeis *et al.*); WO93/14188 dated July 22, 1993 (Clarke *et al.*), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438).

In a specific embodiment, viral vectors that contains nucleic acid sequences encoding an antibody of the invention are used. For example, a retroviral vector can be used (see Miller *et al.*, 1993, Meth. Enzymol. 217:581-599). These retroviral vectors have been to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The nucleic acid sequences encoding the antibody to be used in gene therapy are cloned into one or more vectors, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen *et al.*, 1994, Biotherapy 6:291-302, which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes *et al.*, 1994, J. Clin. Invest. 93:644-651; Kiem *et al.*, 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, Current Opinion in Genetics and Development 3:499-503 present a review of adenovirus-based gene therapy. Bout *et al.*, 1994, Human Gene Therapy 5:3-10 demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld *et al.*, 1991, Science 252:431-434; Rosenfeld *et al.*, 1992, Cell 68:143- 155; Mastrangeli *et al.*, 1993, J. Clin. Invest. 91:225-234; PCT Publication WO94/12649; and Wang *et al.*, 1995, Gene Therapy 2:775-783. In a preferred embodiment, adenovirus vectors are used.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh *et al.*, 1993, Proc. Soc. Exp. Biol. Med. 204:289-300; U.S. Patent No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (*see, e.g.*, Loeffler and Behr, 1993, *Meth. Enzymol. 217*:599-618; Cohen *et al.*, 1993, *Meth. Enzymol. 217*:618-644; Cline, 1985, *Pharmac. Ther. 29*:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited

to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T-lymphocytes, B-lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding an antibody are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention (*see, e.g.*, PCT Publication WO 94/08598, dated April 28, 1994; Stemple and Anderson, 1992, *Cell 71*:973-985; Rheinwald, 1980, *Meth. Cell Bio. 21A*:229; and Pittelkow and Scott, 1986, *Mayo Clinic Proc. 61*:771).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

Modes of Administration

The invention provides methods of treatment, inhibition and prophylaxis by administration to a subject of an effective amount of a compound or pharmaceutical composition of the invention, preferably an antibody of the invention. In a preferred aspect, the compound is substantially purified (*e.g.*, substantially free from substances that limit its effect or produce undesired side-

effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid or an immunoglobulin are described above; additional appropriate formulations and routes of administration can be selected from among those described herein below.

The agonist or antagonists described herein can be administered *in vitro*, *ex vivo*, or *in vivo* to cells which express the receptor of the present invention. By administration of an "effective amount" of an agonist or antagonist is intended an amount of the compound that is sufficient to enhance or inhibit a cellular response to a TNF-family ligand and include polypeptides. In particular, by administration of an "effective amount" of an agonist or antagonists is intended an amount effective to enhance or inhibit DR4 mediated apoptosis. Of course, where apoptosis is to be enhanced, an agonist according to the present invention can be co-administered with a TNF-family ligand. One of ordinary skill will appreciate that effective amounts of an agonist or antagonist can be determined empirically and may be employed in pure form or in pharmaceutically acceptable salt, ester or prodrug form. The agonist or antagonist may be administered in compositions in combination with one or more pharmaceutically acceptable excipients.

It will be understood that, when administered to a human patient, the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgement. The specific therapeutically effective dose level for any particular patient will depend upon factors well known in the medical arts.

As a general proposition, the total pharmaceutically effective amount of DR4 polypeptide administered parenterally per dose will be in the range of about 1 μg/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1

mg/kg/day for the hormone. If given continuously, the DR4 agonists or antagonists is typically administered at a dose rate of about 1 μg/kg/hour to about 50 μg/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed.

Dosaging may also be arranged in a patient specific manner to provide a predetermined concentration of an agonist or antagonist in the blood, as determined by the RIA technique. Thus patient dosaging may be adjusted to achieve regular on-going trough blood levels, as measured by RIA, on the order of from 50 to 1000 ng/ml, preferably 150 to 500 ng/ml.

Pharmaceutical compositions of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use.

In addition to soluble DR4 polypeptides, DR4 polypeptide containing the transmembrane region can also be used when appropriately solubilized by including detergents, such as CHAPS or NP-40, with buffer.

The compounds or pharmaceutical compositions of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays to demonstrate the therapeutic or prophylactic utility of a compound or pharmaceutical composition include, the effect of a compound on a cell line or a patient tissue sample. The effect of the compound or composition on the cell line and/or tissue sample can be determined utilizing techniques known to those of skill in the art including, but not limited to, rosette formation assays and cell lysis assays. In accordance with the invention, *in vitro* assays which can be used to determine whether administration of a specific compound is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a compound, and the effect of such compound upon the tissue sample is observed.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In particular embodiments, pharmaceutical compositions are provided comprising an agonist or antagonist and a pharmaceutically acceptable carrier or excipient, which may be administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), bucally, or as an oral or nasal spray. Importantly, by co-administering an agonist and a TNF-family ligand, clinical side effects can be reduced by using lower doses of both the ligand and the agonist. It will be understood that the agonist can be "co-administered" either before, after, or simultaneously with the TNF-family ligand, depending on the exigencies of a particular therapeutic application. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol,

water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc.,

and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

As indicated above, the compositions of the invention may be administered alone or in combination with other therapeutic agents. Therapeutic agents that may be administered in combination with the compositions of the invention, include but are not limited to, other members of the TNF family, chemotherapeutic agents, antibiotics, steroidal and non-steroidal anti-inflammatories, conventional immunotherapeutic agents, cytokines, chemokines and/or growth factors. Combinations may be administered either concomitantly, *e.g.*, as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture, and also procedures in which the combined agents are administered separately but simultaneously, *e.g.*, as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

In one embodiment, the compositions of the invention are administered in combination with other members of the TNF family. TNF, TNF-related or TNF-like molecules that may be administered with the compositions of the invention include, but are not limited to, soluble forms of TNF-alpha, lymphotoxin-alpha (LT-alpha, also known as TNF-beta), LT-beta (found in complex heterotrimer LT-alpha2-beta), OPGL, FasL, CD27L, CD30L, CD40L, 4-1BBL, DcR3, OX40L, TNF-gamma (International Publication No. WO 96/14328), (International Publication No. WO 96/14328), TNF-γ-α, TNF-γ-β (International Publication No. WO 00/08139), TRAIL, AIM-II (International Publication No. WO 97/34911), APRIL (J. Exp. Med. 188(6):1185-1190), endokine-alpha (International Publication No. WO 98/07880), TR6 (International Publication No. WO 98/30694), OPG, and neutrokine-alpha (International Publication No. WO 98/18921, OX40, and nerve growth factor (NGF), and soluble forms of Fas,

CD30, CD27, CD40 and 4-IBB, TR2 (International Publication No. WO 96/34095), DR3 (International Publication No. WO 97/33904), TR5 (International Publication No. WO 98/30693), TR6 (International Publication No. WO 98/30694), TR7 (International Publication No. WO 98/41629), TRANK, TR9 (International Publication No. WO 98/56892), TR10 (International Publication No. WO 98/54202), 312C2 (International Publication No. WO 98/06842), and TR12, AIM-I (International Publication No. WO 97/33899), and soluble forms CD154, CD70, and CD153.

In another embodiment, the compositions of the invention are administered in combination with CD40 ligand (CD40L), a soluble form of CD40L (*e.g.*, AVREND™), biologically active fragments, variants, or derivatives of CD40L, anti-CD40L antibodies (*e.g.*, agonistic or antagonistic antibodies), and/or anti-CD40 antibodies (*e.g.*, agonistic or antagonistic antibodies).

In yet another embodiment, the compositions of the invention are administered in combination with one, two, three, four, five, or more of the following compositions: tacrolimus (Fujisawa), thalidomide (*e.g.*, Celgene), anti-Tac(Fv)-PE40 (*e.g.*, Protein Design Labs), inolimomab (Biotest), MAK-195F (Knoll), ASM-981 (Novartis), interleukin-1 receptor (*e.g.*, Immunex), interleukin-4 receptor (*e.g.*, Immunex), ICM3 (ICOS), BMS-188667 (Bristol-Myers Squibb), anti-TNF Ab (*e.g.*, Therapeutic antibodies), CG-1088 (Celgene), anti-B7 monoclonal antibody (*e.g.*, Innogetics), MEDI-507 (BioTransplant), ABX-CBL (Abgenix).

According to the invention, a patient susceptible to both Fas ligand (Fas-L) mediated and TRAIL mediated cell death may be treated with both an agent that inhibits TRAIL/TRAIL-R interactions and an agent that inhibits Fas-L/Fas interactions. Suitable agents for blocking binding of Fas-L to Fas include, but are not limited to, soluble Fas polypeptides; oligomeric forms of soluble Fas polypeptides (*e.g.*, dimers of sFas/Fc); anti-Fas antibodies that bind Fas without transducing the biological signal that results in apoptosis; anti-Fas-L antibodies that block binding of Fas-L to Fas; and muteins of Fas-L that bind Fas

but do not transduce the biological signal that results in apoptosis. Preferably, the antibodies employed according to this method are monoclonal antibodies. Examples of suitable agents for blocking Fas-L/Fas interactions, including blocking anti-Fas monoclonal antibodies, are described in WO 95/10540, hereby incorporated by reference.

In certain embodiments, compositions of the invention are administered in combination with antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors. Nucleoside reverse transcriptase inhibitors that may be administered in combination with the compositions of the invention, include, but are not limited to, RETROVIR™ (zidovudine/AZT), VIDEX™ (didanosine/ddI), HIVID™ (zalcitabine/ddC), ZERIT™ (stavudine/d4T), EPIVIR™ (lamivudine/3TC), and COMBIVIR™ (zidovudine/lamivudine). Non-nucleoside reverse transcriptase inhibitors that may be administered in combination with the compositions of the invention, include, but are not limited to, VIRAMUNE™ (nevirapine), RESCRIPTOR™ (delavirdine), and SUSTIVA™ (efavirenz). Protease inhibitors that may be administered in combination with the compositions of the invention, include, but are not limited to, CRIXIVAN™ (indinavir), NORVIR™ (ritonavir), INVIRASE™ (saquinavir), and VIRACEPT™ (nelfinavir). In a specific embodiment, antiretroviral agents, nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and/or protease inhibitors may be used in any combination with compositions of the invention to treat AIDS and/or to prevent or treat HIV infection.

In other embodiments, compositions of the invention may be administered in combination with anti-opportunistic infection agents. Anti-opportunistic agents that may be administered in combination with the compositions of the invention, include, but are not limited to, TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™, PENTAMIDINE™, ATOVAQUONE™, ISONIAZID™, RIFAMPIN™, PYRAZINAMIDE™, ETHAMBUTOL™, RIFABUTIN™, CLARITHROMYCIN™, AZITHROMYCIN™, GANCICLOVIR™,

FOSCARNET™, CIDOFOVIR™, FLUCONAZOLE™, ITRACONAZOLE™, KETOCONAZOLE™, ACYCLOVIR™, FAMCICOLVIR™, PYRIMETHAMINE™, LEUCOVORIN™, NEUPOGEN™ (filgrastim/G-CSF), and LEUKINE™ (sargramostim/GM-CSF). In a specific embodiment, compositions of the invention are used in any combination with TRIMETHOPRIM-SULFAMETHOXAZOLE™, DAPSONE™, PENTAMIDINE™, and/or ATOVAQUONE™ to prophylactically treat and/or prevent an opportunistic *Pneumocystis carinii* pneumonia infection. In another specific embodiment, compositions of the invention are used in any combination with ISONIAZID™, RIFAMPIN™, PYRAZINAMIDE™, and/or ETHAMBUTOL™ to prophylactically treat and/or prevent an opportunistic *Mycobacterium avium* complex infection. In another specific embodiment, compositions of the invention are used in any combination with RIFABUTIN™, CLARITHROMYCIN™, and/or AZITHROMYCIN™ to prophylactically treat and/or prevent an opportunistic *Mycobacterium tuberculosis* infection. In another specific embodiment, compositions of the invention are used in any combination with GANCICLOVIR™, FOSCARNET™, and/or CIDOFOVIR™ to prophylactically treat and/or prevent an opportunistic cytomegalovirus infection. In another specific embodiment, compositions of the invention are used in any combination with FLUCONAZOLE™, ITRACONAZOLE™, and/or KETOCONAZOLE™ to prophylactically treat and/or prevent an opportunistic fungal infection. In another specific embodiment, compositions of the invention are used in any combination with ACYCLOVIR™ and/or FAMCICOLVIR™ to prophylactically treat and/or prevent an opportunistic herpes simplex virus type I and/or type II infection. In another specific embodiment, compositions of the invention are used in any combination with PYRIMETHAMINE™ and/or LEUCOVORIN™ to prophylactically treat and/or prevent an opportunistic *Toxoplasma gondii* infection. In another specific embodiment, compositions of the invention are used in any combination with LEUCOVORIN™ and/or NEUPOGEN™ to prophylactically treat and/or prevent an opportunistic bacterial

infection.

In a further embodiment, the compositions of the invention are administered in combination with an antiviral agent. Antiviral agents that may be administered with the compositions of the invention include, but are not limited to, acyclovir, ribavirin, amantadine, and remantidine.

In a further embodiment, the compositions of the invention are administered in combination with an antibiotic agent. Antibiotic agents that may be administered with the compositions of the invention include, but are not limited to, amoxicillin, aminoglycosides, beta-lactam (glycopeptide), beta-lactamases, Clindamycin, chloramphenicol, cephalosporins, ciprofloxacin, ciprofloxacin, erythromycin, fluoroquinolones, macrolides, metronidazole, penicillins, quinolones, rifampin, streptomycin, sulfonamide, tetracyclines, trimethoprim, trimethoprim-sulfamthoxazole, and vancomycin.

Conventional nonspecific immunosuppressive agents, that may be administered in combination with the compositions of the invention include, but are not limited to, steroids, cyclosporine, cyclosporine analogs, cyclophosphamide methylprednisone, prednisone, azathioprine, FK-506, 15-deoxyspergualin, and other immunosuppressive agents that act by suppressing the function of responding T-cells.

In specific embodiments, compositions of the invention are administered in combination with immunosuppressants. Immunosuppressants preparations that may be administered with the compositions of the invention include, but are not limited to, ORTHOCLONE™ (OKT3), SANDIMMUNE™/NEORAL™/ SANGDYA™ (cyclosporin), PROGRAF™ (tacrolimus), CELLCEPT™ (mycophenolate), Azathioprine, glucorticosteroids, and RAPAMUNE™ (sirolimus). In a specific embodiment, immunosuppressants may be used to prevent rejection of organ or bone marrow transplantation.

In an additional embodiment, compositions of the invention are administered alone or in combination with one or more intravenous immune globulin preparations. Intravenous immune globulin preparations that may be

administered with the compositions of the invention include, but not limited to, GAMMAR™, IVEEGAM™, SANDOGLOBULIN™, GAMMAGARD S/D™, and GAMIMUNE™. In a specific embodiment, compositions of the invention are administered in combination with intravenous immune globulin preparations in transplantation therapy (*e.g.*, bone marrow transplant).

In a further embodiment, the compositions of the invention are administered in combination with an antibiotic agent. Antibiotic agents that may be administered with the compositions of the invention include, but are not limited to, tetracycline, metronidazole, amoxicillin, beta-lactamases, aminoglycosides, macrolides, quinolones, fluoroquinolones, cephalosporins, erythromycin, ciprofloxacin, and streptomycin.

In an additional embodiment, the compositions of the invention are administered alone or in combination with an anti-inflammatory agent. Anti-inflammatory agents that may be administered with the compositions of the invention include, but are not limited to, glucocorticoids and the nonsteroidal anti-inflammatories, aminoarylcarboxylic acid derivatives, arylacetic acid derivatives, arylbutyric acid derivatives, arylcarboxylic acids, arylpropionic acid derivatives, pyrazoles, pyrazolones, salicylic acid derivatives, thiazinecarboxamides, e-acetamidocaproic acid, S-adenosylmethionine, 3-amino-4-hydroxybutyric acid, amixetrine, bendazac, benzydamine, bucolome, difenpiramide, ditazol, emorfazone, guaiazulene, nabumetone, nimesulide, orgotein, oxaceprol, paranyline, perisoxal, pifoxime, proquazone, proxazole, and tenidap.

In one embodiment, the compositions of the invention are administered in combination with steroid therapy. Steroids that may be administered in combination with the compositions of the invention, include, but are not limited to, oral corticosteroids, prednisone, and methylprednisolone (*e.g.*, IV methylprednisolone). In a specific embodiment, compositions of the invention are administered in combination with prednisone. In a further specific embodiment, the compositions of the invention are administered in combination with

prednisone and an immunosuppressive agent. Immunosuppressive agents that may be administered with the compositions of the invention and prednisone are those described herein, and include, but are not limited to, azathioprine, cylophosphamide, and cyclophosphamide IV. In a another specific embodiment, compositions of the invention are administered in combination with methylprednisolone. In a further specific embodiment, the compositions of the invention are administered in combination with methylprednisolone and an immunosuppressive agent. Immunosuppressive agents that may be administered with the compositions of the invention and methylprednisolone are those described herein, and include, but are not limited to, azathioprine, cylophosphamide, and cyclophosphamide IV.

In another embodiment, the compositions of the invention are administered in combination with an antimalarial. Antimalarials that may be administered with the compositions of the invention include, but are not limited to, hydroxychloroquine, chloroquine; and/or quinacrine.

In yet another embodiment, the compositions of the invention are administered in combination with an NSAID.

In a nonexclusive embodiment, the compositions of the invention are administered in combination with one, two, three, four, five, ten, or more of the following drugs: NRD-101 (Hoechst Marion Roussel), diclofenac (Dimethaid), oxaprozin potassium (Monsanto), mecasermin (Chiron), T-614 (Toyama), pemetrexed disodium (Eli Lilly), atreleuton (Abbott), valdecoxib (Monsanto), eltenac (Byk Gulden), campath, AGM-1470 (Takeda), CDP-571 (Celltech Chiroscience), CM-101 (CarboMed), ML-3000 (Merckle), CB-2431 (KS Biomedix), CBF-BS2 (KS Biomedix), IL-1Ra gene therapy (Valentis), JTE-522 (Japan Tobacco), paclitaxel (Angiotech), DW-166HC (Dong Wha), darbufelone mesylate (Warner-Lambert), soluble TNF receptor 1 (synergen; Amgen), IPR-6001 (Institute for Pharmaceutical Research), trocade (Hoffman-La Roche), EF-5 (Scotia Pharmaceuticals), BIIL-284 (Boehringer Ingelheim), BIIF-1149 (Boehringer Ingelheim), LeukoVax (Inflammatics), MK-663 (Merck), ST-1482

(Sigma-Tau), and butixocort propionate (WarnerLambert).

In yet another embodiment, the compositions of the invention are administered in combination with one, two, three, four, five or more of the following drugs: methotrexate, sulfasalazine, sodium aurothiomalate, auranofin, cyclosporine, penicillamine, azathioprine, an antimalarial drug (*e.g.*, as described herein), cyclophosphamide, chlorambucil, gold, ENBREL™ (Etanercept), anti-TNF antibody, and prednisolone. In a more preferred embodiment, the compositions of the invention are administered in combination with an antimalarial, methotrexate, anti-TNF antibody, ENBREL™ and/or suflasalazine.

In one embodiment, the compositions of the invention are administered in combination with methotrexate. In another embodiment, the compositions of the invention are administered in combination with anti-TNF antibody. In another embodiment, the compositions of the invention are administered in combination with methotrexate and anti-TNF antibody. In another embodiment, the compositions of the invention are administered in combination with suflasalazine. In another specific embodiment, the compositions of the invention are administered in combination with methotrexate, anti-TNF antibody, and suflasalazine. In another embodiment, the compositions of the invention are administered in combination ENBREL™. In another embodiment, the compositions of the invention are administered in combination with ENBREL™ and methotrexate. In another embodiment, the compositions of the invention are administered in combination with ENBREL™, methotrexate and suflasalazine. In another embodiment, the compositions of the invention are administered in combination with ENBREL™, methotrexate and suflasalazine. In other embodiments, one or more antimalarials is combined with one of the above-recited combinations. In a specific embodiment, the compositions of the invention are administered in combination with an antimalarial (*e.g.*, hydroxychloroquine), ENBREL™, methotrexate and suflasalazine. In another specific embodiment, the compositions of the invention are administered in combination with an antimalarial (*e.g.*, hydroxychloroquine), sulfasalazine, anti-

TNF antibody, and methotrexate.

In another embodiment, compositions of the invention are administered in combination with a chemotherapeutic agent. Chemotherapeutic agents that may be administered with the compositions of the invention include, but are not limited to, antibiotic derivatives (*e.g.*, doxorubicin, bleomycin, daunorubicin, and dactinomycin); antiestrogens (*e.g.*, tamoxifen); antimetabolites (*e.g.*, fluorouracil, 5-FU, methotrexate, floxuridine, interferon alpha-2b, glutamic acid, plicamycin, mercaptopurine, and 6-thioguanine); cytotoxic agents (*e.g.*, carmustine, BCNU, lomustine, CCNU, cytosine arabinoside, cyclophosphamide, estramustine, hydroxyurea, procarbazine, mitomycin, busulfan, cis-platin, and vincristine sulfate); hormones (*e.g.*, medroxyprogesterone, estramustine phosphate sodium, ethinyl estradiol, estradiol, megestrol acetate, methyltestosterone, diethylstilbestrol diphosphate, chlorotrianisene, and testolactone); nitrogen mustard derivatives (*e.g.*, mephalen, chorambucil, mechlorethamine (nitrogen mustard) and thiotepa); steroids and combinations (*e.g.*, bethamethasone sodium phosphate); and others (*e.g.*, dicarbazine, asparaginase, mitotane, vincristine sulfate, vinblastine sulfate, and etoposide).

In an additional embodiment, the compositions of the invention are administered in combination with cytokines. Cytokines that may be administered with the compositions of the invention include, but are not limited to, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, anti-CD40, CD40L, IFN-gamma and TNF-alpha.

In an additional embodiment, the compositions of the invention are administered in combination with angiogenic proteins. Angiogenic proteins that may be administered with the compositions of the invention include, but are not limited to,. Glioma Derived Growth Factor (GDGF), as disclosed in European Patent Number EP-399816; Platelet Derived Growth Factor-A (PDGF-A), as disclosed in European Patent Number EP-682110; Platelet Derived Growth Factor-B (PDGF-B), as disclosed in European Patent Number EP-282317; Placental Growth Factor (PlGF), as disclosed in International Publication Number

WO 92/06194; Placental Growth Factor-2 (PlGF-2), as disclosed in Hauser *et al.*, Growth Factors, 4:259-268 (1993); Vascular Endothelial Growth Factor (VEGF), as disclosed in International Publication Number WO 90/13649; Vascular Endothelial Growth Factor-A (VEGF-A), as disclosed in European Patent Number EP-506477; Vascular Endothelial Growth Factor-2 (VEGF-2), as disclosed in International Publication Number WO 96/39515; Vascular Endothelial Growth Factor B-186 (VEGF-B186), as disclosed in International Publication Number WO 96/26736; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/02543; Vascular Endothelial Growth Factor-D (VEGF-D), as disclosed in International Publication Number WO 98/07832; and Vascular Endothelial Growth Factor-E (VEGF-E), as disclosed in German Patent Number DE19639601. The above mentioned references are incorporated herein by reference herein.

In an additional embodiment, the compositions of the invention are administered in combination with Fibroblast Growth Factors. Fibroblast Growth Factors that may be administered with the compositions of the invention include, but are not limited to, FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7, FGF-8, FGF-9, FGF-10, FGF-11, FGF-12, FGF-13, FGF-14, and FGF-15.

In additional embodiments, the compositions of the invention are administered in combination with other therapeutic or prophylactic regimens, such as, for example, radiation therapy.

In one embodiment, the compositions of the invention are administered in combination with one or more chemokines. In specific embodiments, the compositions of the invention are administered in combination with an α(CxC) chemokine selected from the group consisting of gamma-interferon inducible protein-10 (γIP-10), interleukin-8 (IL-8), platelet factor-4 (PF4), neutrophil activating protein (NAP-2), GRO-α, GRO-β, GRO-γ, neutrophil-activating peptide (ENA-78), granulocyte chemoattractant protein-2 (GCP-2), and stromal cell-derived factor-1 (SDF-1, or pre-B-cell stimulatory factor (PBSF)); and/or a β (CC) selected from the group consisting of: RANTES (regulated on activation,

normal T expressed and secreted), macrophage inflammatory protein-1 alpha (MIP-1 α), macrophage inflammatory protein-1 beta (MIP-1 β), monocyte chemotactic protein-1 (MCP-1), monocyte chemotactic protein-2 (MCP-2), monocyte chemotactic protein-3 (MCP-3), monocyte chemotactic protein-4 (MCP-4) macrophage inflammatory protein-1 gamma (MIP-1 γ), macrophage inflammatory protein-3 alpha (MIP-3 α), macrophage inflammatory protein-3 beta (MIP-3 β), macrophage inflammatory protein-4 (MIP-4/DC-CK-1/PARC), eotaxin, Exodus, and I-309; and/or the γ(C) chemokine, lymphotactin.

Various delivery systems are known and can be used to administer a compound of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, 1987, J. *Biol. Chem. 262*:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compounds or compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction

with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a protein, including an antibody, of the invention, care must be taken to use materials to which the protein does not absorb.

In another embodiment, the compound or composition can be delivered in a vesicle, in particular a liposome (see Langer, 1990, *Science 249*:1527-1533; Treat *et al.*, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid.)

In yet another embodiment, the compound or composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1987, *CRC Crit. Ref. Biomed. Eng. 14*:201; Buchwald *et al.*, 1980, Surgery 88:507; Saudek *et al.*, 1989, *N. Engl. J. Med. 321*:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J., 1983, *Macromol. Sci. Rev. Macromol. Chem. 23*:61; see also Levy *et al.*, 1985, *Science 228*:190; During *et al.*, 1989, *Ann. Neurol. 25*:351; Howard *et al.*, 1989, *J. Neurosurg. 71*:105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the brain, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533).

In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate

nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox- like peptide which is known to enter the nucleus (*see, e.g.*, Joliot *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The amount of the compound of the invention which will be effective in the treatment, inhibition and prevention of a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

For antibodies, the dosage administered to a patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, more preferably 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention may be reduced by enhancing uptake and tissue penetration (*e.g.*, into the brain) of the antibodies by modifications such as, for example, lipidation.

In one embodiment, the invention provides a method of delivering compositions containing the polypeptides of the invention (*e.g.*, compositions

containing DR4 polypeptides or anti-DR4 antibodies associated with heterologous polypeptides, heterologous nucleic acids, toxins, or prodrugs) to targeted cells, expressing the membrane-bound form of DR4 on their surface. DR4 polypeptides or anti-DR4 antibodies of the invention may be associated with heterologous polypeptides, heterologous nucleic acids, toxins, or prodrugs via hydrophobic, hydrophilic, ionic and/or covalent interactions.

In one embodiment, the invention provides a method for the specific delivery of compositions of the invention to cells by administering polypeptides of the invention (*e.g.*, DR4 or anti-DR4 antibodies) that are associated with heterologous polypeptides or nucleic acids. In one example, the invention provides a method for delivering a therapeutic protein into the targeted cell. In another example, the invention provides a method for delivering a single stranded nucleic acid (*e.g.*, antisense or ribozymes) or double stranded nucleic acid (*e.g.*, DNA that can integrate into the cell's genome or replicate episomally and that can be transcribed) into the targeted cell.

In another embodiment, the invention provides a method for the specific destruction of cells (*e.g.*, the destruction of tumor cells) by administering polypeptides of the invention (*e.g.*, DR4 polypeptides or anti-DR4 antibodies) in association with toxins or cytotoxic prodrugs.

In a specific embodiment, the invention provides a method for the specific destruction of cells expressing DR4 receptors on their surface (*e.g.*, activated T-cells, cancer cells, or leukemic cells) by administering DR4 polypeptides in association with toxins or cytotoxic prodrugs.

In another specific embodiment, the invention provides a method for the specific destruction of cells expressing the membrane-bound form of DR4 on their surface (*e.g.*, spleen, bone marrow, kidney and PBLs) by administering anti-DR4 antibodies in association with toxins or cytotoxic prodrugs.

By "toxin" is meant compounds that bind and activate endogenous cytotoxic effector systems, radioisotopes, holotoxins, modified toxins, catalytic subunits of toxins, cytotoxins (cytotoxic agents), or any molecules or enzymes not

normally present in or on the surface of a cell that under defined conditions cause the cell's death. Toxins that may be used according to the methods of the invention include, but are not limited to, radioisotopes known in the art, compounds such as, for example, antibodies (or complement fixing containing portions thereof) that bind an inherent or induced endogenous cytotoxic effector system, thymidine kinase, endonuclease, RNAse, alpha toxin, ricin, abrin, *Pseudomonas* exotoxin A, diphtheria toxin, saporin, momordin, gelonin, pokeweed antiviral protein, alpha-sarcin and cholera toxin. "Toxin" also includes a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, *e.g.*, alpha-emitters such as, for example, ^{213}Bi, or other radioisotopes such as, for example, ^{103}Pd, ^{133}Xe, ^{131}I, ^{68}Ge, ^{57}Co, ^{65}Zn, ^{85}Sr, ^{32}P, ^{35}S, ^{90}Y, ^{153}Sm, ^{153}Gd, ^{169}Yb, ^{51}Cr, ^{54}Mn, ^{75}Se, ^{113}Sn, ^{90}Yttrium, ^{117}Tin, ^{186}Rhenium, ^{166}Holmium, and ^{188}Rhenium; luminescent labels, such as luminol; and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Techniques known in the art may be applied to label proetins (including antibodies) of the invention. Such techniques include, but are not limited to, the use of bifunctional conjugating agents (*see, e.g.*, U.S. Patent Nos. 5,756,065; 5,714,631; 5,696,239; 5,652,361; 5,505,931; 5,489,425; 5,435,990; 5,428,139; 5,342,604; 5,274,119; 4,994,560; and 5,808,003; the contents of each of which are hereby incorporated by reference in its entirety). A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin,

mitomycin C, and *cis*-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).

By "cytotoxic prodrug" is meant a non-toxic compound that is converted by an enzyme, normally present in the cell, into a cytotoxic compound. Cytotoxic prodrugs that may be used according to the methods of the invention include, but are not limited to, glutamyl derivatives of benzoic acid mustard alkylating agent, phosphate derivatives of etoposide or mitomycin C, cytosine arabinoside, daunorubicin, and phenoxyacetamide derivatives of doxorubicin.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

Diagnosis and Imaging

Labeled antibodies, and derivatives and analogs thereof, which specifically bind to a polypeptide of interest can be used for diagnostic purposes to detect, diagnose, or monitor diseases and/or disorders associated with the aberrant expression and/or activity of a polypeptide of the invention. The invention provides for the detection of aberrant expression of a polypeptide of interest, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed

polypeptide gene expression level compared to the standard expression level is indicative of aberrant expression.

The invention provides a diagnostic assay for diagnosing a disorder, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of a particular disorder. With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

Antibodies of the invention can be used to assay protein levels in a biological sample using classical immunohistological methods known to those of skill in the art (*e.g.*, see Jalkanen, M. et al., *J. Cell. Biol. 101*:976-985 (1985); Jalkanen, M. *et al., J. Cell. Biol. 105*:3087-3096 (1987)). Other antibody-based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase; radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium (^{99}Tc); luminescent labels, such as luminol; and fluorescent labels, such as fluorescein and rhodamine, and biotin.

One aspect of the invention is the detection and diagnosis of a disease or disorder associated with aberrant expression of a polypeptide of the interest in an animal, preferably a mammal and most preferably a human. In one embodiment, diagnosis comprises: a) administering (for example, parenterally, subcutaneously,

or intraperitoneally) to a subject an effective amount of a labeled molecule which specifically binds to the polypeptide of interest; b) waiting for a time interval following the administering for permitting the labeled molecule to preferentially concentrate at sites in the subject where the polypeptide is expressed (and for unbound labeled molecule to be cleared to background level); c) determining background level; and d) detecting the labeled molecule in the subject, such that detection of labeled molecule above the background level indicates that the subject has a particular disease or disorder associated with aberrant expression of the polypeptide of interest. Background level can be determined by various methods including, comparing the amount of labeled molecule detected to a standard value previously determined for a particular system.

It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of 99mTc. The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain the specific protein. *In vivo* tumor imaging is described in S.W. Burchiel *et al.*, "Immunopharmacokinetics of Radiolabeled Antibodies and Their Fragments." (Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S.W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982)).

Depending on several variables, including the type of label used and the mode of administration, the time interval following the administration for permitting the labeled molecule to preferentially concentrate at sites in the subject and for unbound labeled molecule to be cleared to background level is 6 to 48 hours or 6 to 24 hours or 6 to 12 hours. In another embodiment the time interval following administration is 5 to 20 days or 5 to 10 days.

In an embodiment, monitoring of the disease or disorder is carried out by repeating the method for diagnosing the disease or disease, for example, one month after initial diagnosis, six months after initial diagnosis, one year after initial

diagnosis, etc.

Presence of the labeled molecule can be detected in the patient using methods known in the art for *in vivo* scanning. These methods depend upon the type of label used. Skilled artisans will be able to determine the appropriate method for detecting a particular label. Methods and devices that may be used in the diagnostic methods of the invention include, but are not limited to, computed tomography (CT), whole body scan such as position emission tomography (PET), magnetic resonance imaging (MRI), and sonography.

In a specific embodiment, the molecule is labeled with a radioisotope and is detected in the patient using a radiation responsive surgical instrument (Thurston *et al.*, U.S. Patent No. 5,441,050). In another embodiment, the molecule is labeled with a fluorescent compound and is detected in the patient using a fluorescence responsive scanning instrument. In another embodiment, the molecule is labeled with a positron emitting metal and is detected in the patent using positron emission-tomography. In yet another embodiment, the molecule is labeled with a paramagnetic label and is detected in a patient using magnetic resonance imaging (MRI).

Kits

The present invention provides kits that can be used in the above methods. In one embodiment, a kit comprises an antibody of the invention, preferably a purified antibody, in one or more containers. In a specific embodiment, the kits of the present invention contain a substantially isolated polypeptide comprising an epitope which is specifically immunoreactive with an antibody included in the kit. Preferably, the kits of the present invention further comprise a control antibody which does not react with the polypeptide of interest. In another specific embodiment, the kits of the present invention contain a means for detecting the binding of an antibody to a polypeptide of interest (*e.g.*, the antibody may be conjugated to a detectable substrate such as a fluorescent compound, an

enzymatic substrate, a radioactive compound or a luminescent compound, or a second antibody which recognizes the first antibody may be conjugated to a detectable substrate).

In another specific embodiment of the present invention, the kit is a diagnostic kit for use in screening serum containing antibodies specific against proliferative and/or cancerous polynucleotides and polypeptides. Such a kit may include a control antibody that does not react with the polypeptide of interest. Such a kit may include a substantially isolated polypeptide antigen comprising an epitope which is specifically immunoreactive with at least one anti-polypeptide antigen antibody. Further, such a kit includes means for detecting the binding of said antibody to the antigen (*e.g.*, the antibody may be conjugated to a fluorescent compound such as fluorescein or rhodamine which can be detected by flow cytometry). In specific embodiments, the kit may include a recombinantly produced or chemically synthesized polypeptide antigen. The polypeptide antigen of the kit may also be attached to a solid support.

In a more specific embodiment the detecting means of the above-described kit includes a solid support to which said polypeptide antigen is attached. Such a kit may also include a non-attached reporter-labeled anti-human antibody. In this embodiment, binding of the antibody to the polypeptide antigen can be detected by binding of the said reporter-labeled antibody.

In an additional embodiment, the invention includes a diagnostic kit for use in screening serum containing antigens of the polypeptide of the invention. The diagnostic kit includes a substantially isolated antibody specifically immunoreactive with polypeptide or polynucleotide antigens, and means for detecting the binding of the polynucleotide or polypeptide antigen to the antibody. In one embodiment, the antibody is attached to a solid support. In a specific embodiment, the antibody may be a monoclonal antibody. The detecting means of the kit may include a second, labeled monoclonal antibody. Alternatively, or in addition, the detecting means may include a labeled, competing antigen.

In one diagnostic configuration, test serum is reacted with a solid phase

reagent having a surface-bound antigen obtained by the methods of the present invention. After binding with specific antigen antibody to the reagent and removing unbound serum components by washing, the reagent is reacted with reporter-labeled anti-human antibody to bind reporter to the reagent in proportion to the amount of bound anti-antigen antibody on the solid support. The reagent is again washed to remove unbound labeled antibody, and the amount of reporter associated with the reagent is determined. Typically, the reporter is an enzyme which is detected by incubating the solid phase in the presence of a suitable fluorometric, luminescent or colorimetric substrate (Sigma, St. Louis, MO).

The solid surface reagent in the above assay is prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, 96-well plate or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein, typically through a free amine group, to a chemically reactive group on the solid support, such as an activated carboxyl, hydroxyl, or aldehyde group. Alternatively, streptavidin coated plates can be used in conjunction with biotinylated antigen(s).

Thus, the invention provides an assay system or kit for carrying out this diagnostic method. The kit generally includes a support with surface-bound recombinant antigens, and a reporter-labeled anti-human antibody for detecting surface-bound anti-antigen antibody.

Chromosome Assays

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

In certain preferred embodiments in this regard, the cDNA herein disclosed

is used to clone genomic DNA of a DR4 gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA the is used for *in situ* chromosome mapping using well known techniques for this purpose.

In addition, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes.

Fluorescence *in situ* hybridization ("FISH") of a cDNA to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60. For a review of this technique, see Verma *et al., Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man*, available on line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes)).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Example 1

Expression and Purification in E. coli

The deposited cDNA encoding the mature DR4 protein (ATCC No. 97853) is amplified using PCR oligonucleotide primers specific to the amino terminal sequences of the DR4 protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning are added to the 5' and 3' sequences respectively.

The following primers are used for expression of DR4 extracellular domain in *E. coli* 5' primer 5'-GCGGCATGCATGATCAATCAATTGGCAC-3' (SEQ ID NO:8) contains the underlined *Sph*I site. 3' primer: 5'-GCGAAGCTTTCAATTATGTCCATTGCCTG-3' (SEQ ID NO:9) contains the underlined *Hind*III site. Vector is pQE60.

The restriction sites are convenient to restriction enzyme sites in the bacterial expression vector pQE60, which are used for bacterial expression in these examples. (Qiagen, Inc. 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Amp^r") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS").

The amplified DR4 DNA and the vector pQE60 both are digested with *Sph*I and *Hind*III and the digested DNAs are then ligated together. Insertion of the DDCR protein DNA into the restricted pQE60 vector places the DR4 protein coding region downstream of and operably linked to the vector's IPTG-inducible promoter and in-frame with an initiating AUG appropriately positioned for translation of DR4 protein.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures. Such procedures are described in Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses lac repressor

and confers kanamycin resistance ("Kan^r"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing DR4 protein, is available commercially from Qiagen.

Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 μg/ml) and kanamycin (25 μg/ml).

The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:100 to 1:250. The cells are grown to an optical density at 600nm ("OD600") of between 0.4 and 0.6. Isopropyl-B-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from *lac* repressor sensitive promoters, by inactivating the *lac*I repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation and disrupted, by standard methods. Inclusion bodies are purified from the disrupted cells using routine collection techniques, and protein is solubilized from the inclusion bodies into 8M urea. The 8M urea solution containing the solubilized protein is passed over a PD-10 column in 2X phosphate-buffered saline ("PBS"), thereby removing the urea, exchanging the buffer and refolding the protein. The protein is purified by a further step of chromatography to remove endotoxin. Then, it is sterile filtered. The sterile filtered protein preparation is stored in 2X PBS at a concentration of 95 μg/ml.

Example 2
Expression in Mammalian Cells

Most of the vectors used for the transient expression of a given gene sequence in mammalian cells carry the SV40 origin of replication. This allows the replication of the vector to high copy numbers in cells (*e.g.*, COS cells) which express the T antigen required for the initiation of viral DNA synthesis. Any other mammalian cell line can also be utilized for this purpose.

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, *e.g.* RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, also cellular signals can be used (*e.g.*, human actin, promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC67109). Mammalian host cells that could be used include, human HeLa, 283, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1 African green monkey cells, quail QC1-3 cells, mouse L cells and Chinese hamster ovary cells.

Alternatively, a gene of interest can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) is a useful marker to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Using this marker, the mammalian cells are grown in increasing

amounts of methotrexate for selection and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) cells are often used for the production of proteins.

The expression vectors pCl and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al., Molecular and Cellular Biology 438*:44701 (1985)), plus a fragment of the CMV-enhancer (Boshart *et al., Cell 41*:521-530 (1985)). Multiple cloning sites, *e.g.* with the restriction enzyme cleavage sites *Bam*HI, *Xba*I and *Asp*718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Cloning and Expression in CHO Cells

The vector pC4 is used for the expression of DR4 polypeptide. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, *e.g.*, Alt, F. W., *et al., J. Biol. Chem. 253*:1357-1370 (1978); Hamlin, J. L. and Ma, C. *Biochem. et Biophys. Acta, 1097*:107-143 (1990); Page, M. J. and Sydenham, M. A. *Biotechnology 9*:64-68 (1991)). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach may be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is

withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains for expressing the gene of interest the strong promoter of the long terminal repeat (LTR) of the Rouse Sarcoma Virus (Cullen, *et al., Molecular and Cellular Biology*, March 1985:438-447) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart *et al., Cell 41*:521-530 (1985)). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: *Bam*HI, *Xba* I, and *Asp*718. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of the rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, *e.g.*, the human ß-actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, *e.g.*, HIV and HTLVI. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the DR4 polypeptide in a regulated way in mammalian cells (Goshen, M., & Bujard, H. *Proc. Natl. Acad. Sci. USA 89*:5547-5551 (1992)). For the polyadenylation of the mRNA other signals, *e.g.*, from the human growth hormone or globin genes can be used as well. Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, *e.g.*, G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzyme *Bam*HI and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the complete polypeptide is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the desired portion of the gene. The 5' primer containing the underlined *Bam*HI site, a Kozak sequence, and an AUG start codon, has the following sequence: 5' GCGGGATCCGCCATCATGGCGCCACCACCAGCTAGA 3' (SEQ ID NO:10). The 3' primer, containing the underlined *Bam*HI site, has the following

sequence: 5' GCGGGATCCTCACTCCAAGGACACGGCAGAGCC 3' (SEQ ID NO:11).

The amplified fragment is digested with the endonuclease *Bam*HI and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. Five μg of the expression plasmid pC4 is cotransfected with 0.5 μg of the plasmid pSVneo using lipofectin (Felgner *et al.*, *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of Methotrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μM, 2 μM, 5 μM, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100-200 μM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

Example 3
Protein Fusions of DR4

DR4 polypeptides of the invention are optionally fused to other proteins. These fusion proteins can be used for a variety of applications. For example, fusion of DR4 polypeptides to His-tag, HA-tag, protein A, IgG domains, and maltose binding protein facilitates purification. (See EP A 394,827; Traunecker, *et al., Nature 331*:84-86 (1988)). Similarly, fusion to IgG-1, IgG-3, and albumin increases the half-life time *in vivo*. Nuclear localization signals fused to DR4 polypeptides can target the protein to a specific subcellular localization, while covalent heterodimer or homodimers can increase or decrease the activity of a fusion protein. Fusion proteins can also create chimeric molecules having more than one function. Finally, fusion proteins can increase solubility and/or stability of the fused protein compared to the non-fused protein. All of the types of fusion proteins described above can be made using techniques known in the art or by using or routinely modifying the following protocol, which outlines the fusion of a polypeptide to an IgG molecule.

Briefly, the human Fc portion of the IgG molecule can be PCR amplified, using primers that span the 5' and 3' ends of the sequence described in SEQ ID NO:13. These primers also preferably contain convenient restriction enzyme sites that will facilitate cloning into an expression vector, preferably a mammalian expression vector.

For example, if the pC4 (Accession No. 209646) expression vector is used, the human Fc portion can be ligated into the *Bam*HI cloning site. Note that the 3' *Bam*HI site should be destroyed. Next, the vector containing the human Fc portion is re-restricted with *Bam*HI, linearizing the vector, and DR4 polynucleotide, isolated by the PCR protocol described in Example 1, is ligated into this *Bam*HI site. Note that the polynucleotide is cloned without a stop codon, otherwise a fusion protein will not be produced.

If the naturally occurring signal sequence is used to produce the secreted protein, pC4 does not need a second signal peptide. Alternatively, if the naturally

occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (*See, e.g.*, WO 96/34891.)

Example 4

Cloning and expression of the soluble extracellular domain of DR4 in a baculovirus expression system

The depsited cDNA encoding the soluble extracellular domain of DR4 protein (ATCC No. 97853) is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer for DR4 has the sequence:

5' GCGGGATCCGCCATCATGGCGCCACCACCAGCTAGA 3' (SEQ ID NO:10) containing the underlined *Bam*HI restriction enzyme site. Inserted into an expression vector, as described below, the 5' end of the amplified fragment encoding DR4 provides an efficient cleavage signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol. 196*:947-950 (1987) is appropriately located in the vector portion of the construct.

The 3' primer for both DR4 has the sequence:

5' GCGGGATCCTCAATTATGTCCATTGCCTG 3' (SEQ ID NO:12) containing the underlined *Bam*HI restriction followed by nucleotides complementary to the DR4 nucleotide sequence set out in SEQ ID NO:1, followed by the stop codon.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean, " BIO 101 Inc., La Jolla, Ca.) The fragment then is digested with *Bam*HI and *Asp*718 and again is purified on a 1% agarose gel.

The vector pA2 is used to express the DR4 protein in the baculovirus expression system, using standard methods, such as those described in Summers *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedron promoter of the Autograph

californica nuclear polyhedrosis virus (ACMNPV) followed by convenient restriction sites. For an easy selection of recombinant virus the beta-galactosidase gene from *E. coli* is inserted in the same orientation as the polyhedron promoter and is followed by the polyadenylation signal of the polyhedron gene. The polyhedron sequences are flanked at both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of pA2, such as pAc373, pVL941 and pAcIM1 provided, as those of skill readily will appreciate, that construction provides appropriately located signals for transcription, translation, trafficking and the like, such as an in-frame AUG and a signal peptide, as required. Such vectors are described in Luckow *et al., Virology 170*:31-39, among others.

The plasmid is digested with the restriction enzyme *Bam*HI and then is dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.).

Fragment and the dephosphorylated plasmid are ligated together with T4 DNA ligase. *E. coli* HB101 cells are transformed with ligation mix and spread on culture plates. Bacteria are identified that contain the plasmid with the human DDCR gene by digesting DNA from individual colonies using *Bam*HI and then analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBac DR4.

5 μg of the plasmid pBac DR4 is co-transfected with 1.0 μg of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.), using the lipofection method described by Felgner *et al., Proc. Natl. Acad. Sci. USA 84*:7413-7417 (1987). 1 μg of BaculoGold™ virus DNA and 5 μg of the plasmid pBac DR4 are mixed in a sterile well of a microliter plate containing 50 μl of serum free Grace's medium

(Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27° C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27° C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, cited above. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after serial dilution, the virus is added to the cells. After appropriate incubation, blue stained plaques are picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses is then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar is removed by a brief centrifugation and the supernatant containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4° C. A clone containing properly inserted DR4 is identified by DNA analysis including restriction mapping and sequencing. This is designated herein as V- DR4.

Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V- DR4 at a multiplicity of infection ("MOI") of about 2 (about 1 to about 3). Six hours later the medium is removed and is replaced with SF900 II medium minus

methionine and cysteine (available from Life Technologies Inc., Gaithersburg). 42 hours later, 5 μCi of ^{35}S-methionine and 5 μCi ^{35}S cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation, lysed and the labeled proteins are visualized by SDS-PAGE and autoradiography.

Example 5
DR4 Induced Apoptosis in Mammalian Cells

Overexpression of Fas/APO-1 and TNFR-1 in mammalian cells mimics receptor activation (M. Muzio, *et al.*, *Cell 85*, 817-827 (1996); M. P. Boldin, *et al.*, *Cell 85*, 803-815 (1996)). Thus, this system was utilized to study the functional role of DR4. Transient expression of DR4 in MCF7 human breast carcinoma cells and 293 human embryonic kidney cells induced rapid apoptosis.

Cell death assays are performed essentially as previously described (A.M. Chinnaiyan, *et al.*, *Cell 81*, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem 270*, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO 14*, 5579-5588 (1995); A.M. Chinnaiyan, *et al.*, *J Biol Chem 271*, 4961-4965 (1996)). Briefly, MCF-7 human breast carcinoma clonal cell lines stably transfected with either vector alone or a CrmA expression construct (M. Tewari, *et al.*, *J Biol Chem 270*, 3255-60 (1995)), are transiently transfected with pCMV-DR4-galactosidase (or pCMV-DR4-galactosidase (lacking the death domain)) in the presence of a ten-fold excess of pcDNA3 expression constructs encoding the indicated proteins using lipofectamine (GIBCO-BRL). 293 cells are likewise transfected using the $CaPO_4$ method. The ICE family inhibitor z-VAD-fmk (Enzyme Systems Products, Dublin, CA) is added to the cells at a concentration of 10μM, 5 hrs after transfection. 32 hours following transfection, cells are fixed and stained with X-Gal as previously described (A.M. Chinnaiyan, *et al.*, *Cell 81*, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem 270*, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO 14*, 5579-5588 (1995)).

The cells displayed morphological alterations typical of cells undergoing

apoptosis, becoming rounded, condensed and detaching from the dish. Similar to TNFR-1 and Fas/APO-1 (M. Muzio, *et al.*, *Cell 85*, 817-827 (1996); M. P. Boldin, *et al.*, *Cell 85*, 803-815 (1996); M. Tewari, *et al.*, *J Biol Chem 270*, 3255-60 (1995)), DR4-induced apoptosis was blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk.

Example 6

The Extracellular Domain of DR4 Binds the Cytotoxic Ligand, TRAIL, and Blocks TRAIL-Induced Apoptosis

This example shows that the present receptor, DR4, binds TRAIL. The soluble extracellular ligand binding domain of DR4 was expressed as a fusion to the Fc portion of human immunoglobulin (IgG). cDNA encoding the extracellular domain of DR4 (amino acids 110 to 239) was obtained by polymerase chain reaction and cloned into a modified pCMV1FLAG vector that allowed for in-frame fusion with the Fc portion of human IgG.

As shown in Figure 6A and 6B, DR4-Fc specifically bound TRAIL, but not the related cytotoxic ligand TNFα. In this experiment, we transfected 293 cells with constructs encoding the Fc-extracellular domains of DR4, TNFR1, or Fas, and the corresponding ligands, and the conditioned media was harvested 72 to 80 hours later, clarified by centrifugation, divided, and stored at -80°C. For binding assays, equal amounts of receptor-Fc- and ligand containing conditioned media were mixed in buffer containing 50 mM Hepes, pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.5 % NP-40, and a protease inhibitor mixture, and the sample was incubated at 4°C with continuous rotation for 4 hours. Receptor-Fc-ligand complexes were precipitated with protein G-Sepharose, extensively washed with the above buffer, boiled in SDS sample buffer, and resolved on a 12% SDS-polyacrylamide gel and co-precipitated soluble ligands were detected by immunoblotting with anti-Flag (Babco) or anti-myc-HRP (BMB) or to FasL (Pharmingen).

Additionally, DR4-Fc blocked the ability of TRAIL to induce apoptosis

(Figure 6A). MCF7 cells were treated with soluble TRAIL (400 ng/ml) in the presence of equal amounts of Fc-fusions or Fc alone. Five hours later cells were fixed with formaldehyde, and the nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy with a fluorescein isothiocyanate range barrier filter cube. The data (mean 6 ± SD) shown in Figure 6A are the percentage of apoptotic nuclei among total nuclei counted (n=3).

Finally, DR4-Fc had no effect on apoptosis TNFα-induced cell death under conditions where TNFR1-Fc completely abolished TNFα killing (Figure 6B). MCF7 cells were treated with TNFα (40 ng/ml; Genentech, Inc.) in the presence of equal amounts of Fc-fusions or Fc alone. Nuclei were stained and examined 15 to 18 hours later.

Example 7

Assays to detect stimulation or inhibition of B-cell proliferation and differentiation

Generation of functional humoral immune responses requires both soluble and cognate signaling between B-lineage cells and their microenvironment. Signals may impart a positive stimulus that allows a B-lineage cell to continue its programmed development, or a negative stimulus that instructs the cell to arrest its current developmental pathway. To date, numerous stimulatory and inhibitory signals have been found to influence B-cell responsiveness including IL-2, IL-4, IL-5, IL6, IL-7, IL-10, IL-13, IL-14 and IL-15. Interestingly, these signals are by themselves weak effectors but can, in combination with various co-stimulatory proteins, induce activation, proliferation, differentiation, homing, tolerance and death among B-cell populations. One of the best studied classes of B-cell co-stimulatory proteins is the TNF-superfamily. Within this family CD40, CD27, and CD30 along with their respective ligands CD154, CD70, and CD153 have been found to regulate a variety of immune responses. Assays which allow for the detection and/or observation of the proliferation and differentiation of these B-cell populations and their precursors are valuable tools in determining the effects

various proteins may have on these B-cell populations in terms of proliferation and differentiation. Listed below are two assays designed to allow for the detection of the differentiation, proliferation, or inhibition of B-cell populations and their precursors.

Experimental Procedure

In Vitro assay-Purified DR4 protein, or truncated forms thereof, is assessed for its ability to induce activation, proliferation, differentiation or inhibition and/or death in B-cell populations and their precursors. The activity of DR4 protein on purified human tonsillar B-cells, measured qualitatively over the dose range from 0.1 to 10,000 ng/mL, is assessed in a standard B-lymphocyte co-stimulation assay in which purified tonsillar B-cells are cultured in the presence of either formalin-fixed *Staphylococcus aureus* Cowan I (SAC) or immobilized anti-human IgM antibody as the priming agent. Second signals such as IL-2 and IL-15 synergize with SAC and IgM crosslinking to elicit B-cell proliferation as measured by tritiated-thymidine incorporation. Novel synergizing agents can be readily identified using this assay. The assay involves isolating human tonsillar B-cells by magnetic bead (MACS) depletion of CD3-positive cells. The resulting cell population is greater than 95% B-cells as assessed by expression of CD45R(B220). Various dilutions of each sample are placed into individual wells of a 96-well plate to which are added 10^5 B-cells suspended in culture medium (RPMI 1640 containing 10% FBS, 5 X 10^{-5}M βME, 100U/ml penicillin, 10 μg/ml streptomycin, and 10^{-5} dilution of SAC) in a total volume of 150 μl. Proliferation or inhibition is quantitated by a 20 hour pulse (1 μCi/well) with ^{3}H-thymidine (6.7 Ci/mM) beginning 72 hour post factor addition. The positive and negative controls are IL-2 and medium respectively.

In Vivo assay-BALB/c mice are injected (i.p.) twice per day with buffer only, or 2 mg/Kg of DR4 protein, or truncated forms thereof. Mice receive this treatment for 4 consecutive days, at which time they are sacrificed and various

tissues and serum collected for analyses. Comparison of H&E sections from normal and DR4 protein-treated spleens identify the results of the activity of DR4 protein on spleen cells, such as the diffusion of peri-arterial lymphatic sheaths, and/or significant increases in the nucleated cellularity of the red pulp regions, which may indicate the activation of the differentiation and proliferation of B-cell populations. Immunohistochemical studies using a B-cell marker, anti-CD45R(B220), are used to determine whether any physiological changes to splenic cells, such as splenic disorganization, are due to increased B-cell representation within loosely defined B-cell zones that infiltrate established T-cell regions.

Flow cytometric analyses of the spleens from DR4 protein-treated mice is used to indicate whether DR4 protein specifically increases the proportion of ThB+, CD45R(B220)dull B-cells over that which is observed in control mice.

Likewise, a predicted consequence of increased mature B-cell representation *in vivo* is a relative increase in serum Ig titers. Accordingly, serum IgM and IgA levels are compared between buffer and DR4 protein-treated mice.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 8
T-Cell Proliferation Assay

A CD3-induced proliferation assay is performed on PBMCs and is measured by the uptake of ^{3}H-thymidine. The assay is performed as follows. Ninety-six well plates are coated with 100 µl/well of monoclonal antibody to CD3 (HIT3a, Pharmingen) or isotype-matched control monoclonal antibody (B33.1) overnight at 4°C (1 µg/ml in 0.05M bicarbonate buffer, pH 9.5), then washed three times with PBS. PBMC are isolated by F/H gradient centrifugation from human peripheral blood and added to quadruplicate wells (5 x 10^{4}/well) of

monoclonal antibody coated plates in RPMI containing 10% FCS and P/S in the presence of varying concentrations of DR4 protein (total volume 200 µl). Relevant protein buffer and medium alone are controls. After 48 hours culture at 37°C, plates are spun for 2 minutes at 1000 rpm and 100 µl of supernatant is removed and stored -20°C for measurement of IL-2 (or other cytokines) if effect on proliferation is observed. Wells are supplemented with 100 µl of medium containing 0.5 µCi of ^{3}H-thymidine and cultured at 37°C for 18-24 hr. Wells are harvested and incorporation of ^{3}H-thymidine used as a measure of proliferation. Anti-CD3 alone is the positive control for proliferation. IL-2 (100 U/ml) is also used as a control which enhances proliferation. Control antibody which does not induce proliferation of T-cells is used as the negative controls for the effects of DR4 proteins.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 9

Effect of DR4 on the Expression of MHC Class II, Costimulatory and Adhesion Molecules and Cell Differentiation of monocytes and Monocyte-Derived Human Dendritic Cells

Dendritic cells are generated by the expansion of proliferating precursors found in the peripheral blood: adherent PBMC or elutriated monocytic fractions are cultured for 7-10 days with GM-CSF (50 ng/ml) and IL-4 (20 ng/ml). These dendritic cells have the characteristic phenotype of immature cells (expression of CD1, CD80, CD86, CD40 and MHC class II antigens). Treatment with activating factors, such as TNF-α, causes a rapid change in surface phenotype (increased expression of MHC class I and II, costimulatory and adhesion molecules, downregulation of FCγRII, upregulation of CD83). These changes correlate with increased antigen-presenting capacity and with functional maturation of the

dendritic cells.

FACS analysis of surface antigens is performed as follows. Cells are treated 1-3 days with increasing concentrations of DR4 or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4°C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

Effect on the production of cytokines

Cytokines generated by dendritic cells, in particular IL-12, are important in the initiation of T-cell dependent immune responses. IL-12 strongly influences the development of Thl helper T-cell immune response, and induces cytotoxic T and NK cell function. An ELISA is used to measure the IL-12 release as follows. Dendritic cells (10^6/ml) are treated with increasing concentrations of DR4 for 24 hours. LPS (100 ng/ml) is added to the cell culture as positive control. Supernatants from the cell cultures are then collected and analyzed for IL-12 content using commercial ELISA kit (*e.g.*, R & D Systems (Minneapolis, MN)). The standard protocols provided with the kits are used.

Effect on the expression of MHC Class II, costimulatory and adhesion molecules

Three major families of cell surface antigens can be identified on monocytes: adhesion molecules, molecules involved in antigen presentation, and Fc receptor. Modulation of the expression of MHC class II antigens and other costimulatory molecules, such as B7 and ICAM-1, may result in changes in the antigen presenting capacity of monocytes and ability to induce T-cell activation. Increase expression of Fc receptors may correlate with improved monocyte cytotoxic activity, cytokine release and phagocytosis.

FACS analysis is used to examine the surface antigens as follows.

Monocytes are treated 1-5 days with increasing concentrations of DR4 or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4°C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

Monocyte activation and/or increased survival:

Assays for molecules that activate (or alternatively, inactivate) monocytes and/or increase monocyte survival (or alternatively, decrease monocyte survival) are known in the art and may routinely be applied to determine whether a molecule of the invention functions as an inhibitor or activator of monocytes. DR4, agonists, or antagonists of DR4 can be screened using the three assays described below. For each of these assays, Peripheral blood mononuclear cells (PBMC) are purified from single donor leukopacks (American Red Cross, Baltimore, MD) by centrifugation through a Histopaque gradient (Sigma). Monocytes are isolated from PBMC by counterflow centrifugal elutriation.

1. Monocyte Survival Assay. Human peripheral blood monocytes progressively lose viability when cultured in absence of serum or other stimuli. Their death results from internally regulated process (apoptosis). Addition to the culture of activating factors, such as TNF-alpha dramatically improves cell survival and prevents DNA fragmentation. Propidium iodide (PI) staining is used to measure apoptosis as follows. Monocytes are cultured for 48 hours in polypropylene tubes in serum-free medium (positive control), in the presence of 100 ng/ml TNF-alpha (negative control), and in the presence of varying concentrations of DR4. Cells are suspended at a concentration of 2 x 10^6/ml in PBS containing PI at a final concentration of 5 μg/ml, and then incubated at room temperature for 5 minutes before FAC Scan analysis. PI uptake has been demonstrated to correlate with DNA fragmentation in this experimental paradigm.

2. Effect on cytokine release. An important function of monocytes/ macrophages is their regulatory activity on other cellular populations of the immune system through the release of cytokines after stimulation. An ELISA to

measure cytokine release is performed as follows. Human monocytes are incubated at a density of $5x10^5$ cells/ml with increasing concentrations of DR4 and under the same conditions, but in the absence of DR4. For IL-12 production, the cells are primed overnight with IFN-γ (100 U/ml) in presence of DR4. LPS (10 ng/ml) is then added. Conditioned media are collected after 24h and kept frozen until use. Measurement of TNF-α, IL-10, MCP-1 and IL-8 is then performed using a commercially available ELISA kit (*e.g.*, R & D Systems (Minneapolis, MN)) applying the standard protocols provided with the kit.

3. Oxidative burst. Purified monocytes are plated in 96-well plate at $2\text{-}1x10^5$ cell/well. Increasing concentrations of DR4 are added to the wells in a total volume of 0.2 ml culture medium (RPMI 1640 + 10% FCS, glutamine and antibiotics). After 3 days incubation, the plates are centrifuged and the medium is removed from the wells. To the macrophage monolayers, 0.2 ml per well of phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 19 U/ml of HRPO) is added, together with the stimulant (200 nM PMA). The plates are incubated at 37°C for 2 hours and the reaction is stopped by adding 20 µl 1N NaOH per well. The absorbance is read at 610 nm. To calculate the amount of H_2O_2 produced by the macrophages, a standard curve of a H_2O_2 solution of known molarity is performed for each experiment.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 10

The Effect of DR4 on the Growth of Vascular Endothelial Cells

On day 1, human umbilical vein endothelial cells (HUVEC) are seeded at $2\text{-}5x10^4$ cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth

supplements (ECGS, Biotechnique, Inc.). On day 2, the medium is replaced with M199 containing 10% FBS, 8 units/ml heparin. DR4 protein of SEQ ID NO. 2, and positive controls, such as VEGF and basic FGF (bFGF) are added, at varying concentrations. On days 4 and 6, the medium is replaced. On day 8, cell number is determined with a Coulter Counter.

An increase in the number of HUVEC cells after treatment with DR4 indicates that DR4 may proliferate vascular endothelial cells.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 11
Stimulatory Effect of DR4 on the Proliferation of Vascular Endothelial Cells

For evaluation of mitogenic activity of growth factors, the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium) assay with the electron coupling reagent PMS (phenazine methosulfate) was performed (CellTiter 96 AQ, Promega). Cells are seeded in a 96-well plate (5,000 cells/well) in 0.1 ml serum-supplemented medium and are allowed to attach overnight. After serum-starvation for 12 hours in 0.5% FBS, conditions (bFGF, $VEGF_{165}$ or DR4 in 0.5% FBS) with or without Heparin (8 U/ml) are added to wells for 48 hours. 20 mg of MTS/PMS mixture (1:0.05) are added per well and allowed to incubate for 1 hour at 37°C before measuring the absorbance at 490 nm in an ELISA plate reader. Background absorbance from control wells (some media, no cells) is subtracted, and seven wells are performed in parallel for each condition. See, Leak *et al. In Vitro Cell. Dev. Biol. 30A:*512-518 (1994).

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test

the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 12
Inhibition of PDGF-induced Vascular Smooth Muscle Cell Proliferation Stimulatory Effect

HAoSMC proliferation can be measured, for example, by BrdUrd incorporation. Briefly, subconfluent, quiescent cells grown on the 4-chamber slides are transfected with CRP or FITC-labeled AT2-3LP. Then, the cells are pulsed with 10% calf serum and 6 mg/ml BrdUrd. After 24 hours, immunocytochemistry is performed by using BrdUrd Staining Kit (Zymed Laboratories). In brief, the cells are incubated with the biotinylated mouse anti-BrdUrd antibody at 4° C for 2 h after exposing to denaturing solution and then with the streptavidin-peroxidase and diaminobenzidine. After counterstaining with hematoxylin, the cells are mounted for microscopic examination, and the BrdUrd-positive cells are counted. The BrdUrd index is calculated as a percent of the BrdUrd-positive cells to the total cell number. In addition, the simultaneous detection of the BrdUrd staining (nucleus) and the FITC uptake (cytoplasm) is performed for individual cells by the concomitant use of bright field illumination and dark field-UV fluorescent illumination. See, Hayashida *et al., J. Biol. Chem. 6;271(36)*:21985-21992 (1996).

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 13
Stimulation of Endothelial Migration

This example will be used to explore the possibility that DR4 may

stimulate lymphatic endothelial cell migration.

Endothelial cell migration assays are performed using a 48 well microchemotaxis chamber (Neuroprobe Inc., Cabin John, MD; Falk, W., *et al.*, "A 48 well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration." *J. Immunological Methods 33*:239-247 (1980)). Polyvinylpyrrolidone-free polycarbonate filters with a pore size of 8 μm (Nucleopore Corp. Cambridge, MA) are coated with 0.1% gelatin for at least 6 hours at room temperature and dried under sterile air. Test substances are diluted to appropriate concentrations in M199 supplemented with 0.25% bovine serum albumin (BSA), and 25 μl of the final dilution is placed in the lower chamber of the modified Boyden apparatus. Subconfluent, early passage (2-6) HUVEC or BMEC cultures are washed and trypsinized for the minimum time required to achieve cell detachment. After placing the filter between lower and upper chamber, 2.5 x 10^5 cells suspended in 50 μl M199 containing 1% FBS are seeded in the upper compartment. The apparatus is then incubated for 5 hours at 37°C in a humidified chamber with 5% CO2 to allow cell migration. After the incubation period, the filter is removed and the upper side of the filter with the non-migrated cells is scraped with a rubber policeman. The filters are fixed with methanol and stained with a Giemsa solution (Diff-Quick, Baxter, McGraw Park, IL). Migration is quantified by counting cells of three random high-power fields (40x) in each well, and all groups are performed in quadruplicate.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 14

Stimulation of Nitric Oxide Production by Endothelial Cells

Nitric oxide released by the vascular endothelium is believed to be a mediator of vascular endothelium relaxation. Thus, DR4 activity can be assayed by determining nitric oxide production by endothelial cells in response to DR4.

Nitric oxide is measured in 96-well plates of confluent microvascular endothelial cells after 24 hours starvation and a subsequent 4 hr exposure to various levels of a positive control (such as VEGF-1) and DR4. Nitric oxide in the medium is determined by use of the Griess reagent to measure total nitrite after reduction of nitric oxide-derived nitrate by nitrate reductase. The effect of DR4 on nitric oxide release is examined on HUVEC.

Briefly, NO release from cultured HUVEC monolayer is measured with a NO-specific polarographic electrode connected to a NO meter (Iso-NO, World Precision Instruments Inc.). Calibration of the NO element is performed according to the following equation:

$$2\ KNO_2 + 2\ KI + 2\ H_2SO_4\ 6\ 2\ NO + I_2 + 2\ H_2O + 2\ K_2SO_4$$

The standard calibration curve is obtained by adding graded concentrations of KNO_2 (0, 5, 10, 25, 50, 100, 250, and 500 nmol/L) into the calibration solution containing KI and H_2SO_4. The specificity of the Iso-NO electrode to NO is previously determined by measurement of NO from authentic NO gas. The culture medium is removed and HUVECs are washed twice with Dulbecco's phosphate buffered saline. The cells are then bathed in 5 ml of filtered Krebs-Henseleit solution in 6-well plates, and the cell plates are kept on a slide warmer (Lab Line Instruments Inc.) to maintain the temperature at 37°C. The NO sensor probe is inserted vertically into the wells, keeping the tip of the electrode 2 mm under the surface of the solution, before addition of the different conditions. S-nitroso acetyl penicillamin (SNAP) is used as a positive control. The amount of released NO is expressed as picomoles per $1x10^6$ endothelial cells. All values reported are means of four to six measurements in each group (number of cell culture wells). See, Leak *et al. Biochem. and Biophys. Res. Comm. 217*:96-105

(1995).

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 15
Effect of DR4 on Cord Formation in Angiogenesis

Another step in angiogenesis is cord formation, marked by differentiation of endothelial cells. This bioassay measures the ability of microvascular endothelial cells to form capillary-like structures (hollow structures) when cultured *in vitro*.

CADMEC (microvascular endothelial cells) are purchased from Cell Applications, Inc. as proliferating (passage 2) cells and are cultured in Cell Applications' CADMEC Growth Medium and used at passage 5. For the *in vitro* angiogenesis assay, the wells of a 48-well cell culture plate are coated with Cell Applications' Attachment Factor Medium (200 µl/well) for 30 minutes at 37° C. CADMEC are seeded onto the coated wells at 7,500 cells/well and cultured overnight in Growth Medium. The Growth Medium is then replaced with 300 µg Cell Applications' Chord Formation Medium containing control buffer or DR4 (0.1 to 100 ng/ml) and the cells are cultured for an additional 48 hr. The numbers and lengths of the capillary-like chords are quantitated through use of the Boeckeler VIA-170 video image analyzer. All assays are done in triplicate.

Commercial (R&D) VEGF (50 ng/ml) is used as a positive control. b-esteradiol (1 ng/ml) is used as a negative control. The appropriate buffer (without protein) is also utilized as a control.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 16

Angiogenic Effect on Chick Chorioallantoic Membrane

Chick chorioallantoic membrane (CAM) is a well-established system to examine angiogenesis. Blood vessel formation on CAM is easily visible and quantifiable. The ability of DR4 to stimulate angiogenesis in CAM can be examined.

Fertilized eggs of the White Leghorn chick (*Gallus gallus*) and the Japanese quail (*Coturnix coturnix*) are incubated at 37.8°C and 80% humidity. Differentiated CAM of 16-day-old chick and 13-day-old quail embryos is studied with the following methods.

On Day 4 of development, a window is made into the egg shell of chick eggs. The embryos are checked for normal development and the eggs sealed with cellotape. They are further incubated until Day 13. Thermanox coverslips (Nunc, Naperville, IL) are cut into disks of about 5 mm in diameter. Sterile and salt-free growth factors, and DR4, are dissolved in distilled water and about 3.3 mg/5 ml are pipetted on the disks. After air-drying, the inverted disks are applied on CAM. After 3 days, the specimens are fixed in 3% glutaraldehyde and 2% formaldehyde and rinsed in 0.12 M sodium cacodylate buffer. They are photographed with a stereo microscope [Wild M8] and embedded for semi- and ultrathin sectioning as described above. Controls are performed with carrier disks alone.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 17

Angiogenesis Assay Using a Matrigel Implant in Mouse

In order to establish an *in vivo* model for angiogenesis to test DR4 protein activities, mice and rats are implanted subcutaneously with methylcellulose disks containing either 20 mg of BSA (negative control), 1 mg of DR4, or 0.5 mg of VEGF-1 (positive control). The negative control disks should contain little vascularization, while the positive control disks should show signs of vessel formation.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 18

Rescue of Ischemia in Rabbit Lower Limb Model

To study the *in vivo* effects of DR4 on ischemia, a rabbit hind-limb ischemia model is created by surgical removal of one femoral arteries as described previously (Takeshita, S. *et al.*, *Am J. Pathol 147*:1649-1660 (1995)). The excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Consequently, blood flow to the ischemic limb is dependent upon collateral vessels originating from the internal iliac artery (Takeshita, S. *et al.*, *Am J. Pathol 147*:1649-1660 (1995)). An interval of 10 days is allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. At 10 day post-operatively (day 0), after performing a baseline angiogram, the internal iliac artery of the ischemic limb is transfected with 500 mg naked DR4 expression plasmid by arterial gene transfer technology using a hydrogel-coated balloon catheter as described (Riessen, R. *et al.*, *Hum Gene Ther. 4*:749-758 (1993); Leclerc, G. *et al.*, *J. Clin. Invest. 90*: 936-944 (1992)). When DR4 is used in the treatment, a single bolus of 500 mg DR4 protein or control is delivered into the internal iliac artery of the ischemic limb

over a period of 1 minute through an infusion catheter. On day 30, various parameters are measured in these rabbits: (a) BP ratio - The blood pressure ratio of systolic pressure of the ischemic limb to that of normal limb; (b) Blood Flow and Flow Reserve - Resting FL: the blood flow during undilated condition and Max FL: the blood flow during fully dilated condition (also an indirect measure of the blood vessel amount) and Flow Reserve is reflected by the ratio of max FL: resting FL; (c) Angiographic Score - This is measured by the angiogram of collateral vessels. A score is determined by the percentage of circles in an overlaying grid that with crossing opacified arteries divided by the total number m the rabbit thigh; (d) Capillary density - The number of collateral capillaries determined in light microscopic sections taken from hind-limbs.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 19

Diabetic Mouse and Glucocorticoid-Impaired Wound Healing Models

A. Diabetic db+/db+ Mouse Model

To demonstrate that DR4 accelerates the healing process, the genetically diabetic mouse model of wound healing is used. The full thickness wound healing model in the db+/db+ mouse is a well characterized, clinically relevant and reproducible model of impaired wound healing. Healing of the diabetic wound is dependent on formation of granulation tissue and re-epithelialization rather than contraction (Gartner, M.H. *et al., J. Surg. Res. 52*:389 (1992); Greenhalgh, D.G. *et al., Am. J. Pathol. 136*:1235 (1990)).

The diabetic animals have many of the characteristic features observed in Type II diabetes mellitus. Homozygous (db+/db+) mice are obese in comparison to their normal heterozygous (db+/+m) littermates. Mutant diabetic (db+/db+)

mice have a single autosomal recessive mutation on chromosome 4 (db+) (Coleman *et al. Proc. Natl. Acad. Sci. USA 77*:283-293 (1982)). Animals show polyphagia, polydipsia and polyuria. Mutant diabetic mice (db+/db+) have elevated blood glucose, increased or normal insulin levels, and suppressed cell-mediated immunity (Mandel *et al., J. Immunol. 120*:1375 (1978); Debray-Sachs, M. *et al., Clin. Exp. Immunol. 51(1)*:1-7 (1983); Leiter *et al., Am. J. of Pathol. 114*:46-55 (1985)). Peripheral neuropathy, myocardial complications, and microvascular lesions, basement membrane thickening and glomerular filtration abnormalities have been described in these animals (Norido, F. *et al., Exp. Neurol. 83(2)*:221-232 (1984); Robertson *et al., Diabetes 29(1)*:60-67 (1980); Giacomelli *et al., Lab Invest. 40(4)*:460-473 (1979); Coleman, D.L., *Diabetes 31 (Suppl)*:1-6 (1982)). These homozygous diabetic mice develop hyperglycemia that is resistant to insulin analogous to human type II diabetes (Mandel *et al., J. Immunol. 120*:1375-1377 (1978)).

The characteristics observed in these animals suggests that healing in this model may be similar to the healing observed in human diabetes (Greenhalgh, *et al., Am. J. of Pathol. 136*:1235-1246 (1990)).

Genetically diabetic female C57BL/KsJ (db+/db+) mice and their non-diabetic (db+/+m) heterozygous littermates are used in this study (Jackson Laboratories). The animals are purchased at 6 weeks of age and were 8 weeks old at the beginning of the study. Animals are individually housed and received food and water *ad libitum*. All manipulations are performed using aseptic techniques. The experiments are conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

Wounding protocol is performed according to previously reported methods (Tsuboi, R. and Rifkin, D.B., *J. Exp. Med. 172*:245-251 (1990)). Briefly, on the day of wounding, animals are anesthetized with an intraperitoneal injection of Avertin (0.01 mg/mL), 2,2,2-tribromoethanol and 2-methyl-2-butanol dissolved in deionized water. The dorsal region of the animal is shaved and the

skin washed with 70% ethanol solution and iodine. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is then created using a Keyes tissue punch. Immediately following wounding, the surrounding skin is gently stretched to eliminate wound expansion. The wounds are left open for the duration of the experiment. Application of the treatment is given topically for 5 consecutive days commencing on the day of wounding. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of surgery and at two day intervals thereafter. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

DR4 is administered using at a range different doses of DR4, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology and immunohistochemistry. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

Three groups of 10 animals each (5 diabetic and 5 non-diabetic controls) are evaluated: 1) Vehicle placebo control, 2) DR4, 3) positive control.

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total square area of the wound. Contraction is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 was 64mm^2, the corresponding size of the dermal punch. Calculations were made using the following formula:

[Open area on day 8] - [Open area on day 1] / [Open area on day 1]

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using a Reichert-Jung microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds are used to assess whether the healing process and the morphologic appearance of the repaired skin is altered by treatment with DR4. This assessment included verification of the presence of cell accumulation, inflammatory cells, capillaries, fibroblasts, re-epithelialization and epidermal maturity (Greenhalgh, D.G. *et al., Am. J. Pathol. 136:*1235 (1990)). A calibrated lens micrometer is used by a blinded observer.

Tissue sections are also stained immunohistochemically with a polyclonal rabbit anti-human keratin antibody using ABC Elite detection system. Human skin is used as a positive tissue control while non-immune IgG is used as a negative control. Keratinocyte growth is determined by evaluating the extent of reepithelialization of the wound using a calibrated lens micrometer.

Proliferating cell nuclear antigen/cyclin (PCNA) in skin specimens is demonstrated by using anti-PCNA antibody (1:50) with an ABC Elite detection system. Human colon cancer served as a positive tissue control and human brain tissue is used as a negative tissue control. Each specimen included a section with omission of the primary antibody and substitution with non-immune mouse IgG. Ranking of these sections is based on the extent of proliferation on a scale of 0-8, the lower side of the scale reflecting slight proliferation to the higher side reflecting intense proliferation.

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

B. Steroid Impaired Rat Model

The inhibition of wound healing by steroids has been well documented in various *in vitro* and *in vivo* systems (Wahl, S.M. *Glucocorticoids and Wound healing. In*: Anti-Inflammatory Steroid Action: Basic and Clinical Aspects. 280-302 (1989); Wahl, S.M. *et al., J. Immunol. 115*: 476-481 (1975); Werb, Z. *et al., J. Exp. Med. 147*:1684-1694 (1978)). Glucocorticoids retard wound healing by inhibiting angiogenesis, decreasing vascular permeability (Ebert, R.H., *et al., An. Intern. Med. 37*:701-705 (1952)), fibroblast proliferation, and collagen synthesis (Beck, L.S. *et al., Growth Factors. 5:*295-304 (1991); Haynes, B.F. *et al., J. Clin. Invest. 61:* 703-797 (1978)) and producing a transient reduction of circulating monocytes (Haynes, B.F., *et al., J. Clin. Invest. 61:*703-797 (1978); Wahl, S. M., *Glucocorticoids and wound healing, In:* Anti-inflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989)). The systemic administration of steroids to impaired wound healing is a well establish phenomenon in rats (Beck, L.S. *et al., Growth Factors. 5:*295-304 (1991); Haynes, B.F., *et al., J. Clin. Invest. 61:*703-797 (1978); Wahl, S. M., *Glucocorticoids and wound healing, In*: Anti-inflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989); Pierce, K.F. *et al., Proc. Natl. Acad. Sci. USA 86*: 2229-2233 (1989)).

To demonstrate that DR4 can accelerate the healing process, the effects of multiple topical applications of DR4 on full thickness excisional skin wounds in rats in which healing has been impaired by the systemic administration of methylprednisolone is assessed.

Young adult male Sprague Dawley rats weighing 250-300g (Charles River Laboratories) are used in this example. The animals are purchased at 8 weeks of age and were 9 weeks old at the beginning of the study. The healing response of rats is impaired by the systemic administration of methylprednisolone (17mg/kg/rat intramuscularly) at the time of wounding. Animals are individually housed and received food and water *ad libitum*. All manipulations are performed using

aseptic techniques. This study is conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

The wounding protocol is followed according to section A, above. On the day of wounding, animals are anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The dorsal region of the animal is shaved and the skin washed with 70% ethanol and iodine solutions. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is created using a Keyes tissue punch. The wounds are left open for the duration of the experiment. Applications of the testing materials are given topically once a day for 7 consecutive days commencing on the day of wounding and subsequent to methylprednisolone administration. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of wounding and at the end of treatment. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue was no longer visible and the wound is covered by a continuous epithelium.

DR4 is administered using at a range different doses of DR4, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

Four groups of 10 animals each (5 with methylprednisolone and 5 without glucocorticoid) were evaluated: 1) Untreated group 2) Vehicle placebo control 3) DR4 treated groups.

Wound closure is analyzed by measuring the area in the vertical and

horizontal axis and obtaining the total area of the wound. Closure is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 was 64mm^2, the corresponding size of the dermal punch. Calculations were made using the following formula:

[Open area on day 8] - [Open area on day 1] / [Open area on day 1]

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using an Olympus microtome. Routine hematoxylin-eosin (H&E) staining was performed on cross-sections of bisected wounds. Histologic examination of the wounds allows assessment of whether the healing process and the morphologic appearance of the repaired skin was improved by treatment with DR4. A calibrated lens micrometer is used by a blinded observer to determine the distance of the wound gap.

Experimental data are analyzed using an unpaired t test. A p value of <0.05 is considered significant.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 20
Lymphadema Animal Model

The purpose of this experimental approach is to create an appropriate and consistent lymphedema model for testing the therapeutic effects of DR4 in lymphangiogenesis and re-establishment of the lymphatic circulatory system in the rat hind limb. Effectiveness is measured by swelling volume of the affected limb, quantification of the amount of lymphatic vasculature, total blood plasma protein,

and histopathology. Acute lymphedema is observed for 7-10 days. Perhaps more importantly, the chronic progress of the edema is followed for up to 3-4 weeks.

Prior to beginning surgery, blood sample is drawn for protein concentration analysis. Male rats weighing approximately ~350g are dosed with Pentobarbital. Subsequently, the right legs are shaved from knee to hip. The shaved area is swabbed with gauze soaked in 70% ethanol. Blood is drawn for serum total protein testing. Circumference and volumetric measurements are made prior to injecting dye into paws after marking 2 measurement levels (0.5 cm above heel, at mid-pt of dorsal paw). The intradermal dorsum of both right and left paws are injected with 0.05 ml of 1% Evan's Blue. Circumference and volumetric measurements are then made following injection of dye into paws.

Using the knee joint as a landmark, a mid-leg inguinal incision is made circumferentially allowing the femoral vessels to be located. Forceps and hemostats are used to dissect and separate the skin flaps. After locating the femoral vessels, the lymphatic vessel that runs along side and underneath the vessel(s) is located. The main lymphatic vessels in this area are then electrically coagulated or suture ligated.

Using a microscope, muscles in back of the leg (near the semitendinosis and adductors) are bluntly dissected. The popliteal lymph node is then located. The 2 proximal and 2 distal lymphatic vessels and distal blood supply of the popliteal node are then located and ligated by suturing. The popliteal lymph node, and any accompanying adipose tissue, is then removed by cutting connective tissues.

Care is taken to control any mild bleeding resulting from this procedure. After lymphatics are occluded, the skin flaps are sealed by using liquid skin (Vetbond) (AJ Buck). The separated skin edges are sealed to the underlying muscle tissue while leaving a gap of ~0.5 cm around the leg. Skin also may be anchored by suturing to underlying muscle when necessary.

To avoid infection, animals are housed individually with mesh (no bedding). Recovering animals are checked daily through the optimal edematous

peak, which typically occurred by day 5-7. The plateau edematous peak are then observed. To evaluate the intensity of the lymphedema, the circumference and volumes of 2 designated places on each paw before operation and daily for 7 days are measured. The effect plasma proteins on lymphedema is determined and whether protein analysis is a useful testing perimeter is also investigated. The weights of both control and edematous limbs are evaluated at 2 places. Analysis is performed in a blind manner.

Circumference Measurements: Under brief gas anesthetic to prevent limb movement, a cloth tape is used to measure limb circumference. Measurements are done at the ankle bone and dorsal paw by 2 different people then those 2 readings are averaged. Readings are taken from both control and edematous limbs.

Volumetric Measurements: On the day of surgery, animals are anesthetized with pentobarbital and are tested prior to surgery. For daily volumetrics animals are under brief halothane anesthetic (rapid immobilization and quick recovery), both legs are shaved and equally marked using waterproof marker on legs. Legs are first dipped in water, then dipped into instrument to each marked level then measured by Buxco edema software (Chen/Victor). Data is recorded by one person, while the other is dipping the limb to marked area.

Blood-plasma protein measurements: Blood is drawn, spun, and serum separated prior to surgery and then at conclusion for total protein and Ca2+ comparison.

Limb Weight Comparison: After drawing blood, the animal is prepared for tissue collection. The limbs were amputated using a quillitine, then both experimental and control legs were cut at the ligature and weighed. A second weighing is done as the tibio-cacaneal joint was disarticulated and the foot was weighed.

Histological Preparations: The transverse muscle located behind the knee (popliteal) area is dissected and arranged in a metal mold, filled with freezeGel, dipped into cold methylbutane, placed into labeled sample bags at - 80EC until sectioning. Upon sectioning, the muscle was observed under fluorescent

microscopy for lymphatics. Other immuno/histological methods are currently being evaluated.

The studies described in this example test the activity in DR4 protein. However, one skilled in the art could easily modify the exemplified studies to test the activity of DR4 polynucleotides (*e.g.*, gene therapy), agonists, and/or antagonists of DR4.

Example 21
Production of an Antibody

A. Hybridoma Technology

The antibodies of the present invention can be prepared by a variety of methods. (*See*, Current Protocols, Chapter 2.) As one example of such methods, cells expressing DR4 are administered to an animal to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of DR4 protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

Monoclonal antibodies specific for protein DR4 are prepared using hybridoma technology. (Kohler *et al.*, Nature 256:495 (1975); Kohler *et al.*, *Eur. J. Immunol.* *6*:511 (1976); Kohler *et al.*, *Eur. J. Immunol.* *6*:292 (1976); Hammerling *et al.*, in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981)). In general, an animal (preferably a mouse) is immunized with DR4 polypeptide or, more preferably, with a secreted DR4 polypeptide-expressing cell. Such polypeptide-expressing cells are cultured in any suitable tissue culture medium, preferably in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (inactivated at about 56°C), and supplemented with about 10 g/l of nonessential amino acids, about 1,000 U/ml of penicillin, and about 100 µg/ml of streptomycin.

The splenocytes of such mice are extracted and fused with a suitable

myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the ATCC. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands *et al.* (*Gastroenterology 80*:225-232 (1981). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the DR4 polypeptide.

Alternatively, additional antibodies capable of binding to DR4 polypeptide can be produced in a two-step procedure using anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the DR4 protein-specific antibody can be blocked by DR4. Such antibodies comprise anti-idiotypic antibodies to the DR4 protein-specific antibody and are used to immunize an animal to induce formation of further DR4 protein-specific antibodies.

For *in vivo* use of antibodies in humans, an antibody is "humanized". Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric and humanized antibodies are known in the art and are discussed *infra*. (See, for review, Morrison, *Science 229*:1202 (1985); Oi *et al.*, *BioTechniques 4*:214 (1986); Cabilly *et al.*, U.S. Patent No. 4,816,567; Taniguchi *et al.*, EP 171496; Morrison *et al.*, EP 173494; Neuberger *et al.*, WO 8601533; Robinson *et al.*, WO 8702671; Boulianne *et al.*, *Nature 312*:643 (1984); Neuberger *et al.*, *Nature 314*:268 (1985).)

B. Isolation Of Antibody Fragments Directed Against DR4-V1 and DR4 From A Library Of scFvs

Naturally occurring V-genes isolated from human PBLs are constructed into a large library of antibody fragments which contain reactivities against polypeptides of the present invention to which the donor may or may not have been exposed (*see, e.g.*, U.S. Patent 5,885,793 incorporated herein in its entirety by reference).

Rescue of the library

A library of scFvs is constructed from the RNA of human PBLs as described in WO92/01047. To rescue phage displaying antibody fragments, approximately 10^9 *E. coli* harboring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100 μg/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture is used to innoculate 50 ml of 2xTY-AMP-GLU, $2x10^8$ TU of delta gene 3 helper phage (M13 gene III, see WO92/01047) are added and the culture incubated at 37° C for 45 minutes without shaking and then at 37° C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 minutes and the pellet resuspended in 2 liters of 2xTY containing 100 μg/ml ampicillin and 50 μg/ml kanamycin and grown overnight. Phage are prepared as described in WO92/01047.

M13 gene III is prepared as follows: M13 gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater avidity of binding to antigen. Infectious M13 gene III particles are made by growing the helper phage in cells harboring a pUC19 derivative supplying the wild type gene III protein during phage morphogenesis. The culture is incubated for 1 hour at 37° C without shaking and then for a further hour at 37° C with shaking. Cells are pelleted (IEC-Centra 8, 4000 revs/min for 10 min), resuspended in 300 ml 2xTY broth containing 100 μg ampicillin/ml and 25 μg kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking at 37° C. Phage

particles are purified and concentrated from the culture medium by two PEG-precipitations (Sambrook *et al.*, 1990), resuspended in 2 ml PBS and passed through a 0.45 μm filter (Minisart NML; Sartorius) to give a final concentration of approximately 10^{13} transducing units/ml (ampicillin-resistant clones).

Panning of the library

Immunotubes (Nunc) are coated overnight in PBS with 4 ml of either 100 mg/ml or 10 mg/ml of a polypeptide of the present invention. Tubes are blocked with 2% Marvel-PBS for 2 hours at 37° C and then washed 3 times in PBS. Approximately 10^{13} TU of phage are applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log *E. coli* TG1 by incubating eluted phage with bacteria for 30 minutes at 37° C. The *E. coli* are then plated on TYE plates containing 1% glucose and 100 μg/ml ampicillin. The resulting bacterial library is then rescued with M13 gene III helper phage as described above to prepare phage for a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

Characterization of binders

Eluted phage from the 3rd and 4th rounds of selection are used to infect *E. coli* HB 2151 and soluble scFv is produced (Marks, *et al.*, 1991) from single colonies for assay. ELISAs are performed with microtiter plates coated with either 10 pg/ml of the polypeptide of the present invention in 50 mM bicarbonate

pH 9.6. Clones positive in ELISA are further characterized by PCR fingerprinting (see *e.g.*, WO92/01047) and then by sequencing.

Example 22
Tissue distribution of DR4 gene expression

Northern blot analysis is carried out to examine DR4 gene (ATCC No. 97853) expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of the DR4 protein (SEQ ID NO:1) is labeled with ^{32}P using the *redi*prime™ DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified using a CHROMA SPIN-100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for DR4 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) are obtained from Clontech and are examined with labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70° C overnight, and films developed according to standard procedures. Expression of DR4 was detected in tissues enriched in lymphocytes including amniotic cells, heart, liver cancer, kidney, peripheral blood leukocytes, activated T-cell, K562 plus PMA, W138 cells, Th2 cells, human tonsils, and CD34 depleted buffy coat (cord blood). It can be envisaged that DR4 plays a role in lymphocyte homeostasis.

Example 23
Method of Determining Alterations in the DR4 Gene

RNA is isolated from entire families or individual patients presenting with a phenotype of interest (such as a disease). cDNA is then generated from these RNA samples using protocols known in the art. (See, Sambrook.) The cDNA is then used as a template for PCR, employing primers surrounding regions of interest in SEQ ID NO:1. Suggested PCR conditions consist of 35 cycles at 95° C for 30 seconds; 60-120 seconds at 52-58° C; and 60-120 seconds at 70° C, using buffer solutions described in Sidransky, D., *et al., Science 252*:706 (1991).

PCR products are then sequenced using primers labeled at their 5' end with T4 polynucleotide kinase, employing SequiTherm Polymerase. (Epicentre Technologies). The intron-exon borders of selected exons of DR4 are also determined and genomic PCR products analyzed to confirm the results. PCR products harboring suspected mutations in DR4 is then cloned and sequenced to validate the results of the direct sequencing.

PCR products of DR4 are cloned into T-tailed vectors as described in Holton, T.A. and Graham, M.W., *Nucleic Acids Research, 19*:1156 (1991) and sequenced with T7 polymerase (United States Biochemical). Affected individuals are identified by mutations in DR4 not present in unaffected individuals.

Genomic rearrangements are also observed as a method of determining alterations in the DR4 gene. Genomic clones isolated using techniques known in the art are nick-translated with digoxigenindeoxy-uridine 5'-triphosphate (Boehringer Manheim), and FISH performed as described in Johnson, C. *et al., Methods Cell Biol. 35*:73-99 (1991). Hybridization with the labeled probe is carried out using a vast excess of human cot-1 DNA for specific hybridization to the DR4 genomic locus.

Chromosomes are counterstained with 4,6-diamino-2-phenylidole and propidium iodide, producing a combination of C- and R-bands. Aligned images for precise mapping are obtained using a triple-band filter set (Chroma Technology, Brattleboro, VT) in combination with a cooled charge-coupled

device camera (Photometrics, Tucson, AZ) and variable excitation wavelength filters. (Johnson, C. *et al.*, Genet. Anal. Tech. Appl., 8:75 (1991).) Image collection, analysis and chromosomal fractional length measurements are performed using the ISee Graphical Program System. (Inovision Corporation, Durham, NC.) Chromosome alterations of the genomic region of DR4 (hybridized by the probe) are identified as insertions, deletions, and translocations. These DR4 alterations are used as a diagnostic marker for an associated disease.

Example 24

Method of Detecting Abnormal Levels of DR4 in a Biological Sample

DR4 polypeptides can be detected in a biological sample, and if an increased or decreased level of DR4 is detected, this polypeptide is a marker for a particular phenotype. Methods of detection are numerous, and thus, it is understood that one skilled in the art can modify the following assay to fit their particular needs.

For example, antibody-sandwich ELISAs are used to detect DR4 in a sample, preferably a biological sample. Wells of a microtiter plate are coated with specific antibodies to DR4, at a final concentration of 0.2 to 10 μg/ml. The antibodies are either monoclonal or polyclonal and are produced using technique known in the art. The wells are blocked so that non-specific binding of DR4 to the well is reduced.

The coated wells are then incubated for > 2 hours at RT with a sample containing DR4. Preferably, serial dilutions of the sample should be used to validate results. The plates are then washed three times with deionized or distilled water to remove unbounded DR4.

Next, 50 μl of specific antibody-alkaline phosphatase conjugate, at a concentration of 25-400 ng, is added and incubated for 2 hours at room temperature. The plates are again washed three times with deionized or distilled water to remove unbounded conjugate.

75 µl of 4-methylumbelliferyl phosphate (MUP) or p-nitrophenyl phosphate (NPP) substrate solution is then added to each well and incubated 1 hour at room temperature to allow cleavage of the substrate and flourescence. The flourescence is measured by a microtiter plate reader. A standard curve is prepared using the experimental results from serial dilutions of a control sample with the sample concentration plotted on the X-axis (log scale) and fluorescence or absorbance on the Y-axis (linear scale). The DR4 polypeptide concentration in a sample is then interpolated using the standard curve based on the measured flourescence of that sample.

Example 25
Method of Treating Decreased Levels of DR4

The present invention relates to a method for treating an individual in need of a decreased level of DR4 biological activity in the body comprising, administering to such an individual a composition comprising a therapeutically effective amount of DR4 antagonist. Preferred antagonists for use in the present invention are DR4-specific antibodies.

Moreover, it will be appreciated that conditions caused by a decrease in the standard or normal expression level of DR4 in an individual can be treated by administering DR4, preferably in a soluble and/or secreted form. Thus, the invention also provides a method of treatment of an individual in need of an increased level of DR4 polypeptide comprising administering to such an individual a pharmaceutical composition comprising an amount of DR4 to increase the biological activity level of DR4 in such an individual.

For example, a patient with decreased levels of DR4 polypeptide receives a daily dose 0.1-100 µg/kg of the polypeptide for six consecutive days. Preferably, the polypeptide is in a soluble and/or secreted form.

Example 26
Method of Treating Increased Levels of DR4

The present invention also relates to a method for treating an individual in need of an increased level of DR4 biological activity in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of DR4 or an agonist thereof.

Antisense technology is used to inhibit production of DR4. This technology is one example of a method of decreasing levels of DR4 polypeptide, preferably a soluble and/or secreted form, due to a variety of etiologies, such as cancer.

For example, a patient diagnosed with abnormally increased levels of DR4 is administered intravenously antisense polynucleotides at 0.5, 1.0, 1.5, 2.0 and 3.0 mg/kg day for 21 days. This treatment is repeated after a 7-day rest period if the is determined to be well tolerated.

Example 27
Method of Treatment Using Gene Therapy - Ex Vivo

One method of gene therapy transplants fibroblasts, which are capable of expressing soluble and/or mature DR4 polypeptides, onto a patient. Generally, fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (*e.g.*, Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37° C for approximately one week.

At this time, fresh media is added and subsequently changed every several

days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. *et al., DNA, 7*:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with *Eco*RI and *Hind*III and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding DR4 can be amplified using PCR primers which correspond to the 5' and 3' end encoding sequences respectively. Preferably, the 5' primer contains an *Eco*RI site and the 3' primer includes a *Hind*III site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified *Eco*RI and *Hind*III fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is then used to transform *E. coli* HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector contains properly inserted DR4.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the DR4 gene is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the DR4 gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a Millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or

his. Once the fibroblasts have been efficiently infected, the fibroblasts are analyzed to determine whether DR4 protein is produced.

The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads.

Example 28

Method of Treatment Using Gene Therapy - In Vivo

Another aspect of the present invention is using *in vivo* gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) DR4 sequences into an animal to increase or decrease the expression of the DR4 polypeptide. The DR4 polynucleotide may be operatively linked to a promoter or any other genetic elements necessary for the expression of the DR4 polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, WO90/11092, WO98/11779; U.S. Patent NO. 5693622, 5705151, 5580859; Tabata H. *et al., Cardiovasc. Res. 35*:470-479 (1997); Chao J. *et al., Pharmacol. Res. 35*:517-522 (1997); Wolff J.A. *Neuromuscul. Disord. 7*:314-318 (1997); Schwartz B. *et al., Gene Ther. 3*:405-411 (1996); Tsurumi Y. *et al., Circulation 94*:3281-3290 (1996) (incorporated herein by reference).

The DR4 polynucleotide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The DR4 polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

The term "naked" polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, the DR4 polynucleotides may also be delivered in liposome formulations (such as those taught in Felgner

P.L. *et al. Ann. NY Acad. Sci. 772*:126-139 (1995) and Abdallah B. *et al. Biol. Cell 85*(1):1-7 (1995)) which can be prepared by methods well known to those skilled in the art.

The DR4 polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene therapies techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

The DR4 polynucleotide construct can be delivered to the interstitial space of tissues within the an animal, including of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. *In vivo* muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked DR4 polynucleotide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 μg/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked DR4 polynucleotide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The dose response effects of injected DR4 polynucleotide in muscle *in vivo* is determined as follows. Suitable DR4 template DNA for production of mRNA coding for DR4 polypeptide is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is made on the anterior thigh, and the quadriceps muscle is directly visualized. The DR4 template DNA is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is closed with stainless steel clips.

After an appropriate incubation time (*e.g.*, 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 μm cross-section of the

individual quadriceps muscles is histochemically stained for DR4 protein expression. A time course for DR4 protein expression may be done in a similar fashion except that quadriceps from different mice are harvested at different times. Persistence of DR4 DNA in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be use to extrapolate proper dosages and other treatment parameters in humans and other animals using DR4 naked DNA.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

Further, the Sequence Listing submitted herewith, in both computer and paper forms, is hereby incorporated by reference in its entirety.

Applicant's or agent's file reference number 1488.130PC06	International application No. (TO BE ASSIGNED)

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 8, line 10.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
American Type Culture Collection

Address of depositary institution *(including postal code and country)*
10801 University Boulevard
Manassas, Virginia 20110-2209
United States of America

Date of deposit	Accession Number
January 21, 1997	97853

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

DNA Plasmid 1921583

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer SIGFRED ROSTAD INTERNATIONAL DIVISION 703-305-3680	Authorized officer

What Is Claimed Is:

1. A method for treating graft versus host disease, viral infection, cancer, leukemia, immunodeficiency, or an autoimmune disorder comprising administering to an individual therapeutically effective amounts of:

(a) a first therapeutic agent comprising an antibody which binds to a polypeptide consisting of amino acids 1 to 468 of SEQ ID NO:2; and

(b) a second therapeutic agent selected from the group consisting of:

(i) TRAIL;

(ii) a tumor necrosis factor;

(iii) a tumor necrosis factor blocking agent;

(iv) an immunosuppressive agent;

(v) an antibiotic;

(vi) an anti-inflammatory agent;

(vii) a chemotherapeutic agent; and

(viii) a cytokine.

2. The method of claim 1, wherein said first therapeutic agent comprises an antibody which binds to a polypeptide consisting of amino acids 24 to 238 of SEQ ID NO:2.

3. The method of claim 1, wherein said antibody is a monoclonal antibody.

4. The method of claim 1, wherein said antibody is a polyclonal antibody.

5. The method of claim 1, wherein said antibody is a chimeric antibody.

6. The method of claim 1, wherein said antibody is a humanized antibody.

7. The method of claim 1, wherein said antibody is a single-chain Fv antibody.

8. The method of claim 1, wherein said antibody is an Fab antibody fragment.

9. The method of claim 1, wherein said first and second therapeutic agents are administered to the individual at the same time.

10. The method of claim 1, wherein said first and second therapeutic agents are administered to the individual at different times.

11. The method of claim 1, wherein said second therapeutic agent is TRAIL.

12. The method of claim 1, wherein said tumor necrosis factor blocking agent comprises an antibody which binds to a protein selected from the group consisting of:

(a) TNF-α;

(b) TNF-β;

(c) TNF-γ;

(d) TNF-γ-α; and

(e) TNF-γ-β.

13. The method of claim 1, wherein said immunosuppressive agent is selected from the group consisting of:

(a) cyclosporine;

(b) cyclophosphamide;

(c) methylprednisone;

(d) prednisone;

(e) azathioprine;

(f) FK-506; and

(g) 15-deoxyspergualin.

14. The method of claim 1, wherein said cytokine is selected from the group consisting of:

(a) IL-2;

(b) IL-3;

(c) IL-4;

(d) IL-5;

(e) IL-6;

(f) IL-7;

(g) IL-10;

(h) IL-12;

(i) IL-13;

(j) IL-15; and

(k) IFN-γ.

15. A composition comprising:

(a) a first therapeutic agent comprising an antibody which binds to a polypeptide consisting of amino acids 1 to 468 of SEQ ID NO:2; and

(b) a second therapeutic agent selected from the group consisting of:

(i) TRAIL;

(ii) a tumor necrosis factor;

(iii) a tumor necrosis factor blocking agent;

(iv) an immunosuppressive agent;

(v) an antibiotic;

(vi) an anti-inflammatory agent;

(vii) a chemotherapeutic agent; and

(viii) a cytokine.

16. The composition of claim 15, which further comprises a pharmaceutically acceptable carrier or excipient.

17. An isolated polypeptide comprising an amino acid sequence at least 90% identical to amino acids 24 to 238 of SEQ ID NO:2;

wherein said polypeptide is covalently attached to polyethylene glycol, said polyethylene glycol having an average molecule weight selected from the group consisting of 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 15,000, and 20,000.

18. The polypeptide of claim 17, comprising an amino acid sequence at least 95% identical to amino acids 24 to 238 of SEQ ID NO:2.

19. The polypeptide of claim 18, wherein said amino acid sequence comprises amino acids 24 to 238 of SEQ ID NO:2.

20. The polypeptide of claim 17, wherein said polypeptide has an average degree of substitution with polyethylene glycol which falls within a range selected from the group consisting of 1-3, 2-4, 3-5, 4-6, 5-7, 6-8, 7-9, 8-10, 9-11, and 10-12.

21. The polypeptide of claim 17, which is produced by a recombinant host cell.

22. The polypeptide of claim 20, wherein said recombinant host cell is a eukaryotic host cell.

23. The polypeptide of claim 17, which comprises a heterologous polypeptide.

24. The polypeptide of claim 23, wherein said heterologous polypeptide comprises an Fc portion of an antibody.

25. A composition comprising the polypeptide of claim 17 and a pharmaceutically acceptable carrier.

Figure 1

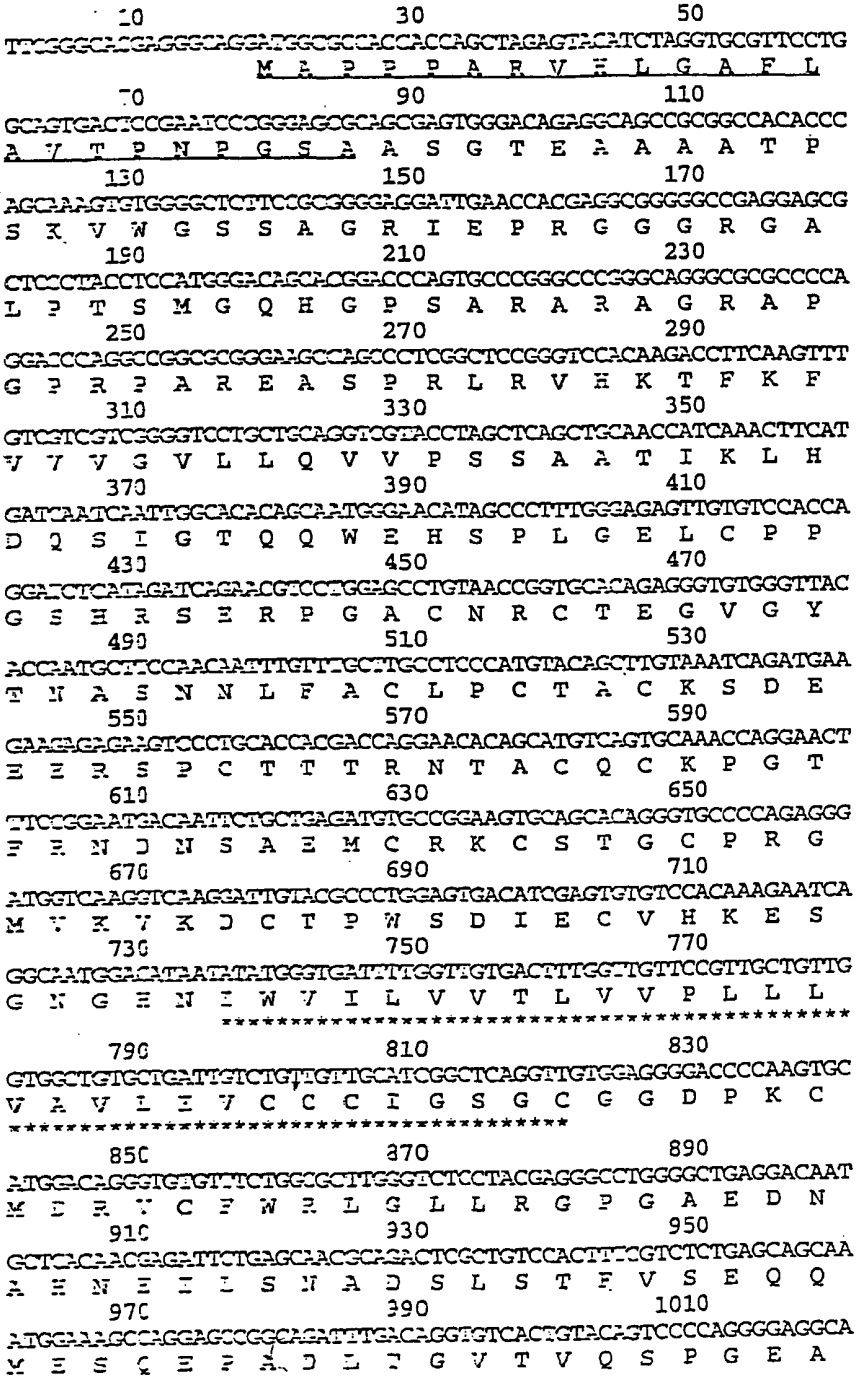
10 30 50
M A P P P A R V H L G A F L
70 90 110
A V T P N P G S A A S G T E A A A T P
130 150 170
S K V W G S S A G R I E P R G G G R G A
190 210 230
L P T S M G Q H G P S A R A R A G R A P
250 270 290
G P R P A R E A S P R L R V H K T F K F
310 330 350
V V V G V L L Q V V P S S A A T I K L H
370 390 410
D Q S I G T Q Q W E H S P L G E L C P P
430 450 470
G S H R S E R P G A C N R C T E G V G Y
490 510 530
T N A S N N L F A C L P C T A C K S D E
550 570 590
E E R S P C T T T R N T A C Q C K P G T
610 630 650
F E N D N S A E M C R K C S T G C P R G
670 690 710
M V K V K D C T P W S D I E C V H K E S
730 750 770
G N G E N I W V I L V V T L V V P L L L
****
790 810 830
V A V L I Y C C C I G S G C G G D P K C
****
850 870 890
M D R Y C F W R L G L L R G P G A E D N
910 930 950
A H N E I L S N A D S L S T F V S E Q Q
970 990 1010
M E S Q E P A D L I G V T V Q S P G E A

Figure 1 (cont.)

```
      1030                1050                1070
CAGTGTCTGCTGGGACCGGCAGAAGCTGAAGGGTCTCAGAGGAGGAGGCTGCTGGTTCCA
Q  C  L  L  G  P  A  E  A  E  G  S  Q  R  R  R  L  L  V  P
      1090                1110                1130
GCAAATGGTGCTGACCCCACTGAGACTCTGATGCTGTTCTTTGACAAGTTTGCAAACATC
A  N  G  A  D  P  T  E  T  L  M  L  F  F  D  K  F  A  N  I
      1150                1170                1190
GTGCCCTTTGACTCCTGGGACCAGCTCATGAGGCAGCTGGACCTCACGAAAAATGAGATC
V  P  F  D  S  W  D  Q  L  M  R  Q  L  D  L  T  K  N  E  I
      1210                1230                1250
GATGTGGTCAGAGCTGGTACAGCAGGCCCAGGGGATGCCTTGTATGCAATGCTGATGAAA
D  V  V  R  A  G  T  A  G  P  G  D  A  L  Y  A  M  L  M  K
      1270                1290                1310
TGGGTCAACAAAACTGGACGGAACGCCTCGATCCACACCCTGCTGGATGCCTTGGAGAGG
W  V  N  K  T  G  R  N  A  S  I  H  T  L  L  D  A  L  E  R
      1330                1350                1370
ATGGAAGAGAGACATGCAAAAGAGAAGATTCAGGACCTCTTGGTGGACTCTGGAAAGTTC
M  E  E  R  H  A  K  E  K  I  Q  D  L  L  V  D  S  G  K  F
      1390                1410                1430
ATCTACTTAGAAGATGGCACAGGCTCTGCCGTGTCCTTGGAGTGAAAGACTCTTTTTACC
I  Y  L  E  D  G  T  F  S  A  V  S  L  E
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AGAGGTTTCCTCTTAGGTGTTAGGAGTTAATACATATTAGGTTTTTTTTTTTTTAACAT
      1510                1530                1550
GTATACAAAGTAAATTCTTAGCCACGTGTATTGGCTCCTGCCTGTAATCCCATCACTTTG
      1570                1590                1610
GGAGGCTGACGCCGGTGGATCCACTTGAGGTCCGAAGTTCCAAGACCAGCCCTGAACCAA
      1630                1650                1670
CATCGTGGAAATGCCCGTCTTTTACAAAAAAATACCAAAAATTCAACTGGAATGTGCATG
      1690                1710                1730
GTGTGTGCCATCATTTCCTCGGCTAACTACGGGAGGTCTGAGGCCAGGAGAATCCACTTG
      1750                1770                1790
AACCCCACGAAGGACAGTGTAGACTGCAGATTGCACCACTGCACTCCCAGCCTGGGAACA
      1810                1830                1850
CAGAGCAAGACTCTGTCTCAAGATAAAATAAAATAAACTTGAAAGAATTATTGCCCGACT
      1870                1890                1910
GAGGCTCACATGCCAAAGGAAAATCTGGTTCTCCCCTGAGCTGGCCTCCGTGTGTTTCCT
      1930                1950                1970
TATCATGGTGGTCAATTGGAGGTGTTAATTTGAATGGATTAAGGAACACCTAGAACACTG
      1990                2010                2030
GTAAGGCATTATTTCTGGGACATTATTTCTGGGCATGTCTTCGAGGGTGTTTCCAGAGGG
      2050                2070                2090
GATTGGCATGCGATCGGGTGGACTGAGTGGAAAAGACCTACCCTTAATTTGGGGGGGCAC
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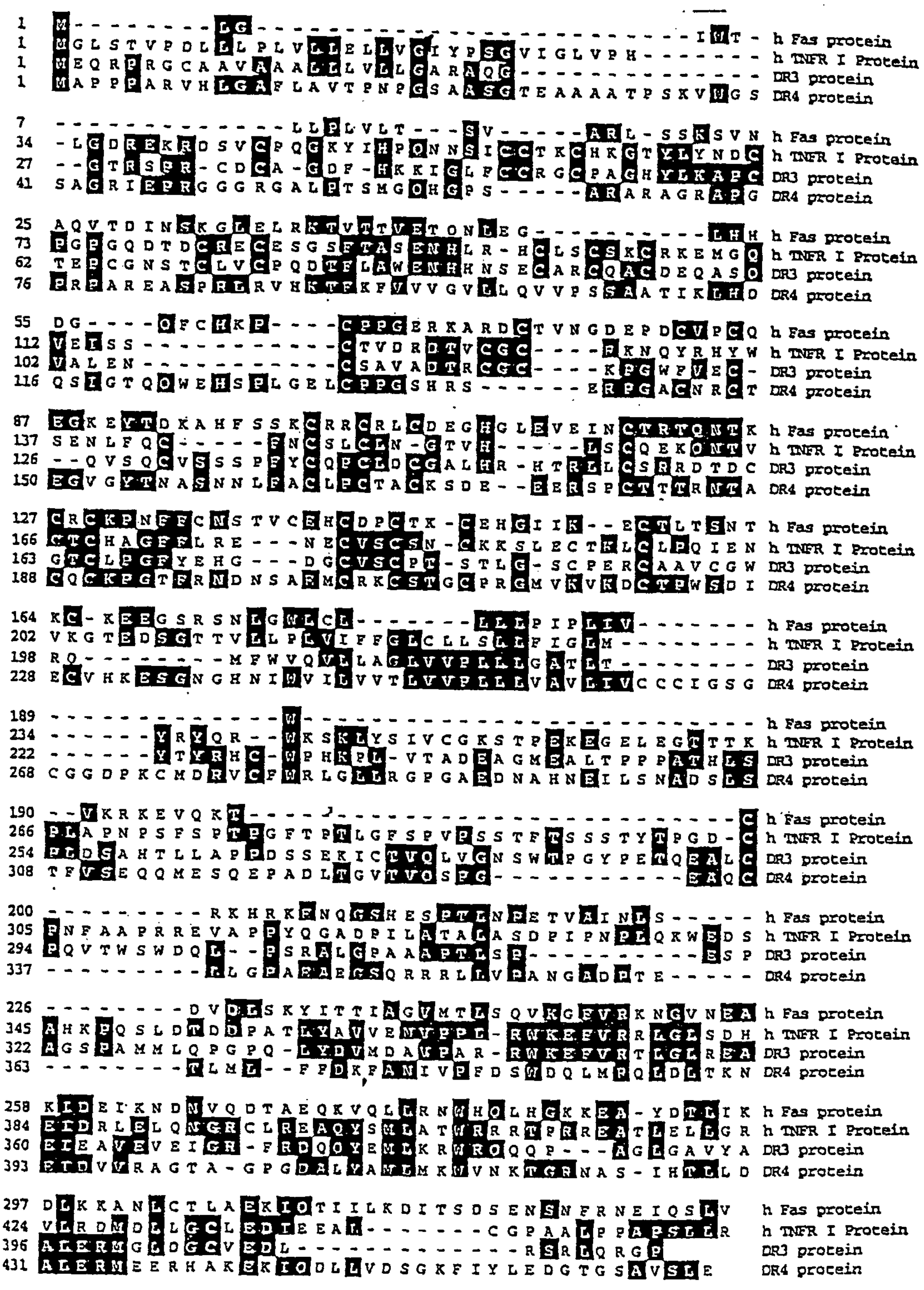
Figure 2



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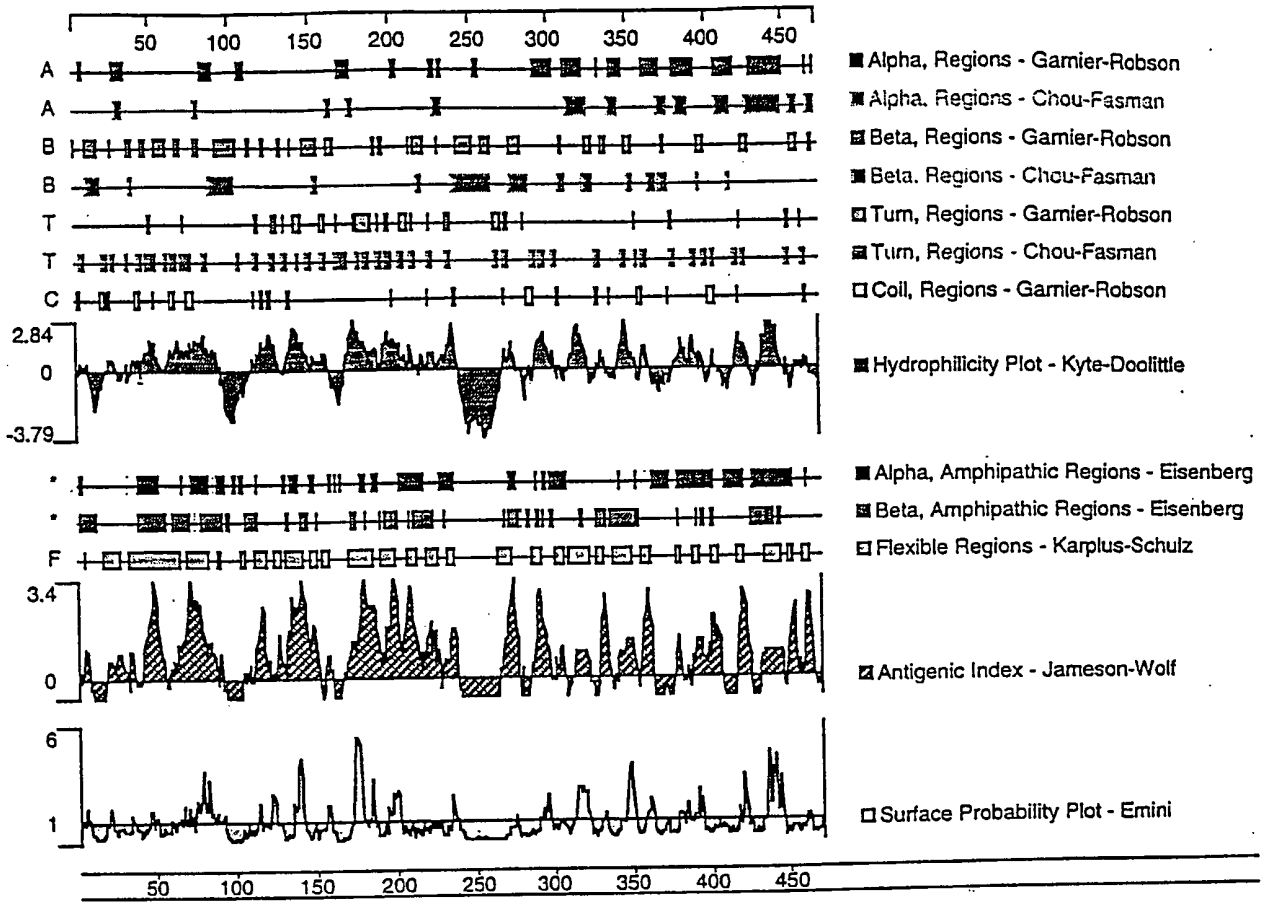
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Alpha, Regions - Chou-Fasman
Beta, Regions - Garnier-Robson
Beta, Regions - Chou-Fasman
Turn, Regions - Garnier-Robson
Turn, Regions - Chou-Fasman
Coil, Regions - Garnier-Robson
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0
-3.79
Hydrophilicity Plot - Kyte-Doolittle
Alpha, Amphipathic Regions - Eisenberg
Beta, Amphipathic Regions - Eisenberg
F
Flexible Regions - Karplus-Schulz
3.4
0
Antigenic Index - Jameson-Wolf
6
1
Surface Probability Plot - Emini
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100
150
200
250
300
350
400
450

FIGURE 4

HTOIY07R

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 51 TCAATTGGCA CACAGCAATG GGAAACATAG CCCTTTGGAA GANTTGTNTC
101 CACCAGGATC TCATAGATCA AAACATCCTG GGAGCCTGTT AACCGGTGCC
151 CCAAAGGNTG GTCAAGGTCA AGGAATTGTT NCGCCCTGGA AGTGAACATC
201 GAGTGTNTCC ACAAAGGATT CAGGCAATGG GACATAAATA TATGGGTGAA
251 TTTTGGTTGT GAACTTTGGT TGNTCCCGTT GNTGTTGNTG GCTGTGCTGA
301 TTGTTTGTTG TTGCATCGGC TTCAGGTTNT GGAGGGGGAC CCAAGTGCAT
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401 GCANGTTCAC AAGGGTTTTA GCAANG
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HTXEY80R

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151 TGAGTTGGGG ACAGGCCCTT GCAAGACCTT GTGAGGCAGG GGGTGAAGGC
201 CATGNCTCGG CTTCNNNTGG TCAAAGGGGA AGTGGAGCCT GAGGGAGATG
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301 AAGGGGGACA GGCATTTTTN CAAATGTGTG CTTNTTGGT
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Figure 5A

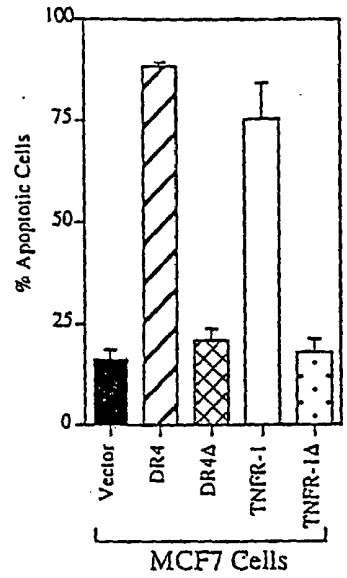


Figure 5B

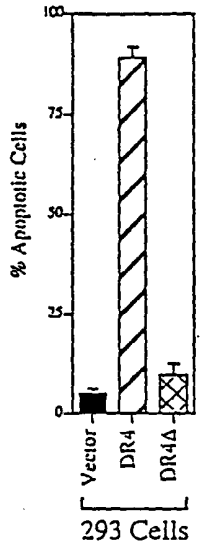


Figure 5C

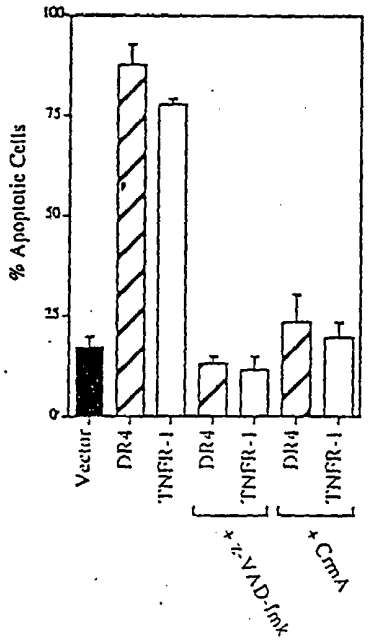


Figure 6A

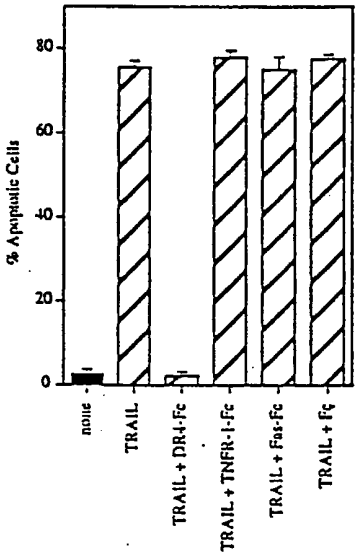
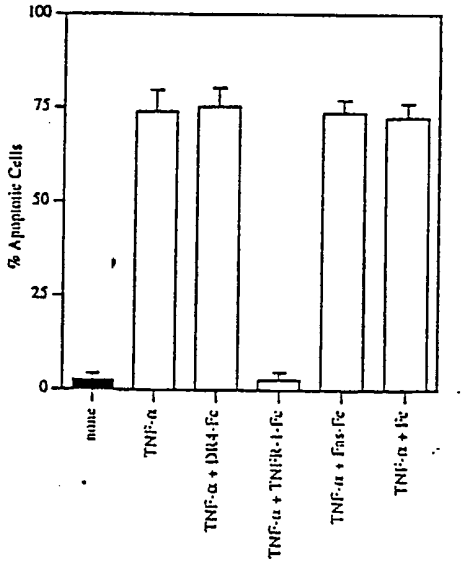


Figure 6B



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The Regents of the University of Michigan
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Rosen, Craig A.
Pan, James G.
Gentz, Reiner L.
Dixit, Vishva M.

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Ala Phe Leu Ala Val Thr Pro Asn Pro Gly Ser Ala Ala Ser Gly Thr
             15                  20                  25

gag gca gcc gcg gcc aca ccc agc aaa gtg tgg ggc tct tcc gcg ggg   147
Glu Ala Ala Ala Ala Thr Pro Ser Lys Val Trp Gly Ser Ser Ala Gly
         30                  35                  40

agg att gaa cca cga ggc ggg ggc cga gga gcg ctc cct acc tcc atg   195
Arg Ile Glu Pro Arg Gly Gly Gly Arg Gly Ala Leu Pro Thr Ser Met
     45                  50                  55

gga cag cac gga ccc agt gcc cgg gcc cgg gca ggg cgc gcc cca gga   243
Gly Gln His Gly Pro Ser Ala Arg Ala Arg Ala Gly Arg Ala Pro Gly
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ccc agg ccg gcg cgg gaa gcc agc cct cgg ctc cgg gtc cac aag acc   291
Pro Arg Pro Ala Arg Glu Ala Ser Pro Arg Leu Arg Val His Lys Thr
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ttc aag ttt gtc gtc gtc ggg gtc ctg ctg cag gtc gta cct agc tca   339
Phe Lys Phe Val Val Val Gly Val Leu Leu Gln Val Val Pro Ser Ser
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gct gca acc atc aaa ctt cat gat caa tca att ggc aca cag caa tgg   387
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        110                 115                 120

gaa cat agc cct ttg gga gag ttg tgt cca cca gga tct cat aga tca   435
Glu His Ser Pro Leu Gly Glu Leu Cys Pro Pro Gly Ser His Arg Ser
    125                 130                 135

gaa cgt cct gga gcc tgt aac cgg tgc aca gag ggt gtg ggt tac acc   483
Glu Arg Pro Gly Ala Cys Asn Arg Cys Thr Glu Gly Val Gly Tyr Thr
140                 145                 150                 155

aat gct tcc aac aat ttg ttt gct tgc ctc cca tgt aca gct tgt aaa   531
Asn Ala Ser Asn Asn Leu Phe Ala Cys Leu Pro Cys Thr Ala Cys Lys
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tca gat gaa gaa gag aga agt ccc tgc acc acg acc agg aac aca gca   579
Ser Asp Glu Glu Glu Arg Ser Pro Cys Thr Thr Thr Arg Asn Thr Ala
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tgt cag tgc aaa cca gga act ttc cgg aat gac aat tct gct gag atg   627
Cys Gln Cys Lys Pro Gly Thr Phe Arg Asn Asp Asn Ser Ala Glu Met
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Cys Arg Lys Cys Ser Thr Gly Cys Pro Arg Gly Met Val Lys Val Lys
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gat tgt acg ccc tgg agt gac atc gag tgt gtc cac aaa gaa tca ggc   723
Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His Lys Glu Ser Gly
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aat gga cat aat ata tgg gtg att ttg gtt gtg act ttg gtt gtt ccg   771
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                240                 245                 250

ttg ctg ttg gtg gct gtg ctg att gtc tgt tgt tgc atc ggc tca ggt   819
Leu Leu Leu Val Ala Val Leu Ile Val Cys Cys Cys Ile Gly Ser Gly
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tgt gga ggg gac ccc aag tgc atg gac agg gtg tgt ttc tgg cgc ttg   867
Cys Gly Gly Asp Pro Lys Cys Met Asp Arg Val Cys Phe Trp Arg Leu
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ggt ctc cta cga ggg cct ggg gct gag gac aat gct cac aac gag att   915
Gly Leu Leu Arg Gly Pro Gly Ala Glu Asp Asn Ala His Asn Glu Ile
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ctg agc aac gca gac tcg ctg tcc act ttc gtc tct gag cag caa atg   963
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300                 305                 310                 315

gaa agc cag gag ccg gca gat ttg aca ggt gtc act gta cag tcc cca  1011
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ggg gag gca cag tgt ctg ctg gga ccg gca gaa gct gaa ggg tct cag  1059
Gly Glu Ala Gln Cys Leu Leu Gly Pro Ala Glu Ala Glu Gly Ser Gln
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tgg gac cag ctc atg agg cag ctg gac ctc acg aaa aat gag atc gat   1203
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gtg gtc aga gct ggt aca gca ggc cca ggg gat gcc ttg tat gca atg   1251
Val Val Arg Ala Gly Thr Ala Gly Pro Gly Asp Ala Leu Tyr Ala Met
                400                 405                 410

ctg atg aaa tgg gtc aac aaa act gga cgg aac gcc tcg atc cac acc   1299
Leu Met Lys Trp Val Asn Lys Thr Gly Arg Asn Ala Ser Ile His Thr
            415                 420                 425

ctg ctg gat gcc ttg gag agg atg gaa gag aga cat gca aaa gag aag   1347
Leu Leu Asp Ala Leu Glu Arg Met Glu Glu Arg His Ala Lys Glu Lys
        430                 435                 440

att cag gac ctc ttg gtg gac tct gga aag ttc atc tac tta gaa gat   1395
Ile Gln Asp Leu Leu Val Asp Ser Gly Lys Phe Ile Tyr Leu Glu Asp
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Gly Thr Gly Ser Ala Val Ser Leu Glu
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         35                  40                  45

Gly Gly Gly Arg Gly Ala Leu Pro Thr Ser Met Gly Gln His Gly Pro
     50                  55                  60

Ser Ala Arg Ala Arg Ala Gly Arg Ala Pro Gly Pro Arg Pro Ala Arg
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Glu Ala Ser Pro Arg Leu Arg Val His Lys Thr Phe Lys Phe Val Val
                 85                  90                  95

Val Gly Val Leu Leu Gln Val Val Pro Ser Ser Ala Ala Thr Ile Lys
            100                 105                 110

Leu His Asp Gln Ser Ile Gly Thr Gln Gln Trp Glu His Ser Pro Leu
        115                 120                 125

Gly Glu Leu Cys Pro Pro Gly Ser His Arg Ser Glu Arg Pro Gly Ala
    130                 135                 140

Cys Asn Arg Cys Thr Glu Gly Val Gly Tyr Thr Asn Ala Ser Asn Asn
145                 150                 155                 160

Leu Phe Ala Cys Leu Pro Cys Thr Ala Cys Lys Ser Asp Glu Glu Glu
                165                 170                 175

Arg Ser Pro Cys Thr Thr Thr Arg Asn Thr Ala Cys Gln Cys Lys Pro
            180                 185                 190

Gly Thr Phe Arg Asn Asp Asn Ser Ala Glu Met Cys Arg Lys Cys Ser
        195                 200                 205

Thr Gly Cys Pro Arg Gly Met Val Lys Val Lys Asp Cys Thr Pro Trp
    210                 215                 220

Ser Asp Ile Glu Cys Val His Lys Glu Ser Gly Asn Gly His Asn Ile
225                 230                 235                 240

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

Val Leu Ile Val Cys Cys Cys Ile Gly Ser Gly Cys Gly Gly Asp Pro
            260                 265                 270

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

Pro Gly Ala Glu Asp Asn Ala His Asn Glu Ile Leu Ser Asn Ala Asp
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Ser Leu Ser Thr Phe Val Ser Glu Gln Gln Met Glu Ser Gln Glu Pro
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Ala Asp Leu Thr Gly Val Thr Val Gln Ser Pro Gly Glu Ala Gln Cys
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370 375 380

Arg Gln Leu Asp Leu Thr Lys Asn Glu Ile Asp Val Val Arg Ala Gly
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Thr Ala Gly Pro Gly Asp Ala Leu Tyr Ala Met Leu Met Lys Trp Val
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Asn Lys Thr Gly Arg Asn Ala Ser Ile His Thr Leu Leu Asp Ala Leu
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Asp Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His
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Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Glu Gly His Gly
100 105 110

Leu Glu Val Glu Ile Asn Cys Thr Arg Thr Gln Asn Thr Lys Cys Arg
115 120 125

Cys Lys Pro Asn Phe Phe Cys Asn Ser Thr Val Cys Glu His Cys Asp

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    130                 135                 140

Pro Cys Thr Lys Cys Glu His Gly Ile Ile Lys Glu Cys Thr Leu Thr
145                 150                 155                 160

Ser Asn Thr Lys Cys Lys Glu Glu Gly Ser Arg Ser Asn Leu Gly Trp
                165                 170                 175

Leu Cys Leu Leu Leu Leu Pro Ile Pro Leu Ile Val Trp Val Lys Arg
            180                 185                 190

Lys Glu Val Gln Lys Thr Cys Arg Lys His Arg Lys Glu Asn Gln Gly
        195                 200                 205

Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr Val Ala Ile Asn Leu
    210                 215                 220

Ser Asp Val Asp Leu Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met
225                 230                 235                 240

Thr Leu Ser Gln Val Lys Gly Phe Val Arg Lys Asn Gly Val Asn Glu
                245                 250                 255

Ala Lys Ile Asp Glu Ile Lys Asn Asp Asn Val Gln Asp Thr Ala Glu
            260                 265                 270

Gln Lys Val Gln Leu Leu Arg Asn Trp His Gln Leu His Gly Lys Lys
        275                 280                 285

Glu Ala Tyr Asp Thr Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys
    290                 295                 300

Thr Leu Ala Glu Lys Ile Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser
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Asp Ser Glu Asn Ser Asn Phe Arg Asn Glu Ile Gln Ser Leu Val
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His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
        35                  40                  45

Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
    50                  55                  60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
65                  70                  75                  80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
                85                  90                  95
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Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
        115                 120                 125

Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
    130                 135                 140

Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
145                 150                 155                 160

Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu
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Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr
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Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
        195                 200                 205

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu
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225                 230                 235                 240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu
                245                 250                 255

Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser
            260                 265                 270

Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val
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Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr Pro Gly Asp Cys
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Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly
305                 310                 315                 320

Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala Ser Asp Pro Ile Pro Asn
                325                 330                 335

Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp
            340                 345                 350

Thr Asp Asp Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro
        355                 360                 365

Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu
    370                 375                 380

Ile Asp Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln
385                 390                 395                 400

Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala
                405                 410                 415

Thr Leu Glu Leu Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly
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Arg Gly Cys Pro Ala Gly His Tyr Leu Lys Ala Pro Cys Thr Glu Pro
     50                  55                  60

Cys Gly Asn Ser Thr Cys Leu Val Cys Pro Gln Asp Thr Phe Leu Ala
 65                  70                  75                  80

Trp Glu Asn His His Asn Ser Glu Cys Ala Arg Cys Gln Ala Cys Asp
                 85                  90                  95

Glu Gln Ala Ser Gln Val Ala Leu Glu Asn Cys Ser Ala Val Ala Asp
            100                 105                 110

Thr Arg Cys Gly Cys Lys Pro Gly Trp Phe Val Glu Cys Gln Val Ser
        115                 120                 125

Gln Cys Val Ser Ser Ser Pro Phe Tyr Cys Gln Pro Cys Leu Asp Cys
    130                 135                 140

Gly Ala Leu His Arg His Thr Arg Leu Leu Cys Ser Arg Arg Asp Thr
145                 150                 155                 160

Asp Cys Gly Thr Cys Leu Pro Gly Phe Tyr Glu His Gly Asp Gly Cys
                165                 170                 175

Val Ser Cys Pro Thr Ser Thr Leu Gly Ser Cys Pro Glu Arg Cys Ala
            180                 185                 190

Ala Val Cys Gly Trp Arg Gln Met Phe Trp Val Gln Val Leu Leu Ala
        195                 200                 205

Gly Leu Val Val Pro Leu Leu Leu Gly Ala Thr Leu Thr Tyr Thr Tyr
    210                 215                 220

Arg His Cys Trp Pro His Lys Pro Leu Val Thr Ala Asp Glu Ala Gly
225                 230                 235                 240

Met Glu Ala Leu Thr Pro Pro Pro Ala Thr His Leu Ser Pro Leu Asp
                245                 250                 255
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Ser Ala His Thr Leu Leu Ala Pro Pro Asp Ser Ser Glu Lys Ile Cys
            260                 265                 270

Thr Val Gln Leu Val Gly Asn Ser Trp Thr Pro Gly Tyr Pro Glu Thr
        275                 280                 285

Gln Glu Ala Leu Cys Pro Gln Val Thr Trp Ser Trp Asp Gln Leu Pro
    290                 295                 300

Ser Arg Ala Leu Gly Pro Ala Ala Ala Pro Thr Leu Ser Pro Glu Ser
305                 310                 315                 320

Pro Ala Gly Ser Pro Ala Met Met Leu Gln Pro Gly Pro Gln Leu Tyr
                325                 330                 335

Asp Val Met Asp Ala Val Pro Ala Arg Arg Trp Lys Glu Phe Val Arg
            340                 345                 350

Thr Leu Gly Leu Arg Glu Ala Glu Ile Glu Ala Val Glu Val Glu Ile
        355                 360                 365

Gly Arg Phe Arg Asp Gln Gln Tyr Glu Met Leu Lys Arg Trp Arg Gln
    370                 375                 380

Gln Gln Pro Ala Gly Leu Gly Ala Val Tyr Ala Ala Leu Glu Arg Met
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Gly Leu Asp Gly Cys Val Glu Asp Leu Arg Ser Arg Leu Gln Arg Gly
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Pro
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gagggagatg ggacttnagg gggacggngc tgcgtgggga aaaagcagcc accntttgac 300
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<211> 28
<212> DNA
<213> Artificial Sequence

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<223> Description of Artificial Sequence: DNA Primer

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<223> Description of Artificial Sequence: DNA Primer

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<210> 10
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<220>
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tctcccggac tcctgaggtc acatgcgtgg tggtggacgt aagccacgaa gaccctgagg 180
tcaagttcaa ctggtacgtg gacggcgtgg aggtgcataa tgccaagaca aagccgcggg 240
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acaagagcag gtggcagcag gggaacgtct tctcatgctc cgtgatgcat gaggctctgc 660
acaaccacta cacgcagaag agcctctccc tgtctccggg taaatgagtg cgacggccgc 720
gactctagag gat 733

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12163

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 39/395; C07K 1/100, 14/00, 17/00
US CL : 424/130.1;530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/130.1;530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST,STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PAN ET AL. "The receptor for the cytotoxic ligand TRAIL." SCIENCE, April 1997, Vol 276, pages 111-113.	1-10,15-25
Y	WO 98/32856 (GENTZ ET AL.) 30 July 1998 (30.07.98) pages 3, 21,28,30,34,36,37	1-10,15-16,21-23,25
Y	Database Medline, US National Library of Medicine (Bethesda, MD, USA), No. 97238921, PAN et al. "The receptor for the cytotoxic ligand TRAIL", April 1997.	1-10,15-25
Y	US 4,179,337 (DAVIS et al.) 18 December 1979 (18.12.1979) abstract	17
Y	DELGADO et al., "Quantitative analysis of polyethylene glycol (PEG) in PEG-modified proteins/cytokines by aqueous two-phase systems". J.Biochem.Biophys.Methods, 1994, Vol. 29, pages 237-250.	17,20
Y	DILLMAN, R.O. "Monoclonal Antibodies for Treating Cancer". Annals of Internal Medicine. 1989, Vol. 111. pages 592-603, especially page 597	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
11 August 2000 (11.08.2000)

Date of mailing of the international search report
29 AUG 2000

Name and mailing address of the ISA/US
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Gary Nickol
Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/12163

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim Nos.: 11-14
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 11-14 were part of groups not paid for in the request for additonal fees

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
SEE INVITATION TO PAY ADDITIONAL FEES

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.: 1-10,15-25

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.