ANTI-DLL4 ANTIBODIES AND METHODS USING SAME

FIELD OF THE INVENTION

The present invention relates generally to the field of molecular biology. More specifically, the invention concerns anti-DLL4 antibodies, and uses of same.

BACKGROUND OF THE INVENTION

Development of a vascular supply is a fundamental requirement for many physiological and pathological processes. Actively growing tissues such as embryos and tumors require adequate blood supply. They satisfy this need by producing pro-angiogenic factors, which promote new blood vessel formation via a process called angiogenesis. Vascular tube formation is a complex but orderly biological event involving all or many of the following steps: a) endothelial cells (ECs) proliferate from existing ECs or differentiate from progenitor cells; b) ECs migrate and coalesce to form cord-like structures; c) vascular cords then undergo tubulogenesis to form vessels with a central lumen; d) existing cords or vessels send out sprouts to form secondary vessels; e) primitive vascular plexus undergo further remodeling and reshaping; and f) peri-endothelial cells are recruited to encase the endothelial tubes, providing maintenance and modulatory functions to the vessels; such cells including pericytes for small capillaries, smooth muscle cells for larger vessels, and myocardial cells in the heart. Hanahan, *Science* 277:48-50 (1997); Hogan & Kolodziej, *Nat. Rev. Genet.* 3:513-23 (2002); Lubarsky & Krasnow, *Cell* 112:19-28 (2003).

It is now well established that angiogenesis is implicated in the pathogenesis of a variety of disorders. These include solid tumors and metastasis, atherosclerosis, retrolental fibroplasia, hemangiomas, chronic inflammation, intraocular neovascular diseases such as proliferative retinopathies, e.g., diabetic retinopathy, age-related macular degeneration (AMD), neovascular glaucoma, immune rejection of transplanted corneal tissue and other tissues, rheumatoid arthritis, and psoriasis. Folkman et al., *J. Biol. Chem.* 267:10931-34 (1992); Klagsbrun et al., *Annu. Rev. Physiol.* 53:217-39 (1991); and Garner A., "Vascular diseases," In: Pathobiology of Ocular Disease. A Dynamic Approach, Garner A., Klintworth GK, eds., 2nd Edition (Marcel Dekker, NY, 1994), pp 1625-1710.

In the case of tumor growth, angiogenesis appears to be crucial for the transition from hyperplasia to neoplasia, and for providing nourishment for the growth and metastasis of the tumor. Folkman et al., *Nature* 339:58 (1989). The neovascularization

allows the tumor cells to acquire a growth advantage and proliferative autonomy compared to the normal cells. A tumor usually begins as a single aberrant cell which can proliferate only to a size of a few cubic millimeters due to the distance from available capillary beds, and it can stay 'dormant' without further growth and dissemination for a long period of time. Some tumor cells then switch to the angiogenic phenotype to activate endothelial cells, which proliferate and mature into new capillary blood vessels. These newly formed blood vessels not only allow for continued growth of the primary tumor, but also for the dissemination and recolonization of metastatic tumor cells. Accordingly, a correlation has been observed between density of microvessels in tumor sections and patient survival in breast cancer as well as in several other tumors. Weidner et al., *N. Engl. J. Med.* 324:1-6 (1991); Horak et al., *Lancet* 340:1120-24 (1992); Macchiarini et al., *Lancet* 340:145-46 (1992). The precise mechanisms that control the angiogenic switch is not well understood, but it is believed that neovascularization of tumor mass results from the net balance of a multitude of angiogenesis stimulators and inhibitors (Folkman, *Nat. Med.* 1(1):27-31 (1995)).

The process of vascular development is tightly regulated. To date, a significant number of molecules, mostly secreted factors produced by surrounding cells, have been shown to regulate EC differentiation, proliferation, migration and coalescence into cord-like structures. For example, vascular endothelial growth factor (VEGF) has been identified as the key factor involved in stimulating angiogenesis and in inducing vascular permeability. Ferrara et al., *Endocr. Rev.* 18:4-25 (1997). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system. Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders. Ferrara et al., *Endocr. Rev. supra*. The VEGF mRNA is overexpressed by the majority of human tumors examined. Berkman et al., *J. Clin. Invest.* 91:153-59 (1993); Brown et al., *Human Pathol.* 26:86-91 (1995); Brown et al., *Cancer Res.* 53:4727-35 (1993); Mattern et al., *Brit. J. Cancer* 73:931-34 (1996); Dvorak et al., *Am. J. Pathol.* 146:1029-39 (1995).

Also, the concentration levels of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies. Aiello et al., *N. Engl. J. Med.* 331:1480-87 (1994). Furthermore, studies have demonstrated the localization of VEGF in choroidal neovascular

membranes in patients affected by AMD. Lopez et al., *Invest. Ophthalmol. Vis. Sci.* 37:855-68 (1996).

Anti-VEGF neutralizing antibodies suppress the growth of a variety of human tumor cell lines in nude mice (Kim et al., *Nature* 362:841-44 (1993); Warren et al., *J. Clin. Invest.* 95:1789-97 (1995); Borgström et al., *Cancer Res.* 56:4032-39 (1996); Melnyk et al., *Cancer Res.* 56:921-24 (1996)) and also inhibit intraocular angiogenesis in models of ischemic retinal disorders (Adamis et al., *Arch. Ophthalmol.* 114:66-71 (1996)). Therefore, anti-VEGF monoclonal antibodies or other inhibitors of VEGF action are promising candidates for the treatment of tumors and various intraocular neovascular disorders. Such antibodies are described, for example, in EP 817,648, published January 14, 1998; and in WO 98/45331 and WO 98/45332, both published October 15, 1998. One anti-VEGF antibody, bevacizumab, has been approved by the FDA for use in combination with a chemotherapy regimen to treat specific cancers and an anti-VEGF antibody fragment, ranibizumab, has been approved by the FDA to treat age-related (wet) macular degeneration. Both drugs are being investigated in ongoing clinical trials.

It is clear that there continues to be a need for agents that have clinical attributes that are optimal for development as therapeutic agents. The invention described herein meets this need and provides other benefits.

All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The invention is in part based on the identification of a variety of DLL4 binding agents (such as immunoconjugates, antibodies, and fragments thereof). DLL4 presents as an important and advantageous therapeutic target, and the invention provides compositions and methods based on binding DLL4. DLL4 binding agents of the invention, as described herein, provide important therapeutic and diagnostic agents for use in targeting pathological conditions associated with expression and/or activity of the DLL4-Notch receptor pathways. Accordingly, the invention provides methods, compositions, kits and articles of manufacture related to DLL4 binding.

The present invention provides antibodies that bind (such as specifically bind) to DLL4.

In one aspect, the invention provides an isolated anti-DLL4 antibody, wherein a full length IgG form of the antibody specifically binds human DLL4 with a binding affinity of about 1 nM or better, or about 500 pM or better. As is well-established in the art, binding affinity of a ligand to its receptor can be determined using any of a variety of assays, and expressed in terms of a variety of quantitative values. Accordingly, in one embodiment, the binding affinity is expressed as Kd values and reflects intrinsic binding affinity (e.g., with minimized avidity effects). Generally and preferably, binding affinity is measured in vitro, whether in a cell-free or cell-associated setting. Any of a number of assays known in the art, including those described herein, can be used to obtain binding affinity measurements, including, for example, Biacore®, radioimmunoassay (RIA) and ELISA. In some embodiments, the isolated anti-DLL4 antibody binds to both human and mouse DLL4 with similar affinity, i.e. the binding affinity for human DLL4 is no more than 100-fold more or less than the binding affinity for mouse DLL4. In some embodiments, the binding affinity for human DLL4 is no more than 10-fold more or less than the binding affinity for mouse DLL4. In some embodiments, the antibody specifically binds mouse DLL4 with a binding affinity of about 1 nM or better, or about 500 pM or better.

In one aspect, the invention provides an isolated anti-DLL4 antibody, wherein a full length IgG form of the antibody specifically binds human DLL4 with a k_{on} of about 2 X 10^5 or better, or about 1 X 10^5 or better. As is well-established in the art, the k_{on} of binding of a ligand to its receptor can be determined using any of a variety of assays and expressed in terms of a variety of quantitative values.

In one aspect, the invention provides an isolated antibody that binds a ligand binding region of DLL4. In some embodiments, the isolated antibody binds a polypeptide comprising, consisting of or consisting essentially of the DLL4 extracellular domain. In some embodiments, the isolated antibody binds a polypeptide comprising, consisting of or consisting essentially of amino acids 252-282, 1-252, 1-286, 1-324, and/or 219-286 of human DLL4.

In one aspect, the invention provides an isolated anti-DLL4 antibody that competes with Notch receptor for binding of DLL4.

In one aspect, the invention provides an isolated anti-DLL4 antibody that inhibits, reduces, and/or blocks DLL4 biological activity.

In one aspect, an anti-DLL4 antibody of the invention comprises:

(a) at least one, two, three, four, five or six hypervariable region (HVR) sequences selected from the group consisting of:

(i) HVR-L1 comprising sequence A1-A11, wherein A1-A11 is RASQDVSTAVA (SEQ ID NO: 10)

(ii) HVR-L2 comprising sequence B1-B7, wherein B1-B7 is SASFLYS (SEQ ID NO:11)

(iii) HVR-L3 comprising sequence C1-C9, wherein C1-C9 is QQSYNGPST (SEQ ID NO:15)

(iv) HVR-H1 comprising sequence D1-D10, wherein D1-D10 is GFTFTDNWIS (SEQ ID NO:1)

(v) HVR-H2 comprising sequence E1-E18, wherein E1-E18 is GVINPNSGATEYADSVKG (SEQ ID NO:5) and

(vi) HVR-H3 comprising sequence F1-F15, wherein F1-F15 is VYYCARDNFGGYFDY (SEQ ID NO:9) ; and

(b) at least one variant HVR, wherein the variant HVR sequence comprises modification of at least one residue of the sequence depicted in SEQ ID NOs:1-18.

In one aspect, an anti-DLL4 antibody of the invention comprises:

(a) at least one, two, three, four or five hypervariable region (HVR) sequences selected from the group consisting of:

(i) HVR-L1 comprising sequence A1-A11, wherein A1-A11 is RASQDVSTAVA (SEQ ID NO: 10)

(ii) HVR-L2 comprising sequence B1-B7, wherein B1-B7 is SASFLYS (SEQ ID NO:11)

(iii) HVR-L3 comprising sequence C1-C9, wherein C1-C9 is QQSVNGPAT (SEQ ID NO:14)

(iv) HVR-H1 comprising sequence D1-D10, wherein D1-D10 is GFSFRDNWIS (SEQ ID NO:2);

(v) HVR-H2 comprising sequence E1-E18, wherein E1-E18 is GVINPNSGSTDYADSVKG (SEQ ID NO:3);

(vi) HVR-H3 comprising sequence F1-F15, wherein F1-F15 is VYYCARDNFGGYFDY (SEQ ID NO:9); and

(b) at least one variant HVR, wherein the variant HVR sequence comprises modification of at least one residue of the sequence depicted in SEQ ID NOs:1-18.

In one aspect, an anti-DLL4 antibody of the invention comprises:

(a) at least one, two, three, four or five hypervariable region (HVR) sequences selected from the group consisting of:

(i) HVR-L1 comprising sequence A1-A11, wherein A1-A11 is RASQDVSTAVA (SEQ ID NO: 10)

(ii) HVR-L2 comprising sequence B1-B7, wherein B1-B7 is SASFLYS (SEQ ID NO:11)

(iii) HVR-L3 comprising sequence C1-C9, wherein C1-C9 is QQSYTGTVT (SEQ ID NO:18)

(iv) HVR-H1 comprising sequence D1-D10, wherein D1-D10 is GFTFTDNWIS (SEQ ID NO:1);

(v) HVR-H2 comprising sequence E1-E18, wherein E1-E18 is GYISPNSGFTYYADSVKG (SEQ ID NO:8) and

(vi) HVR-H3 comprising sequence F1-F15, wherein F1-F15 is VYYCARDNFGGYFDY (SEQ ID NO:9) ; and

(b) at least one variant HVR, wherein the variant HVR sequence comprises modification of at least one residue of the sequence depicted in SEQ ID NOs:1-18.

In one aspect, the invention provides an antibody comprising one, two, three, four, five or six HVRs, wherein each HVR comprises, consists or consists essentially of a sequence selected from the group consisting of SEQ ID NOs: 1-18, and wherein SEQ ID NO:10 corresponds to an HVR-L1, SEQ ID NO:11 corresponds to an HVR-L2, SEQ ID NO:12, 13, 14, 15, 16, 17, or 18 correspond to an HVR-L3, SEQ ID NO:1 or 2 correspond to an HVR-H1, SEQ ID NO:3, 4, 5, 6, 7 or 8 correspond to an HVR-H2, and SEQ ID NO:9 corresponds to an HVR-H3. In one embodiment, an antibody of the invention comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 12, 1, 3, 9. In one embodiment, an antibody of the invention comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 13, 1, 4, 9. In one embodiment, an antibody of the invention comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 14, 2, 3, 9. In one embodiment, an antibody of the invention comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 15, 1, 5, 9. In one embodiment, an antibody of the invention comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 16, 1, 6, 9. In one embodiment, an antibody of the invention comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 17, 1, 7, 9. In one embodiment, an antibody of the

invention comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 18, 1, 8, 9.

Variant HVRs in an antibody of the invention can have modifications of one or more (such as two, three, four, five, or more) residues within the HVR.

In one embodiment, a HVR-L3 variant comprises 1-6 (1, 2, 3, 4, 5, or 6) substitutions in any combination of the following positions: 91 (S or W), 92 (Y or F), 93 (T, N or S), 94 (T or G), 95 (P, Q, A or T),and/or 96 (P, S, A, or V).

In one embodiment, a HVR- H2 variant comprises 1-4 (1, 2, 3, or 4) substitutions in any combination of the following positions: 50 (V, L or Y), 52 (N or S), 52a (P or S), or 53 (N, Q, T, or I).

Letter(s) in parenthesis following each position indicates an illustrative substitution (i.e., replacement) amino acid; as would be evident to one skilled in the art, suitability of other amino acids as substitution amino acids in the context described herein can be routinely assessed using techniques known in the art and/or described herein.

In one aspect, the invention provides an antibody comprising a HVR-H1 region comprising the sequence of SEQ ID NO:1 or 2. In one aspect, the invention provides an antibody comprising a HVR-H2 region comprising the sequence of SEQ ID NO:3, 4, 5, 6, 7, or 8. In one aspect, the invention provides an antibody comprising a HVR-H3 region comprising the sequence of SEQ ID NO: 9. In one embodiment, the invention provides an antibody comprising a HVR-L1 region comprising the sequence of SEQ ID NO:10. In one embodiment, the invention provides an antibody comprising a HVR-L2 region comprising the sequence of SEQ ID NO: 11. In one embodiment, the invention provides an antibody comprising a HVR-L3 region comprising the sequence of SEQ ID NO: 12, 13, 14, 15, 16, 17, or 18.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following:

(i) a HVR-H1 sequence comprising the sequence of SEQ ID NO:1;

(ii) a HVR-H2 sequence comprising the sequence of SEQ ID NO: 5;

(iii) a HVR-H3 sequence comprising the sequence of SEQ ID NO: 9.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following:

(i) a HVR-L1 sequence comprising the sequence of SEQ ID NO: 10;

(ii) a HVR-L2 sequence comprising the sequence of SEQ ID NO: 11;

(iii) a HVR-L3 sequence comprising the sequence of SEQ ID NO: 15.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following:

(i) a HVR-H1 sequence comprising the sequence of SEQ ID NO:1;

(ii) a HVR-H2 sequence comprising the sequence of SEQ ID NO: 8;

(iii) a HVR-H3 sequence comprising the sequence of SEQ ID NO: 9.

In one aspect, the invention provides an antibody comprising at least one, at least two, or all three of the following:

(i) a HVR-L1 sequence comprising the sequence of SEQ ID NO: 10;

(ii) a HVR-L2 sequence comprising the sequence of SEQ ID NO: 11;

(iii) a HVR-L3 sequence comprising the sequence of SEQ ID NO: 18.

The amino acid sequences of SEQ ID NOs:1-18 are numbered with respect to individual HVR (i.e., H1, H2 or H3) as indicated in Figures 1a and 1b, the numbering being consistent with the Kabat numbering system as described below.

In one aspect, the invention provides antibodies comprising heavy chain HVR sequences as depicted in Figures 1a and 1b.

In one aspect, the invention provides antibodies comprising light-chain HVR sequences as depicted in Figures 1a and 1b.

Some embodiments of antibodies of the invention comprise a light chain variable domain of humanized 4D5 antibody (huMAb4D5-8) (HERCEPTIN®, Genentech, Inc., South San Francisco, CA, USA) (also referred to in U.S. Pat. No. 6,407,213 and Lee et al., *J. Mol. Biol.* (2004), 340(5):1073-93) as depicted in SEQ ID NO:52 below.

1 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val ***Asn*** Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly Ser ***Arg*** Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln ***His*** Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys 107 (SEQ ID NO: 52) (HVR residues are underlined)

In one embodiment, the huMAb4D5-8 light chain variable domain sequence is modified at one or more of positions 30, 66 and 91 (Asn, Arg and His as indicated in bold/italics above, respectively). In one embodiment, the modified huMAb4D5-8 sequence comprises Ser in position 30, Gly in position 66 and/or Ser in position 91. Accordingly, in one embodiment, an antibody of the invention comprises a

light chain variable domain comprising the sequence depicted in SEQ ID NO:53 below:

1 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val ***Ser*** Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly Ser ***Gly*** Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln ***Ser*** Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys 107 (SEQ ID NO: 53) (HVR residues are underlined)

Substituted residues with respect to huMAb4D5-8 are indicated in bold/italics above.

Antibodies of the invention can comprise any suitable framework variable domain sequence, provided binding activity to DLL4 is substantially retained. For example, in some embodiments, antibodies of the invention comprise a human subgroup III heavy chain framework consensus sequence. In one embodiment of these antibodies, the framework consensus sequence comprises substitution at position 71, 73 and/or 78. In some embodiments of these antibodies, position 71 is A, 73 is T and/or 78 is A. In one embodiment, these antibodies comprise heavy chain variable domain framework sequences of huMAb4D5-8 (HERCEPTIN®, Genentech, Inc., South San Francisco, CA, USA) (also referred to in U.S. Pat. Nos. 6,407,213 & 5,821,337, and Lee et al., *J. Mol. Biol.* (2004), 340(5):1073-93). In one embodiment, these antibodies further comprise a human κI light chain framework consensus sequence. In one embodiment, these antibodies comprise light chain HVR sequences of huMAb4D5-8 as described in U.S. Pat. Nos. 6,407,213 & 5,821,337.) In one embodiment, these antibodies comprise light chain variable domain sequences of huMAb4D5-8 (HERCEPTIN®, Genentech, Inc., South San Francisco, CA, USA) (also referred to in U.S. Pat. Nos. 6,407,213 & 5,821,337, and Lee et al., *J. Mol. Biol.* (2004), 340(5):1073-93).

In one embodiment, an antibody of the invention comprises a heavy chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS: 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, and/or 37, and HVR H1, H2 and H3 sequences are SEQ ID NOS:1, 5, and/or 9, respectively. In one embodiment, an antibody of the invention comprises a light chain variable domain, wherein the framework

sequence comprise the sequence of SEQ ID NOS: 38, 39, 40 and/or 41, and HVR L1, L2 and L3 sequences are SEQ ID NOS: 10, 11 and/or 15, respectively.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS: 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, and/or 37, and HVR H1, H2 and H3 sequences are SEQ ID NOS:2, 3, and/or 9, respectively. In one embodiment, an antibody of the invention comprises a light chain variable domain, wherein the framework sequence comprise the sequence of SEQ ID NOS: 38, 39, 40 and/or 41, and HVR L1, L2 and L3 sequences are SEQ ID NOS: 10, 11 and/or 14, respectively.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS: 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, and/or 37, and HVR H1, H2 and H3 sequences are SEQ ID NOS:1, 8 and/or 9, respectively. In one embodiment, an antibody of the invention comprises a light chain variable domain, wherein the framework sequence comprise the sequence of SEQ ID NOS: 38, 39, 40 and/or 41, and HVR L1, L2 and L3 sequences are SEQ ID NOS: 10, 11 and/or 18, respectively.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS: 46, 47, 48, and/or 49, and HVR H1, H2 and H3 sequences are SEQ ID NOS:1, 5, and/or 9, respectively. In one embodiment, an antibody of the invention comprises a light chain variable domain, wherein the framework sequence comprise the sequence of SEQ ID NOS: 42, 43, 44, and/or 45, and HVR L1, L2 and L3 sequences are SEQ ID NOS: 10, 11 and/or 15, respectively.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS: 46, 47, 48, and/or 49, and HVR H1, H2 and H3 sequences are SEQ ID NOS:2, 3, and/or 9, respectively. In one embodiment, an antibody of the invention comprises a light chain variable domain, wherein the framework sequence comprise the sequence of SEQ ID NOS: 42, 43, 44, and/or 45, and HVR L1, L2 and L3 sequences are SEQ ID NOS: 10, 11 and/or 14, respectively.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain, wherein the framework sequence comprises the sequence of SEQ ID NOS: 46, 47, 48, and/or 49, and HVR H1, H2 and H3 sequences are SEQ ID NOS:1, 8, and/or 9, respectively. In one embodiment, an antibody of the invention comprises a light chain

variable domain, wherein the framework sequence comprise the sequence of SEQ ID NOS: 42, 43, 44, and/or 45, and HVR L1, L2 and L3 sequences are SEQ ID NOS: 10, 11 and/or 18, respectively.

In one embodiment, an antibody of the invention is affinity matured to obtain the target binding affinity desired. In one example, an affinity matured antibody of the invention comprises substitution at one or more of amino acid position H28, H30, H31, H32, H33, L91, L92, L93, L94, L95 and/or L96. In one example an affinity matured antibody of the invention comprises one or more of the following substitutions: (a) in the heavy chain, V50L, V50Y, N52S, P52aS, N53Q, N53T, N53I, S56A, S56F, T57S, D58E, D58I, D58A, D58Y, or (b), in the light chain, S91W, Y92F, T93N, T93S, T94G, P95Q, P95A, P95T, P96S, P96A, P96V.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain comprising the sequence of SEQ ID NO:54. In one embodiment, an antibody of the invention comprises a light chain variable domain comprising the sequence of SEQ ID NO:55. In one embodiment, an antibody of the invention comprises a heavy chain variable domain comprising the sequence of SEQ ID NO:54 and a light chain variable domain comprising the sequence of SEQ ID NO:55.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain comprising the sequence of SEQ ID NO:56. In one embodiment, an antibody of the invention comprises a light chain variable domain comprising the sequence of SEQ ID NO:57. In one embodiment, an antibody of the invention comprises a heavy chain variable domain comprising the sequence of SEQ ID NO:56 and a light chain variable domain comprising the sequence of SEQ ID NO:57.

In one embodiment, an antibody of the invention comprises a heavy chain variable domain comprising the sequence of SEQ ID NO:58. In one embodiment, an antibody of the invention comprises a light chain variable domain comprising the sequence of SEQ ID NO:59. In one embodiment, an antibody of the invention comprises a heavy chain variable domain comprising the sequence of SEQ ID NO:58 and a light chain variable domain comprising the sequence of SEQ ID NO:59.

In one aspect, the invention provides an antibody that competes with any of the above-mentioned antibodies for binding to DLL4. In one aspect, the invention provides an antibody that binds to the same epitope on DLL4 as any of the above-mentioned antibodies.

As is known in the art, and as described in greater detail hereinbelow, the amino acid position/boundary delineating a hypervariable region of an antibody can vary, depending on the context and the various definitions known in the art (as described below). Some positions within a variable domain may be viewed as hybrid hypervariable positions in that these positions can be deemed to be within a hypervariable region under one set of criteria while being deemed to be outside a hypervariable region under a different set of criteria. One or more of these positions can also be found in extended hypervariable regions (as further defined below).

In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the antibody is a polyclonal antibody. In some embodiments, the antibody is selected from the group consisting of a chimeric antibody, an affinity matured antibody, a humanized antibody, and a human antibody. In some embodiments, the antibody is an antibody fragment. In some embodiments, the antibody is a Fab, Fab', Fab'-SH, $F(ab')_2$, or scFv.

In one embodiment, the antibody is a chimeric antibody, for example, an antibody comprising antigen binding sequences from a non-human donor grafted to a heterologous non-human, human or humanized sequence (e.g., framework and/or constant domain sequences). In one embodiment, the non-human donor is a mouse. In one embodiment, an antigen binding sequence is synthetic, e.g. obtained by mutagenesis (e.g., phage display screening, etc.). In one embodiment, a chimeric antibody of the invention has murine V regions and human C region. In one embodiment, the murine light chain V region is fused to a human kappa light chain. In one embodiment, the murine heavy chain V region is fused to a human IgG1 C region.

Humanized antibodies of the invention include those that have amino acid substitutions in the FR and affinity maturation variants with changes in the grafted CDRs. The substituted amino acids in the CDR or FR are not limited to those present in the donor or recipient antibody. In other embodiments, the antibodies of the invention further comprise changes in amino acid residues in the Fc region that lead to improved effector function including enhanced CDC and/or ADCC function and B-cell killing. Other antibodies of the invention include those having specific changes that improve stability. In other embodiments, the antibodies of the invention comprise changes in amino acid residues in the Fc region that lead to decreased effector function, e.g. decreased CDC and/or ADCC function and/or decreased B-cell killing. In some embodiments, the antibodies of the invention are characterized by decreased binding (such as absence of

binding) to human complement factor C1q and/or human Fc receptor on natural killer (NK) cells. In some embodiments, the antibodies of the invention are characterized by decreased binding (such as the absence of binding) to human FcγRI, FcγRIIA, and/or FcγRIIIA. In some embodiments, the antibodies of the invention is of the IgG class (eg, IgG1 or IgG4) and comprises at least one mutation in E233, L234, G236, D265, D270, N297, E318, K320, K322, A327, A330, P331 and/or P329 (numbering according to the EU index). In some embodiments, the antibodies comprise the mutation L234A/L235A or D265A/N297A.

In one aspect, the invention provides anti-DLL4 polypeptides comprising any of the antigen binding sequences provided herein, wherein the anti-DLL4 polypeptides specifically bind to DLL4.

The antibodies of the invention bind (such as specifically bind) DLL4, and in some embodiments, may modulate one or more aspects of DLL4-associated effects, including but not limited to any one or more of reduction or blocking of Notch receptor activation, reduction or blocking of Notch receptor downstream molecular signaling, disruption or blocking of Notch receptor binding to DLL4, and/or promotion of endothelial cell proliferation, and/or inhibition of endothelial cell differentiation, and/or inhibition of arterial differentiation, and/or inhibition of tumor vascular perfusion, and/or treatment and/or prevention of a tumor, cell proliferative disorder or a cancer; and/or treatment or prevention of a disorder associated with DLL4 expression and/or activity and/or treatment or prevention of a disorder associated with Notch receptor expression and/or activity. In some embodiments, the antibody of the invention specifically binds to DLL4. In some embodiments, the antibody specifically binds to the DLL4 extracellular domain (ECD). In some embodiments, the antibody specifically binds to a polypeptide consisting of or consisting essentially of the DLL4 extracellular domain. In some embodiments, the antibody specifically binds DLL4 with a KD of about 1 nM or better, or about 500 pM or better. In some embodiments, the antibody specifically binds human DLL4 with a k_{on} of about 2 X 10^5 or better, or about 1 X 10^5 or better. In some embodiments, the antibody of the invention reduces, inhibits, and/or blocks DLL4 activity in vivo and/or in vitro. In some embodiments, the antibody competes for binding with DLL4-ligand (reduces and/or blocks Notch receptor binding to DLL4).

In one aspect, the invention provides compositions comprising one or more antibodies of the invention and a carrier. In one embodiment, the carrier is pharmaceutically acceptable.

In one aspect, the invention provides nucleic acids encoding an anti-DLL4 antibody of the invention.

In one aspect, the invention provides vectors comprising a nucleic acid of the invention.

In one aspect, the invention provides compositions comprising one or more nucleic acid of the invention and a carrier. In one embodiment, the carrier is pharmaceutically acceptable.

In one aspect, the invention provides host cells comprising a nucleic acid or a vector of the invention. A vector can be of any type, for example a recombinant vector such as an expression vector. Any of a variety of host cells can be used. In one embodiment, a host cell is a prokaryotic cell, for example, E. coli. In one embodiment, a host cell is a eukaryotic cell, for example a mammalian cell such as Chinese Hamster Ovary (CHO) cell.

In one aspect, the invention provides methods of making an antibody of the invention. For example, the invention provides methods of making an anti-DLL4 antibody (which, as defined herein includes full length and fragments thereof) or immunoconjugate, said method comprising expressing in a suitable host cell a recombinant vector of the invention encoding said antibody (or fragment thereof), and recovering said antibody.

In one aspect, the invention provides an article of manufacture comprising a container; and a composition contained within the container, wherein the composition comprises one or more anti-DLL4 antibodies of the invention. In one embodiment, the composition further comprises an anti-angiogenesis agent. In one embodiment the anti-angiogenesis agent is an anti-VEGF antibody, e.g., bevacizumab. In one embodiment, the composition comprises a nucleic acid of the invention. In one embodiment, a composition comprising an antibody further comprises a carrier, which in some embodiments is pharmaceutically acceptable. In one embodiment, a second composition is contained within the container, wherein the second composition comprises an anti-angiogenesis agent. In one embodiment, the anti-angiogenesis agent is an anti-VEGF antibody, e.g. bevacizumab. In one embodiment, an article of manufacture of the invention further comprises instructions for administering the composition(s) (for e.g., the antibody) to a subject (such as instructions for any of the methods described herein).

In one aspect, the invention provides a kit comprising a first container comprising a composition comprising one or more anti-DLL4 antibodies of the invention; and a second container comprising a buffer. In one embodiment, the buffer is pharmaceutically acceptable. In one embodiment, a composition comprising an antibody further comprises a carrier, which in some embodiments is pharmaceutically acceptable. In one embodiment, a kit further comprises instructions for administering the composition (for e.g., the antibody) to a subject.

In one aspect, the invention provides use of an anti-DLL4 antibody of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the disorder is a pathological condition associated with angiogenesis.

In one aspect, the invention provides use of a nucleic acid of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the disorder is a pathological condition associated with angiogenesis.

In one aspect, the invention provides use of an expression vector of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the disorder is a pathological condition associated with angiogenesis.

In one aspect, the invention provides use of a host cell of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the disorder is a pathological condition associated with angiogenesis.

In one aspect, the invention provides use of an article of manufacture of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the disorder is a pathological condition associated with angiogenesis.

In one aspect, the invention provides use of a kit of the invention in the preparation of a medicament for the therapeutic and/or prophylactic treatment of a disorder, such as a cancer, a tumor, and/or a cell proliferative disorder. In some embodiments, the disorder is a pathological condition associated with angiogenesis.

The invention provides methods and compositions useful for modulating disease states associated with expression and/or activity of DLL4, such as increased or decreased expression and/or activity, or undesired expression and/or activity.

In one aspect, the invention provides methods for treating or preventing a tumor, a cancer, and/or a cell proliferative disorder associated with increased expression and/or activity of DLL4, the methods comprising administering an effective amount of an anti-DLL4 antibody to a subject in need of such treatment.

In one aspect, the invention provides methods for reducing, inhibiting, blocking, or preventing growth of a tumor or cancer, the methods comprising administering an effective amount of an anti-DLL4 antibody to a subject in need of such treatment.

In one aspect, the invention provides methods for treating a tumor, a cancer, and/or a cell proliferative disorder comprising administering an effective amount of an anti-DLL4 antibody to a subject in need of such treatment.

In one aspect, the invention provides methods for inhibiting angiogenesis comprising administering an effective amount of an anti-DLL4 antibody to a subject in need of such treatment.

In one aspect, the invention provides methods for treating a pathological condition associated with angiogenesis comprising administering an effective amount of an anti-DLL4 antibody to a subject in need of such treatment. In some embodiments, the pathological condition associated with angiogenesis is a tumor, a cancer, and/or a cell proliferative disorder. In some embodiments, the pathological condition associated with angiogenesis is an intraocular neovascular disease.

Methods of the invention can be used to affect any suitable pathological state. Exemplary disorders are described herein, and include cancers selected from the group consisting of small cell lung cancer, neuroblastomas, melanoma, breast carcinoma, gastric cancer, colorectal cancer (CRC), and hepatocellular carcinoma, including metastatic forms of those cancers.

Methods of the invention can further comprise additional treatment steps. For example, in one embodiment, a method further comprises a step wherein a targeted cell and/or tissue (for e.g., a cancer cell) is exposed to radiation treatment or a chemotherapeutic agent or an anti-angiogenic agent.

In another aspect, the invention provides methods for detection of DLL4, the methods comprising detecting DLL4-anti-DLL4 antibody complex in the sample. The term "detection" as used herein includes qualitative and/or quantitative detection (measuring levels) with or without reference to a control.

In another aspect, the invention provides methods for diagnosing a disorder associated with DLL4 expression and/or activity, the methods comprising detecting DLL4-

anti-DLL4 antibody complex in a biological sample from a patient having or suspected of having the disorder. In some embodiments, the DLL4 expression is increased expression or abnormal expression. In some embodiments, the disorder is a tumor, cancer, and/or a cell proliferative disorder.

In another aspect, the invention provides any of the anti-DLL4 antibodies described herein, wherein the anti-DLL4 antibody comprises a detectable label.

In another aspect, the invention provides a complex of any of the anti-DLL4 antibodies described herein and DLL4. In some embodiments, the complex is in vivo or in vitro. In some embodiments, the complex comprises a cancer cell. In some embodiments, the anti-DLL4 antibody is detectably labeled.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1a, b: Heavy chain and light chain HVR loop sequences of anti-DLL4 antibodies. The figures show the heavy chain HVR sequences, H1, H2, and H3, and light chain HVR sequences, L1, L2 and L3. Sequence numbering is as follows: clone 152.26 (HVR-H1 is SEQ ID NO:1; HVR-H2 is SEQ ID NO:3; HVR-H3 is SEQ ID NO:9; HVR-L1 is SEQ ID NO:10; HVR-L2 is SEQ ID NO:11; HVR-L3 is SEQ ID NO:12); clone 152.26.6 (HVR-H1 is SEQ ID NO:1; HVR-H2 is SEQ ID NO:4; HVR-H3 is SEQ ID NO:9; HVR-L1 is SEQ ID NO:10; HVR-L2 is SEQ ID NO:11; HVR-L3 is SEQ ID NO:13); clone 152.26.14 (HVR-H1 is SEQ ID NO:2; HVR-H2 is SEQ ID NO:3; HVR-H3 is SEQ ID NO:9; HVR-L1 is SEQ ID NO:10; HVR-L2 is SEQ ID NO:11; HVR-L3 is SEQ ID NO:14); clone 152.26.20 (HVR-H1 is SEQ ID NO:1; HVR-H2 is SEQ ID NO:5; HVR-H3 is SEQ ID NO:9; HVR-L1 is SEQ ID NO:10; HVR-L2 is SEQ ID NO:11; HVR-L3 is SEQ ID NO:15); clone 152.26.34 (HVR-H1 is SEQ ID NO:1; HVR-H2 is SEQ ID NO:6; HVR-H3 is SEQ ID NO:9; HVR-L1 is SEQ ID NO:10; HVR-L2 is SEQ ID NO:11; HVR-L3 is SEQ ID NO:16); clone 152.26.40 (HVR-H1 is SEQ ID NO:1; HVR-H2 is SEQ ID NO:7; HVR-H3 is SEQ ID NO:9; HVR-L1 is SEQ ID NO:10; HVR-L2 is SEQ ID NO:11; HVR-L3 is SEQ ID NO:17); and clone 152.26.82 (HVR-H1 is SEQ ID NO:1; HVR-H2 is SEQ ID NO:8; HVR-H3 is SEQ ID NO:9; HVR-L1 is SEQ ID NO:10; HVR-L2 is SEQ ID NO:11; HVR-L3 is SEQ ID NO:18).

Amino acid positions are numbered according to the Kabat numbering system as described below.

FIGURES 2 & 3: depict exemplary acceptor human consensus framework sequences for use in practicing the instant invention with sequence identifiers as follows:

Variable heavy (VH) consensus frameworks (FIG. 2a, b)

human VH subgroup I consensus framework minus Kabat CDRs (SEQ ID NO:19)

human VH subgroup I consensus framework minus extended hypervariable regions (SEQ ID NOs:20-22)

human VH subgroup II consensus framework minus Kabat CDRs (SEQ ID NO:23)

human VH subgroup II consensus framework minus extended hypervariable regions (SEQ ID NOs:24-26)

human VH subgroup II consensus framework minus extended

human VH subgroup III consensus framework minus Kabat CDRs (SEQ ID NO:27)

human VH subgroup III consensus framework minus extended hypervariable regions (SEQ ID NOs:28-30)

human VH acceptor framework minus Kabat CDRs (SEQ ID NO:31)

human VH acceptor framework minus extended hypervariable regions (SEQ ID NOs:32-33)

human VH acceptor 2 framework minus Kabat CDRs (SEQ ID NO:34)

human VH acceptor 2 framework minus extended hypervariable regions (SEQ ID NOs:35-37)

Variable light (VL) consensus frameworks (FIG. 3)

human VL kappa subgroup I consensus framework (SEQ ID NO:38)

human VL kappa subgroup II consensus framework (SEQ ID NO:39)

human VL kappa subgroup III consensus framework (SEQ ID NO:40)

human VL kappa subgroup IV consensus framework (SEQ ID NO:41)

FIGURE 4: depicts framework region sequences of huMAb4D5-8 light and heavy chains. Numbers in superscript/bold indicate amino acid positions according to Kabat.

FIGURE 5: depicts modified/variant framework region sequences of huMAb4D5-8 light and heavy chains. Numbers in superscript/bold indicate amino acid positions according to Kabat.

FIGURE 6: depicts the heavy chain variable regions and light chain variable regions of antibody clones 26.20, 26.14, and 26.82.

FIGURE 7: DLL4-mediated Notch signaling regulated EC proliferation. a-c, f, HUVEC sprouting assays in 3-D fibrin gels. Anti-DLL4 antibody (YW26.82) or DBZ promoted the sprouting of HUVECs (a). Ki67 staining showed that anti-DLL4 antibody or DBZ caused hyperproliferation of HUVECs (b). Anti-DLL4 antibody or DBZ increased the sprouting of HUVECs in the presence of SF conditioned medium (c). d, h, Systemic

delivery of anti-DLL4 antibody caused massive accumulation of ECs in neonatal retinas. Confocal images of low (top) and high magnification (bottom) of retinal vasculature (isolectin staining) (d). Ki67 staining shows increased EC proliferation in the neonatal retinas treated with anti-DLL4 antibody (h). e, Notch activation by immobilized DLL4 inhibited HUVEC proliferation. f, Anti-VEGF antibody inhibited HUVEC sprouting in the presence or absence of DBZ. g, Regulation of VEGFR2 by Notch. Quantitative PCR analysis of VEGFR2 expression in response to, Notch blockade in 3-D fibrin gel culture of HUVECs (7 d) by anti-DLL4 antibody or DBZ (left), or Notch activation in 2-D culture of HUVECs (36 hr) by immobilized DLL4 (right). Anti-DLL4 antibody and DBZ were used at 5 μg/ml and 0.08 μM, respectively (a-c, e-g).

FIGURE 8: DLL4-mediated Notch signaling regulated EC differentiation. a, The lumen-like structures (white arrows) formed by HUVECs growing in fibrin gels were lost in the presence of anti-DLL4 antibody or DBZ. Instead, the spouts were highly packed with cells (black arrows). b, Regulation of TGFβ2 by Notch. Quantitative PCR analysis of TGFβ2 expression in response to, Notch blockade in 3-D fibrin gel culture of HUVECs for (7 d) by anti-DLL4 antibody or DBZ (left), or Notch activation in 2-D culture of HUVECs (36 hr) by immobilized DLL4 (right). c, Anti-DLL4 antibody blocks arterial development. Confocal images of neonatal mouse retinas stained with alpha smooth muscle actin (ASMA) and isolectin. Neonatal mice were treated as described in Fig. 1d. d, Confocal images of adult mouse retinas stained with ASMA and isolectin. 8 week-old mice were treated with PBS or anti-DLL4 antibody (10 mg/kg, twice weekly) for two weeks.

FIGURE 9: Selective blocking of DLL4 and/or VEGF disrupted tumor angiogenesis and inhibits tumor growth. a-f, Results of tumor models: HM7 (a), Colo205 (b), Calu6 (c), MDA-MB-435 (d), MV-522 (e) and WEHI3 (f). Mean tumor volumes with SEs are presented. g-h, Tumor vascular histology studies. Immunohistochemisty of anti-CD31 in EL4 tumor sections from control, anti-DLL4 antibody and anti-VEGF treated mice (g). Lectin perfusion and anti-CD31 staining in EL4 tumor sections (h). i-p. Results of tumor models SK-OV-3X1 (i), LL2 (j), EL4 (k), H1299 (l), SKMES-1(m), MX-1(n), SW620 (o) and LS174T(p).

FIGURE 10: DLL4/Notch was dispensable in the homeostasis of mouse intestine. Immunohistochemical studies of small intestines from control (a, d, g, j), anti-DLL4 antibody (10 mg/kg, twice weekly for 6 weeks) (b, e, h, k), and DBZ treated (30 μmol/kg daily for 5 days) (c, f, i, l) mice. As shown by H&E (a, b, c) and Alcian Blue staining (d, e, f), DBZ caused replacement of the TA population by goblet cells. This change was entirely

absent from anti-DLL4 antibody treatment. Ki67 (g, h, i) and HES-1 (j, k, l) staining further confirmed that anti-DLL4 antibody failed to replicate the effect of DBZ.

FIGURE 11: Characterization of anti-DLL4 antibody. a, Epitope mapping of anti-DLL4 antibody monoclonal antibody (Mab) YW26.82. Schematic representation of a set of DLL4 mutants expressed as C-terminal human placental alkaline phosphatase (AP) fusion proteins. 293T cell conditioned media containing the fusion proteins were tested on 96-well microtiter plates coated with purified anti-DLL4 antibody (YW26.82, 0.5 μg/ml). The bound DLL4.AP was detected using 1-Step PNPP (Pierce) as substrate and OD 405 nm absorbance measurement. b-d, Selective binding of YW26.82 to DLL4. 96-well Nunc MaxiSorp™ plates were coated with purified recombinant proteins as indicated (1 μg/ml). The binding of YW26.82 at indicated concentrations was measured by ELISA assay. Bound antibodies were detected with anti-human antibody HRP conjugate using TMB as substrate and OD 450 nm absorbance measurement. Anti-HER2 and recombinant ErbB2-ECD were used as assay control (b). FACS analysis of 293 cells transiently transfected with vector, full length DLL4, Jag1 or DLL1. Significant binding of YW26.82 was only detected on DLL4 transfected cells (top panel). Expression of Jag1 and DLL1 was confirmed by the binding of recombinant rat Notch1-Fc (rrNotch1-Fc, middle panel) and recombinant rat Notch2-Fc (rrNotch2-Fc, bottom panel), respectively. YW26.82, rrNotch1-Fc or rrNotch2-Fc (R& D system) were used at 2 μg/ml followed by goat anti-human IgG-PE (1:500, Jackson ImmunoResearch) (c). Anti-DLL4 antibody blocked the binding of DLL4-AP, but not DLL1-AP, to coated rNotch1, with a calculated IC50 of ~12 nM (left panel). Anti-DLL4 antibody blocked the binding of DLL4-His, but not Jag1-His, to coated rNotch1, with a calculated IC50 of ~8 nM (right panel) (d). e, Specific binding of YW26.82 to endogenously expressed DLL4. FACS analysis of HUVECs transfected with control or DLL4-specific siRNA. YW26.82 was used at 2 μg/ml, followed by goat anti-human IgG-PE (1:500, Jackson ImmunoResearch) (e).

FIGURE 12: Upregulation of DLL4 by Notch activation. HUVECs were stimulated by immobilized C-terminal His-tagged human DLL4 (amino acids 1-404) in the absence or presence of DBZ (0.08 μM). 36 hr after stimulation, endogenous DLL4 expression was examined by FACS analysis with anti-DLL4 antibody.

DETAILED DESCRIPTION OF THE INVENTION

The invention herein provides anti-DLL4 antibodies, that are useful for, e.g., treatment or prevention of disease states associated with expression and/or activity of DLL4, such as increased expression and/or activity or undesired expression and/or activity. In some embodiments, the antibodies of the invention are used to treat a tumor, a cancer, and/or a cell proliferative disorder. In some embodiments, the antibodies of the invention are used to treat a pathological condition associated with angiogenesis.

In another aspect, the anti-DLL4 antibodies of the invention find utility as reagents for detection and/or isolation of DLL4, such as detection of DLL4 in various tissues and cell type.

The invention further provides methods of making anti-DLL4 antibodies, and polynucleotides encoding anti-DLL4 antibodies.

General techniques

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in Sambrook et al., Molecular Cloning: A Laboratory Manual 3rd. edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (2003)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R. I. Freshney, ed. (1987)).

Definitions

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie™ blue or, preferably, silver stain. Isolated antibody includes

the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Similarly, isolated antibody includes the antibody in medium around recombinant cells. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

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

The term “variable domain residue numbering as in Kabat" or “amino acid position numbering as in Kabat”, and variations thereof, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991). Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a “standard” Kabat numbered sequence.

The phrase “substantially similar,” or “substantially the same”, as used herein, denotes a sufficiently high degree of similarity between two numeric values (generally one associated with an antibody of the invention and the other associated with a reference/comparator antibody) such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is preferably less than about 50%, preferably less than about 40%, preferably less than about 30%, preferably less than

about 20%, preferably less than about 10% as a function of the value for the reference/comparator antibody.

"Binding affinity" generally refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. Specific illustrative embodiments are described in the following.

In one embodiment, the "Kd" or "Kd value" according to this invention is measured by a radiolabeled antigen binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as described by the following assay that measures solution binding affinity of Fabs for antigen by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (Chen, et al., (1999) *J. Mol Biol* 293:865-881). To establish conditions for the assay, microtiter plates (Dynex) are coated overnight with 5 ug/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbant plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (e.g., consistent with assessment of an anti-VEGF antibody, Fab-12, in Presta et al., (1997) *Cancer Res.* 57:4593-4599). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (e.g., 65 hours) to insure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (e.g., for one hour). The solution is then removed and the plate washed eight times with 0.1% Tween®20 in PBS. When the plates have dried, 150 μl/well of scintillant (MicroScint™-20; Packard) is added, and the plates are counted on a TopCount gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are

chosen for use in competitive binding assays. According to another embodiment the Kd or Kd value is measured by using surface plasmon resonance assays using a BIAcore™-2000 or a BIAcore™-3000 (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIAcore Inc.) are activated with N-ethyl-N'- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10mM sodium acetate, pH 4.8, into 5μg/ml (~0.2μM) before injection at a flow rate of 5μl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% Tween®20 (PBST) at 25°C at a flow rate of approximately 25μl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIAcore Evaluation Software version 3.2) by simultaneous fitting the association and dissociation sensorgram. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on}. See, e.g., Chen, Y., et al., (1999) *J. Mol Biol* 293:865-881. If the on-rate exceeds 10^6 M^{-1} S^{-1} by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-Aminco spectrophotometer (ThermoSpectronic) with a stir red cuvette.

An "on-rate" or "rate of association" or "association rate" or "k_{on}" according to this invention can also be determined with the same surface plasmon resonance technique described above using a BIAcore™-2000 or a BIAcore™-3000 (BIAcore, Inc., Piscataway, NJ) at 25C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIAcore Inc.) are activated with N-ethyl-N'- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10mM sodium acetate, pH 4.8, into 5μg/ml (~0.2μM) before injection at a flow rate of 5ul/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are

injected in PBS with 0.05% Tween®20 (PBST) at 25°C at a flow rate of approximately 25µl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIAcore Evaluation Software version 3.2) by simultaneous fitting the association and dissociation sensorgram. The equilibrium dissociation constant (Kd) was calculated as the ratio k_{off}/k_{on}. See, e.g., Chen, Y., et al., (1999) *J. Mol Biol* 293:865-881. However, if the on-rate exceeds 10^6 M^{-1} S^{-1} by the surface plasmon resonance assay above, then the on-rate is preferably determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25°C of a 20nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a a spectrometer, such as a stop-flow equipped spectrophometer (Aviv Instruments) or a 8000-series SLM-Aminco spectrophotometer (ThermoSpectronic) with a stirred cuvette.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "recombinant vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may, at times, be used interchangeably as the plasmid is the most commonly used form of vector.

"Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase, or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide

structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after synthesis, such as by conjugation with a label. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and a basic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), "(O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

"Oligonucleotide," as used herein, generally refers to short, generally single stranded, generally synthetic polynucleotides that are generally, but not necessarily, less than about 200 nucleotides in length. The terms "oligonucleotide" and "polynucleotide"

are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

"Percent (%) amino acid sequence identity" with respect to a peptide or polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the specific peptide or polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or MegAlign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc. and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, California. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino

acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

The term “DLL4” (interchangeably termed “delta-like 4”), as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant (whether native or synthetic) DLL4 polypeptide. The term "native sequence" specifically encompasses naturally occurring truncated or secreted forms (e.g., an extracellular domain sequence), naturally occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants. The term “wild type DLL4” generally refers to a polypeptide comprising the amino acid sequence of a naturally occurring DLL4 protein. The term “wild type DLL4 sequence” generally refers to an amino acid sequence found in a naturally occurring DLL4.

The term “Notch receptor” (interchangeably termed “Notch”), as used herein, refers, unless specifically or contextually indicated otherwise, to any native or variant (whether native or synthetic) Notch receptor polypeptide. Humans have four Notch receptors (Notch1, Notch 2, Notch3, and Notch4). As used herein, the term Notch receptor includes any one of or all four human Notch receptors. The term "native sequence" specifically encompasses naturally occurring truncated or secreted forms (e.g., an extracellular domain sequence), naturally occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants. The term “wild type Notch receptor” generally refers to a polypeptide comprising the amino acid sequence of a naturally occurring Notch receptor protein. The term “wild type Notch receptor sequence” generally refers to an amino acid sequence found in a naturally occurring Notch receptor.

The terms "antibody" and “immunoglobulin” are used interchangeably in the broadest sense and include monoclonal antibodies (e.g., full length or intact monoclonal antibodies), polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibodies so long as they exhibit the desired biological activity) and may also include certain antibody fragments (as described in greater detail herein). An antibody can be human, humanized and/or affinity matured.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly

distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

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

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a

free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

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

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these can be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Antibody fragments" comprise only a portion of an intact antibody, wherein the portion preferably retains at least one, preferably most or all, of the functions normally associated with that portion when present in an intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an *in vivo* half life substantially similar to an intact antibody. For e.g., such an antibody fragment may comprise on antigen binding arm linked to an Fc sequence capable of conferring *in vivo* stability to the fragment.

The term "hypervariable region", "HVR", or "HV", when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six hypervariable regions; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). A number of hypervariable region delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" hypervariable regions are based on an analysis of the available complex crystal structures. The residues from each of these hypervariable regions are noted below.

Loop	Kabat	AbM	Chothia	Contact
L1	L24-L34	L24-L34	L26-L32	L30-L36
L2	L50-L56	L50-L56	L50-L52	L46-L55
L3	L89-L97	L89-L97	L91-L96	L89-L96
H1	H31-H35B	H26-H35B	H26-H32	H30-H35B
	(Kabat Numbering)			
H1	H31-H35	H26-H35	H26-H32	H30-H35
	(Chothia Numbering)			
H2	H50-H65	H50-H58	H53-H55	H47-H58
H3	H95-H102	H95-H102	H96-H101	H93-H101

Hypervariable regions may comprise "extended hypervariable regions" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102 or 95-102 (H3) in the VH. The variable domain residues are numbered according to Kabat *et al.*, *supra* for each of these definitions.

"Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

The term "monoclonal antibody" as used herein refers to an antibody from a population of substantially homogeneous antibodies, i.e., the individual antibodies

comprising the population are identical and/or bind the same epitope(s), except for possible variants that may arise during production of the monoclonal antibody, such variants generally being present in minor amounts. Such monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones or recombinant DNA clones. It should be understood that the selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity *in vivo*, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, the monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler et al., *Nature*, 256:495 (1975); 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)), recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567), phage display technologies (see, e.g., Clackson et al., *Nature*, 352:624-628 (1991); Marks et al., *J. Mol. Biol.*, 222:581-597 (1991); Sidhu et al., *J. Mol. Biol.* 338(2):299-310 (2004); Lee et al., *J.Mol.Biol.*340(5):1073-1093 (2004); Fellouse, *Proc. Nat. Acad. Sci. USA* 101(34):12467-12472 (2004); and Lee et al. *J. Immunol. Methods* 284(1-2):119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551

(1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggemann et al., *Year in Immuno.*, 7:33 (1993); U.S. Patent Nos. 5,545,806; 5,569,825; 5,591,669 (all of GenPharm); 5,545,807; WO 1997/17852; U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks et al., *Bio/Technology*, 10: 779-783 (1992); Lonberg et al., *Nature*, 368: 856-859 (1994); Morrison, *Nature*, 368: 812-813 (1994); Fishwild et al., *Nature Biotechnology*, 14: 845-851 (1996); Neuberger, *Nature Biotechnology*, 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.*, 13: 65-93 (1995).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994).

"Chimeric" antibodies (immunoglobulins) have a portion of the heavy and/or light chain identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological

activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)). Humanized antibody as used herein is a subset of chimeric antibodies.

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

An "antigen" is a predetermined antigen to which an antibody can selectively bind. The target antigen may be polypeptide, carbohydrate, nucleic acid, lipid, hapten or other naturally occurring or synthetic compound. Preferably, the target antigen is a polypeptide.

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

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

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

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.* B cell receptor); and B cell activation.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (*e.g.* Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are absolutely required for such killing. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 or Presta U.S. Patent No. 6,737,056 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g., in a animal model such as that disclosed in Clynes *et al. Proc. Natl. Acad. Sci. USA* 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source, *e.g.* from blood.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ

primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al., Immunomethods* 4:25-34 (1994); and de Haas *et al., J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al., J. Immunol.* 117:587 (1976) and Kim *et al., J. Immunol.* 24:249 (1994)) and regulates homeostasis of immunoglobulins. WO00/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. The content of that patent publication is specifically incorporated herein by reference. See, also, Shields *et al. J. Biol. Chem.* 9(2): 6591-6604 (2001).

Methods of measuring binding to FcRn are known (see, e.g., Ghetie 1997, Hinton 2004). Binding to human FcRn *in vivo* and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates administered with the Fc variant polypeptides.

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, *e.g.* as described in Gazzano-Santoro *et al., J. Immunol. Methods* 202:163 (1996), may be performed.

Polypeptide variants with altered Fc region amino acid sequences and increased or decreased C1q binding capability are described in US patent No. 6,194,551B1 and WO99/51642. The contents of those patent publications are specifically incorporated herein by reference. See, also, Idusogie *et al. J. Immunol.* 164: 4178-4184 (2000).

The term "Fc region-comprising polypeptide" refers to a polypeptide, such as an antibody or immunoadhesin (see definitions below), which comprises an Fc region. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during purification of the polypeptide or by recombinant

engineering the nucleic acid encoding the polypeptide. Accordingly, a composition comprising a polypeptide having an Fc region according to this invention can comprise polypeptides with K447, with all K447 removed, or a mixture of polypeptides with and without the K447 residue.

A "blocking" antibody or an "antagonist" antibody is one which inhibits or reduces biological activity of the antigen it binds. Preferred blocking antibodies or antagonist antibodies substantially or completely inhibit the biological activity of the antigen.

An "agonist antibody", as used herein, is an antibody which mimics at least one of the functional activities of a polypeptide of interest.

An "acceptor human framework" for the purposes herein is a framework comprising the amino acid sequence of a VL or VH framework derived from a human immunoglobulin framework, or from a human consensus framework. An acceptor human framework "derived from" a human immunoglobulin framework or human consensus framework may comprise the same amino acid sequence thereof, or may contain pre-existing amino acid sequence changes. Where pre-existing amino acid changes are present, preferably no more than 5 and preferably 4 or less, or 3 or less, pre-existing amino acid changes are present. Where pre-existing amino acid changes are present in a VH, preferably those changes are only at three, two or one of positions 71H, 73H and 78H; for instance, the amino acid residues at those positions may be 71A, 73T and/or 78A. In one embodiment, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residue in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat *et al.* In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat *et al.* In one embodiment, for the VH, the subgroup is subgroup III as in Kabat *et al.*

A "VH subgroup III consensus framework" comprises the consensus sequence obtained from the amino acid sequences in variable heavy subgroup III of Kabat *et al.* In one embodiment, the VH subgroup III consensus framework amino acid sequence comprises at least a portion or all of each of the following sequences: EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO:42)-H1-WVRQAPGKGLEWV (SEQ ID NO:43)-H2-RFTISRDNSKNTLYLQMNSLRAEDTAVYYC (SEQ ID NO:44)-H3-WGQGTLVTVSS (SEQ ID NO:45).

A "VL subgroup I consensus framework" comprises the consensus sequence obtained from the amino acid sequences in variable light kappa subgroup I of Kabat *et al.* In one embodiment, the VH subgroup I consensus framework amino acid sequence comprises at least a portion or all of each of the following sequences: DIQMTQSPSSLSASVGDRVTITC (SEQ ID NO:46)-L1-WYQQKPGKAPKLLIY (SEQ ID NO:47)-L2-GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC (SEQ ID NO:48)-L3-FGQGTKVEIK (SEQ ID NO:49).

A "disorder" or "disease" is any condition that would benefit from treatment with a substance/molecule or method of the invention. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include malignant and benign tumors; carcinoma, blastoma, and sarcoma.

The terms "cell proliferative disorder" and "proliferative disorder" refer to disorders that are associated with some degree of abnormal cell proliferation. In one embodiment, the cell proliferative disorder is cancer.

"Tumor", as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms "cancer", "cancerous", "cell proliferative disorder", "proliferative disorder" and "tumor" are not mutually exclusive as referred to herein.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer,

endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, gastric cancer, melanoma, and various types of head and neck cancer. Dysregulation of angiogenesis can lead to many disorders that can be treated by compositions and methods of the invention. These disorders include both non-neoplastic and neoplastic conditions. Neoplastics include but are not limited those described above. Non-neoplastic disorders include but are not limited to undesired or aberrant hypertrophy, arthritis, rheumatoid arthritis (RA), psoriasis, psoriatic plaques, sarcoidosis, atherosclerosis, atherosclerotic plaques, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, diabetic macular edema, corneal neovascularization, corneal graft neovascularization, corneal graft rejection, retinal/choroidal neovascularization, neovascularization of the angle (rubeosis), ocular neovascular disease, vascular restenosis, arteriovenous malformations (AVM), meningioma, hemangioma, angiofibroma, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, acute lung injury/ARDS, sepsis, primary pulmonary hypertension, malignant pulmonary effusions, cerebral edema (e.g., associated with acute stroke/ closed head injury/ trauma), synovial inflammation, pannus formation in RA, myositis ossificans, hypertropic bone formation, osteoarthritis (OA), refractory ascites, polycystic ovarian disease, endometriosis, 3rd spacing of fluid diseases (pancreatitis, compartment syndrome, burns, bowel disease), uterine fibroids, premature labor, chronic inflammation such as IBD (Crohn's disease and ulcerative colitis), renal allograft rejection, inflammatory bowel disease, nephrotic syndrome, undesired or aberrant tissue mass growth (non-cancer), hemophilic joints, hypertrophic scars, inhibition of hair growth, Osler-Weber syndrome, pyogenic granuloma retrolental fibroplasias, scleroderma, trachoma, vascular adhesions, synovitis, dermatitis, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion.

As used herein, "treatment" refers to clinical intervention in an attempt to alter the natural course of the individual or cell being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the

disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or disorder.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals (such as cows), sport animals, pets (such as cats, dogs and horses), primates, mice and rats.

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

An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

A "therapeutically effective amount" of a substance/molecule of the invention, agonist or antagonist may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance/molecule, agonist or antagonist to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the substance/molecule, agonist or antagonist are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu), chemotherapeutic agents e.g. methotrexate, adriamicin, vinca alkaloids (vincristine, vinblastine, etoposide), doxorubicin, melphalan, mitomycin C, chlorambucil, daunorubicin or other intercalating agents, enzymes and fragments thereof such as nucleolytic enzymes, antibiotics, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof, and the various antitumor or anticancer agents disclosed below. Other cytotoxic agents are described below. A tumoricidal agent causes destruction of tumor cells.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and CYTOXAN® cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and

piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scopolectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e. g., calicheamicin, especially calicheamicin gamma1I and calicheamicin omegaI1 (see, e.g., Agnew, Chem Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti- adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside;

aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoids, e.g., TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE™ Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, Illinois), and TAXOTERE® doxetaxel (Rhône-Poulenc Rorer, Antony, France); chloranbucil; gemcitabine (GEMZAR®); 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; ibandronate; topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids such as retinoic acid; capecitabine (XELODA®); pharmaceutically acceptable salts, acids or derivatives of any of the above; as well as combinations of two or more of the above such as CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU and leucovovin.

Also included in this definition are anti-hormonal agents that act to regulate, reduce, block, or inhibit the effects of hormones that can promote the growth of cancer, and are often in the form of systemic, or whole-body treatment. They may be hormones themselves. Examples include anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), EVISTA® raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and FARESTON® toremifene; anti-progesterones; estrogen receptor down-regulators (ERDs); agents that function to suppress or shut down the ovaries, for example, leutinizing hormone-releasing hormone (LHRH) agonists such as LUPRON® and ELIGARD® leuprolide acetate, goserelin acetate, buserelin acetate and tripterelin; other

anti-androgens such as flutamide, nilutamide and bicalutamide; and aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® megestrol acetate, AROMASIN® exemestane, formestanie, fadrozole, RIVISOR® vorozole, FEMARA® letrozole, and ARIMIDEX® anastrozole. In addition, such definition of chemotherapeutic agents includes bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), DIDROCAL® etidronate, NE-58095, ZOMETA® zoledronic acid/zoledronate, FOSAMAX® alendronate, AREDIA® pamidronate, SKELID® tiludronate, or ACTONEL® risedronate; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); antisense oligonucleotides, particularly those that inhibit expression of genes in signaling pathways implicated in abherant cell proliferation, such as, for example, PKC-alpha, Raf, H-Ras, and epidermal growth factor receptor (EGF-R); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; lapatinib ditosylate (an ErbB-2 and EGFR dual tyrosine kinase small-molecule inhibitor also known as GW572016); and pharmaceutically acceptable salts, acids or derivatives of any of the above.

A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell (such as a cell expressing DLL4) either *in vitro* or *in vivo*. Thus, the growth inhibitory agent may be one which significantly reduces the percentage of cells (such as a cell expressing DLL4) in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine), taxanes, and topoisomerase II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogenes, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995), especially p. 13. The taxanes (paclitaxel and docetaxel) are anticancer drugs both derived from the yew tree. Docetaxel (TAXOTERE®, Rhone-Poulenc Rorer), derived from the European yew, is a semisynthetic analogue of paclitaxel (TAXOL®, Bristol-Myers

Squibb). Paclitaxel and docetaxel promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization, which results in the inhibition of mitosis in cells.

"Doxorubicin" is an anthracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexapyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-5,12-naphthacenedione.

The term "anti-neoplastic composition" refers to a composition useful in treating cancer comprising at least one active therapeutic agent, e.g., "anti-cancer agent". Examples of therapeutic agents (anti-cancer agents, also termed "anti-neoplastic agent" herein) include, but are limited to, e.g., chemotherapeutic agents, growth inhibitory agents, cytotoxic agents, agents used in radiation therapy, anti-angiogenesis agents, apoptotic agents, anti-tubulin agents, toxins, and other-agents to treat cancer, e.g., anti-VEGF neutralizing antibody, VEGF antagonist, anti-HER-2, anti-CD20, an epidermal growth factor receptor (EGFR) antagonist (e.g., a tyrosine kinase inhibitor), HER1/EGFR inhibitor, erlotinib, a COX-2 inhibitor (e.g., celecoxib), interferons, cytokines, antagonists (e.g., neutralizing antibodies) that bind to one or more of the ErbB2, ErbB3, ErbB4, or VEGF receptor(s), inhibitors for receptor tyrosine kinases for platet-derived growth factor (PDGF) and/or stem cell factor (SCF) (e.g., imatinib mesylate (Gleevec ® Novartis)), TRAIL/Apo2L, and other bioactive and organic chemical agents, etc.

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

drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

An "anti-angiogenesis agent" or "angiogenesis inhibitor" refers to a small molecular weight substance, a polynucleotide (including, e.g., an inhibitory RNA (RNAi or siRNA)), a polypeptide, an isolated protein, a recombinant protein, an antibody, or conjugates or fusion proteins thereof, that inhibits angiogenesis, vasculogenesis, or undesirable vascular permeability, either directly or indirectly. For example, an anti-angiogenesis agent is an antibody or other antagonist to an angiogenic agent as defined above, e.g., antibodies to VEGF, antibodies to VEGF receptors, soluble VEGF receptor fragments or small molecules that block VEGF receptor signaling (e.g., PTK787/ZK2284, SU6668, SUTENT®/SU11248 (sunitinib malate), AMG706, or those described in, e.g., international patent application WO 2004/113304). Anti-angiogensis agents also include native angiogenesis inhibitors, e.g., angiostatin, endostatin, etc. See, e.g., Klagsbrun and D'Amore, *Annu. Rev. Physiol.*, 53:217-39 (1991); Streit and Detmar, *Oncogene*, 22:3172-3179 (2003) (e.g., Table 3 listing anti-angiogenic therapy in malignant melanoma); Ferrara & Alitalo, *Nature Medicine* 5(12):1359-1364 (1999); Tonini et al., *Oncogene*, 22:6549-6556 (2003) (e.g., Table 2 listing antiangiogenic factors); and, Sato *Int. J. Clin. Oncol.*, 8:200-206 (2003) (e.g., Table 1 lists Anti-angiogenic agents used in clinical trials).

Compositions of the invention and methods of making same

This invention encompasses compositions, including pharmaceutical compositions, comprising an anti-DLL4 antibody; and polynucleotides comprising sequences encoding an anti-DLL4 antibody. As used herein, compositions comprise one or more antibodies that bind to DLL4, and/or one or more polynucleotides comprising sequences encoding one or more antibodies that bind to DLL4. These compositions may further comprise suitable carriers, such as pharmaceutically acceptable excipients including buffers, which are well known in the art.

The invention also encompasses isolated antibody and polynucleotide embodiments. The invention also encompasses substantially pure antibody and polynucleotide embodiments.

The anti-DLL4 antibodies of the invention are preferably monoclonal. Also encompassed within the scope of the invention are Fab, Fab', Fab'-SH and $F(ab')_2$ fragments of the anti-DLL4 antibodies provided herein. These antibody fragments can be created by traditional means, such as enzymatic digestion, or may be generated by

recombinant techniques. Such antibody fragments may be chimeric or humanized. These fragments are useful for the diagnostic and therapeutic purposes set forth below.

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

The anti-DLL4 monoclonal antibodies of the invention can be made using the hybridoma method first described by Kohler *et al., Nature,* **256**:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Antibodies to DLL4 generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of DLL4 and an adjuvant. DLL4 may be prepared using methods well-known in the art, some of which are further described herein. For example, recombinant production of DLL4 is described below. In one embodiment, animals are immunized with a derivative of DLL4 that contains the extracellular domain (ECD) of DLL4 fused to the Fc portion of an immunoglobulin heavy chain. In a preferred embodiment, animals are immunized with an DLL4-IgG1 fusion protein. Animals ordinarily are immunized against immunogenic conjugates or derivatives of DLL4 with monophosphoryl lipid A (MPL)/trehalose dicrynomycolate (TDM) (Ribi Immunochem. Research, Inc., Hamilton, MT) and the solution is injected intradermally at multiple sites. Two weeks later the animals are boosted. 7 to 14 days later animals are bled and the serum is assayed for anti-DLL4 titer. Animals are boosted until titer plateaus.

Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the

culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, **133**:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against DLL4. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoadsorbent assay (ELISA).

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

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

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

The anti-DLL4 antibodies of the invention can be made by using combinatorial libraries to screen for synthetic antibody clones with the desired activity or activities. In principle, synthetic antibody clones are selected by screening phage libraries containing phage that display various fragments of antibody variable region (Fv) fused to phage coat protein. Such phage libraries are panned by affinity chromatography against the desired

antigen. Clones expressing Fv fragments capable of binding to the desired antigen are adsorbed to the antigen and thus separated from the non-binding clones in the library. The binding clones are then eluted from the antigen, and can be further enriched by additional cycles of antigen adsorption/elution. Any of the anti-DLL4 antibodies of the invention can be obtained by designing a suitable antigen screening procedure to select for the phage clone of interest followed by construction of a full length anti-DLL4 antibody clone using the Fv sequences from the phage clone of interest and suitable constant region (Fc) sequences described in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3.

The antigen-binding domain of an antibody is formed from two variable (V) regions of about 110 amino acids, one each from the light (VL) and heavy (VH) chains, that both present three hypervariable loops or complementarity-determining regions (CDRs). Variable domains can be displayed functionally on phage, either as single-chain Fv (scFv) fragments, in which VH and VL are covalently linked through a short, flexible peptide, or as Fab fragments, in which they are each fused to a constant domain and interact non-covalently, as described in Winter *et al.*, *Ann. Rev. Immunol.*, **12:** 433-455 (1994). As used herein, scFv encoding phage clones and Fab encoding phage clones are collectively referred to as "Fv phage clones" or "Fv clones".

Repertoires of VH and VL genes can be separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be searched for antigen-binding clones as described in Winter *et al.*, *Ann. Rev. Immunol.*, **12:** 433-455 (1994). Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned to provide a single source of human antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths *et al.*, *EMBO J,* **12**: 725-734 (1993). Finally, naive libraries can also be made synthetically by cloning the unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement in vitro as described by Hoogenboom and Winter, *J. Mol. Biol.*, **227:** 381-388 (1992).

Filamentous phage is used to display antibody fragments by fusion to the minor coat protein pIII. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains are connected on the same polypeptide chain by a flexible polypeptide spacer, e.g. as described by Marks *et al.*, *J. Mol. Biol.,* **222**: 581-597 (1991), or

as Fab fragments, in which one chain is fused to pIII and the other is secreted into the bacterial host cell periplasm where assembly of a Fab-coat protein structure which becomes displayed on the phage surface by displacing some of the wild type coat proteins, e.g. as described in Hoogenboom *et al.*, *Nucl. Acids Res.*, **19**: 4133-4137 (1991).

In general, nucleic acids encoding antibody gene fragments are obtained from immune cells harvested from humans or animals. If a library biased in favor of anti-DLL4 clones is desired, the subject is immunized with DLL4 to generate an antibody response, and spleen cells and/or circulating B cells other peripheral blood lymphocytes (PBLs) are recovered for library construction. In a preferred embodiment, a human antibody gene fragment library biased in favor of anti-DLL4 clones is obtained by generating an anti-DLL4 antibody response in transgenic mice carrying a functional human immunoglobulin gene array (and lacking a functional endogenous antibody production system) such that DLL4 immunization gives rise to B cells producing human antibodies against DLL4. The generation of human antibody-producing transgenic mice is described below.

Additional enrichment for anti-DLL4 reactive cell populations can be obtained by using a suitable screening procedure to isolate B cells expressing DLL4-specific membrane bound antibody, e.g., by cell separation with DLL4 affinity chromatography or adsorption of cells to fluorochrome-labeled DLL4 followed by flow-activated cell sorting (FACS).

Alternatively, the use of spleen cells and/or B cells or other PBLs from an unimmunized donor provides a better representation of the possible antibody repertoire, and also permits the construction of an antibody library using any animal (human or non-human) species in which DLL4 is not antigenic. For libraries incorporating *in vitro* antibody gene construction, stem cells are harvested from the subject to provide nucleic acids encoding unrearranged antibody gene segments. The immune cells of interest can be obtained from a variety of animal species, such as human, mouse, rat, lagomorpha, luprine, canine, feline, porcine, bovine, equine, and avian species, etc.

Nucleic acid encoding antibody variable gene segments (including VH and VL segments) are recovered from the cells of interest and amplified. In the case of rearranged VH and VL gene libraries, the desired DNA can be obtained by isolating genomic DNA or mRNA from lymphocytes followed by polymerase chain reaction (PCR) with primers matching the 5' and 3' ends of rearranged VH and VL genes as described in Orlandi *et al.*, *Proc. Natl. Acad. Sci. (USA),* **86**: 3833-3837 (1989), thereby making diverse V gene repertoires for expression. The V genes can be amplified from cDNA and genomic DNA, with back primers at the 5' end of the exon encoding the mature V-domain and forward

primers based within the J-segment as described in Orlandi *et al.* (1989) and in Ward *et al.*, *Nature*, **341**: 544-546 (1989). However, for amplifying from cDNA, back primers can also be based in the leader exon as described in Jones *et al.*, *Biotechnol.*, **9**: 88-89 (1991), and forward primers within the constant region as described in Sastry *et al.*, *Proc. Natl. Acad. Sci. (USA),* **86**: 5728-5732 (1989). To maximize complementarity, degeneracy can be incorporated in the primers as described in Orlandi *et al.* (1989) or Sastry *et al.* (1989). Preferably, the library diversity is maximized by using PCR primers targeted to each V-gene family in order to amplify all available VH and VL arrangements present in the immune cell nucleic acid sample, e.g. as described in the method of Marks *et al.*, *J. Mol. Biol.*, **222**: 581-597 (1991) or as described in the method of Orum *et al.*, *Nucleic Acids Res.*, **21**: 4491-4498 (1993). For cloning of the amplified DNA into expression vectors, rare restriction sites can be introduced within the PCR primer as a tag at one end as described in Orlandi *et al.* (1989), or by further PCR amplification with a tagged primer as described in Clackson *et al.*, *Nature*, **352:** 624-628 (1991).

Repertoires of synthetically rearranged V genes can be derived *in vitro* from V gene segments. Most of the human VH-gene segments have been cloned and sequenced (reported in Tomlinson *et al.*, *J. Mol. Biol.,* **227**: 776-798 (1992)), and mapped (reported in Matsuda *et al.*, *Nature Genet.,* **3**: 88-94 (1993); these cloned segments (including all the major conformations of the H1 and H2 loop) can be used to generate diverse VH gene repertoires with PCR primers encoding H3 loops of diverse sequence and length as described in Hoogenboom and Winter, *J. Mol. Biol.,* **227:** 381-388 (1992). VH repertoires can also be made with all the sequence diversity focused in a long H3 loop of a single length as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, **89**: 4457-4461 (1992). Human Vκ and Vλ segments have been cloned and sequenced (reported in Williams and Winter, *Eur. J. Immunol.*, **23**: 1456-1461 (1993)) and can be used to make synthetic light chain repertoires. Synthetic V gene repertoires, based on a range of VH and VL folds, and L3 and H3 lengths, will encode antibodies of considerable structural diversity. Following amplification of V-gene encoding DNAs, germline V-gene segments can be rearranged in vitro according to the methods of Hoogenboom and Winter, *J. Mol. Biol.,* **227**: 381-388 (1992).

Repertoires of antibody fragments can be constructed by combining VH and VL gene repertoires together in several ways. Each repertoire can be created in different vectors, and the vectors recombined *in vitro*, e.g., as described in Hogrefe *et al.*, *Gene*, **128**: 119-126 (1993), or *in vivo* by combinatorial infection, e.g., the loxP system described

in Waterhouse *et al.*, *Nucl. Acids Res.*, **21**: 2265-2266 (1993). The *in vivo* recombination approach exploits the two-chain nature of Fab fragments to overcome the limit on library size imposed by *E. coli* transformation efficiency. Naive VH and VL repertoires are cloned separately, one into a phagemid and the other into a phage vector. The two libraries are then combined by phage infection of phagemid-containing bacteria so that each cell contains a different combination and the library size is limited only by the number of cells present (about 10^{12} clones). Both vectors contain *in vivo* recombination signals so that the VH and VL genes are recombined onto a single replicon and are co-packaged into phage virions. These huge libraries provide large numbers of diverse antibodies of good affinity (K_d^{-1} of about 10^{-8} M).

Alternatively, the repertoires may be cloned sequentially into the same vector, e.g. as described in Barbas *et al.*, *Proc. Natl. Acad. Sci. USA,* **88**: 7978-7982 (1991), or assembled together by PCR and then cloned, e.g. as described in Clackson *et al.*, *Nature*, **352:** 624-628 (1991). PCR assembly can also be used to join VH and VL DNAs with DNA encoding a flexible peptide spacer to form single chain Fv (scFv) repertoires. In yet another technique, "in cell PCR assembly" is used to combine VH and VL genes within lymphocytes by PCR and then clone repertoires of linked genes as described in Embleton *et al.*, *Nucl. Acids Res.*, **20**: 3831-3837 (1992).

The antibodies produced by naive libraries (either natural or synthetic) can be of moderate affinity (K_d^{-1} of about 10^6 to 10^7 M^{-1}), but affinity maturation can also be mimicked *in vitro* by constructing and reselecting from secondary libraries as described in Winter *et al.* (1994), *supra*. For example, mutation can be introduced at random in vitro by using error-prone polymerase (reported in Leung *et al.*, *Technique,* **1:** 11-15 (1989)) in the method of Hawkins *et al.*, *J. Mol. Biol.,* **226**: 889-896 (1992) or in the method of Gram *et al.*, *Proc. Natl. Acad. Sci USA*, **89**: 3576-3580 (1992). Additionally, affinity maturation can be performed by randomly mutating one or more CDRs, e.g. using PCR with primers carrying random sequence spanning the CDR of interest, in selected individual Fv clones and screening for higher affinity clones. WO 9607754 (published 14 March 1996) described a method for inducing mutagenesis in a complementarity determining region of an immunoglobulin light chain to create a library of light chain genes. Another effective approach is to recombine the VH or VL domains selected by phage display with repertoires of naturally occurring V domain variants obtained from unimmunized donors and screen for higher affinity in several rounds of chain reshuffling as described in Marks *et al.*,

Biotechnol., **10:** 779-783 (1992). This technique allows the production of antibodies and antibody fragments with affinities in the 10^{-9} M range.

DLL4 nucleic acid and amino acid sequences are known in the art. Nucleic acid sequence encoding the DLL4 can be designed using the amino acid sequence of the desired region of DLL4. Alternatively, the cDNA sequence (or fragments thereof) of GenBank Accession Nos. NM_019074. DLL4 is a transmembrane protein. The extracellular region contains 8 EGF-like repeats, as well as a DSL domain that is conserved among all Notch ligands and is necessary for receptor binding. The predicted protein also contains a transmembrane region, and a cytoplasmic tail lacking any catalytic motifs. Human DLL4 protein is a 685 amino acid protein and contains the following regions: signal peptide (amino acids 1-25); MNNL (amino acids 26-92); DSL (amino acids 155-217); EGF-Like (amino acids 221-251); EGF-Like (amino acids 252-282); EGF-Like (amino acids 284-322); EGF-Like (amino acids 324-360); EGF-Like (amino acids 366-400); EGF-Like (amino acids 402-438); EGF-Like (amino acids 440-476); EGF-Like (amino acids 480-518); transmembrane (amino acids 529-551); cytoplasmic domain (amino acids 552-685). The accession number of human DLL4 is NM_019074, and the accession number of mouse DLL4 is NM_019454.

DNAs encoding DLL4 can be prepared by a variety of methods known in the art. These methods include, but are not limited to, chemical synthesis by any of the methods described in Engels *et al.*, *Agnew. Chem. Int. Ed. Engl.*, **28**: 716-734 (1989), such as the triester, phosphite, phosphoramidite and H-phosphonate methods. In one embodiment, codons preferred by the expression host cell are used in the design of the DLL4 encoding DNA. Alternatively, DNA encoding the DLL4 can be isolated from a genomic or cDNA library.

Following construction of the DNA molecule encoding the DLL4, the DNA molecule is operably linked to an expression control sequence in an expression vector, such as a plasmid, wherein the control sequence is recognized by a host cell transformed with the vector. In general, plasmid vectors contain replication and control sequences which are derived from species compatible with the host cell. The vector ordinarily carries a replication site, as well as sequences which encode proteins that are capable of providing phenotypic selection in transformed cells. Suitable vectors for expression in prokaryotic and eukaryotic host cells are known in the art and some are further described herein. Eukaryotic organisms, such as yeasts, or cells derived from multicellular organisms, such as mammals, may be used.

Optionally, the DNA encoding the DLL4 is operably linked to a secretory leader sequence resulting in secretion of the expression product by the host cell into the culture medium. Examples of secretory leader sequences include stII, ecotin, lamB, herpes GD, lpp, alkaline phosphatase, invertase, and alpha factor. Also suitable for use herein is the 36 amino acid leader sequence of protein A (Abrahmsen *et al.*, *EMBO J.*, **4**: 3901 (1985)).

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

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ precipitation and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell. Methods for transfection are well known in the art, and some are further described herein.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Methods for transformation are well known in the art, and some are further described herein.

Prokaryotic host cells used to produce the DLL4 can be cultured as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the DLL4 can be cultured in a variety of media, which is well known in the art and some of which is described herein.

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

Purification of DLL4 may be accomplished using art-recognized methods, some of which are described herein.

The purified DLL4 can be attached to a suitable matrix such as agarose beads, acrylamide beads, glass beads, cellulose, various acrylic copolymers, hydroxyl methacrylate gels, polyacrylic and polymethacrylic copolymers, nylon, neutral and ionic carriers, and the like, for use in the affinity chromatographic separation of phage display clones. Attachment of the DLL4 protein to the matrix can be accomplished by the methods described in *Methods in Enzymology*, vol. 44 (1976). A commonly employed technique

for attaching protein ligands to polysaccharide matrices, e.g. agarose, dextran or cellulose, involves activation of the carrier with cyanogen halides and subsequent coupling of the peptide ligand's primary aliphatic or aromatic amines to the activated matrix.

Alternatively, DLL4 can be used to coat the wells of adsorption plates, expressed on host cells affixed to adsorption plates or used in cell sorting, or conjugated to biotin for capture with streptavidin-coated beads, or used in any other art-known method for panning phage display libraries.

The phage library samples are contacted with immobilized DLL4 under conditions suitable for binding of at least a portion of the phage particles with the adsorbent. Normally, the conditions, including pH, ionic strength, temperature and the like are selected to mimic physiological conditions. The phages bound to the solid phase are washed and then eluted by acid, e.g. as described in Barbas *et al.*, *Proc. Natl. Acad. Sci USA*, **88**: 7978-7982 (1991), or by alkali, e.g. as described in Marks *et al.*, *J. Mol. Biol.*, **222**: 581-597 (1991), or by DLL4 antigen competition, e.g. in a procedure similar to the antigen competition method of Clackson *et al.*, *Nature*, **352**: 624-628 (1991). Phages can be enriched 20-1,000-fold in a single round of selection. Moreover, the enriched phages can be grown in bacterial culture and subjected to further rounds of selection.

The efficiency of selection depends on many factors, including the kinetics of dissociation during washing, and whether multiple antibody fragments on a single phage can simultaneously engage with antigen. Antibodies with fast dissociation kinetics (and weak binding affinities) can be retained by use of short washes, multivalent phage display and high coating density of antigen in solid phase. The high density not only stabilizes the phage through multivalent interactions, but favors rebinding of phage that has dissociated. The selection of antibodies with slow dissociation kinetics (and good binding affinities) can be promoted by use of long washes and monovalent phage display as described in Bass *et al.*, *Proteins*, **8**: 309-314 (1990) and in WO 92/09690, and a low coating density of antigen as described in Marks *et al.*, *Biotechnol.*, **10**: 779-783 (1992).

It is possible to select between phage antibodies of different affinities, even with affinities that differ slightly, for DLL4. However, random mutation of a selected antibody (e.g. as performed in some of the affinity maturation techniques described above) is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting DLL4, rare high affinity phage could be competed out. To retain all the higher affinity mutants, phages can be incubated with excess biotinylated DLL4, but with the biotinylated DLL4 at a concentration of lower molarity than the target molar affinity

constant for DLL4. The high affinity-binding phages can then be captured by streptavidin-coated paramagnetic beads. Such "equilibrium capture" allows the antibodies to be selected according to their affinities of binding, with sensitivity that permits isolation of mutant clones with as little as two-fold higher affinity from a great excess of phages with lower affinity. Conditions used in washing phages bound to a solid phase can also be manipulated to discriminate on the basis of dissociation kinetics.

Anti-DLL4 clones may be activity selected. In one embodiment, the invention provides anti-DLL4 antibodies that block the binding between a Notch receptor (such as Notch1, Notch2, Notch3 and/or Notch4) and DLL4, but do not block the binding between a Notch receptor and a second protein. Fv clones corresponding to such anti-DLL4 antibodies can be selected by (1) isolating anti-DLL4 clones from a phage library as described above, and optionally amplifying the isolated population of phage clones by growing up the population in a suitable bacterial host; (2) selecting DLL4 and a second protein against which blocking and non-blocking activity, respectively, is desired; (3) adsorbing the anti-DLL4 phage clones to immobilized DLL4; (4) using an excess of the second protein to elute any undesired clones that recognize DLL4-binding determinants which overlap or are shared with the binding determinants of the second protein; and (5) eluting the clones which remain adsorbed following step (4). Optionally, clones with the desired blocking/non-blocking properties can be further enriched by repeating the selection procedures described herein one or more times.

DNA encoding the hybridoma-derived monoclonal antibodies or phage display Fv clones of the invention is readily isolated and sequenced using conventional procedures (e.g. by using oligonucleotide primers designed to specifically amplify the heavy and light chain coding regions of interest from hybridoma or phage DNA template). Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of the desired monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of antibody-encoding DNA include Skerra *et al.*, *Curr. Opinion in Immunol.*, **5**: 256 (1993) and Pluckthun, *Immunol. Revs*, **130**: 151 (1992).

DNA encoding the Fv clones of the invention can be combined with known DNA sequences encoding heavy chain and/or light chain constant regions (e.g. the appropriate DNA sequences can be obtained from Kabat *et al.*, supra) to form clones encoding full or partial length heavy and/or light chains. It will be appreciated that constant regions of any

isotype can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species. A Fv clone derived from the variable domain DNA of one animal (such as human) species and then fused to constant region DNA of another animal species to form coding sequence(s) for "hybrid", full length heavy chain and/or light chain is included in the definition of "chimeric" and "hybrid" antibody as used herein. In a preferred embodiment, a Fv clone derived from human variable DNA is fused to human constant region DNA to form coding sequence(s) for all human, full or partial length heavy and/or light chains.

DNA encoding anti-DLL4 antibody derived from a hybridoma of the invention can also be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of homologous murine sequences derived from the hybridoma clone (e.g. as in the method of Morrison *et al.*, *Proc. Natl. Acad. Sci. USA*, **81**: 6851-6855 (1984)). DNA encoding a hybridoma or Fv clone-derived antibody or fragment can be further modified by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In this manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of the Fv clone or hybridoma clone-derived antibodies of the invention.

Antibody Fragments

The present invention encompasses antibody fragments. In certain circumstances there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved access to solid tumors.

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992); and Brennan et al., Science, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. Fab, Fv and ScFv antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of these fragments. Antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Fab and $F(ab')_2$ fragment with increased *in vivo* half-life comprising a salvage receptor binding epitope residues are described in U.S. Pat.

No. 5,869,046. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Pat. Nos. 5,571,894; and 5,587,458. Fv and sFv are the only species with intact combining sites that are devoid of constant regions; thus, they are suitable for reduced nonspecific binding during in vivo use. sFv fusion proteins may be constructed to yield fusion of an effector protein at either the amino or the carboxy terminus of an sFv. See Antibody Engineering, ed. Borrebaeck, *supra*. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870 for example. Such linear antibody fragments may be monospecific or bispecific.

Humanized Antibodies

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

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework for the humanized antibody (Sims *et al.* (1993) *J. Immunol.* 151:2296; Chothia *et al.* (1987) *J. Mol. Biol.* 196:901. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.* (1992) *Proc. Natl. Acad. Sci. USA*, 89:4285; Presta *et al.* (1993) *J. Immunol.*, 151:2623.

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

Human antibodies

Human anti-DLL4 antibodies of the invention can be constructed by combining Fv clone variable domain sequence(s) selected from human-derived phage display libraries with known human constant domain sequences(s) as described above. Alternatively, human monoclonal anti-DLL4 antibodies of the invention can be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described, for example, by Kozbor *J. Immunol.*, **133**: 3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and Boerner *et al.*, *J. Immunol.*, **147**: 86 (1991).

It is now possible to produce transgenic animals (e.g. mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci USA,* **90**: 2551 (1993); Jakobovits *et al.*, *Nature,* **362**: 255 (1993); Bruggermann *et al.*, *Year in Immunol., 7:* 33 (1993).

Gene shuffling can also be used to derive human antibodies from non-human, e.g. rodent, antibodies, where the human antibody has similar affinities and specificities to the starting non-human antibody. According to this method, which is also called "epitope imprinting", either the heavy or light chain variable region of a non-human antibody fragment obtained by phage display techniques as described above is replaced with a repertoire of human V domain genes, creating a population of non-human chain/human chain scFv or Fab chimeras. Selection with antigen results in isolation of a non-human chain/human chain chimeric scFv or Fab wherein the human chain restores the antigen binding site destroyed upon removal of the corresponding non-human chain in the primary phage display clone, i.e. the epitope governs (imprints) the choice of the human chain partner. When the process is repeated in order to replace the remaining non-human chain, a human antibody is obtained (see PCT WO 93/06213 published April 1, 1993). Unlike traditional humanization of non-human antibodies by CDR grafting, this technique provides completely human antibodies, which have no FR or CDR residues of non-human origin.

Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for DLL4 and the other is for any other antigen. Exemplary bispecific antibodies may bind to two different epitopes of the DLL4 protein. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express DLL4. These antibodies possess an DLL4-binding arm and an arm which binds the cytotoxic agent (*e.g.* saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, *Nature*, **305**: 537 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low.

Similar procedures are disclosed in WO 93/08829 published May 13, 1993, and in Traunecker *et al.*, *EMBO J.,* **10:** 3655 (1991).

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

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al., Methods in Enzymology*, 121:210 (1986).

According to another approach, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This

provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/00373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al., Science*, **229**: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli,* which can be chemically coupled to form bispecific antibodies. Shalaby *et al., J. Exp. Med.*, **175**: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.*, **148(5):**1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody

homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA*, **90**:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the VH and VL domains of one fragment are forced to pair with the complementary VL and VH domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.*, **152:**5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* **147**: 60 (1991).

Multivalent Antibodies

A multivalent antibody may be internalized (and/or catabolized) faster than a bivalent antibody by a cell expressing an antigen to which the antibodies bind. The antibodies of the present invention can be multivalent antibodies (which are other than of the IgM class) with three or more antigen binding sites (e.g. tetravalent antibodies), which can be readily produced by recombinant expression of nucleic acid encoding the polypeptide chains of the antibody. The multivalent antibody can comprise a dimerization domain and three or more antigen binding sites. The preferred dimerization domain comprises (or consists of) an Fc region or a hinge region. In this scenario, the antibody will comprise an Fc region and three or more antigen binding sites amino-terminal to the Fe region. The preferred multivalent antibody herein comprises (or consists of) three to about eight, but preferably four, antigen binding sites. The multivalent antibody comprises at least one polypeptide chain (and preferably two polypeptide chains), wherein the polypeptide chain(s) comprise two or more variable domains. For instance, the polypeptide chain(s) may comprise VD1-(X1)n -VD2-(X2)n -Fc, wherein VD1 is a first variable domain, VD2 is a second variable domain, Fc is one polypeptide chain of an Fc region, X1 and X2 represent an amino acid or polypeptide, and n is 0 or 1. For instance, the polypeptide chain(s) may comprise: VH-CH1-flexible linker-VH-CH1-Fc region chain; or VH-CH1-VH-CH1-Fc region chain. The multivalent antibody herein preferably further comprises at least two (and preferably four) light chain variable domain polypeptides. The multivalent antibody herein may, for instance, comprise from about two to about eight

light chain variable domain polypeptides. The light chain variable domain polypeptides contemplated here comprise a light chain variable domain and, optionally, further comprise a CL domain.

Antibody Variants

In some embodiments, amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of the antibody are prepared by introducing appropriate nucleotide changes into the antibody nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid alterations may be introduced in the subject antibody amino acid sequence at the time that sequence is made.

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. Here, a residue or group of target residues are identified (*e.g.*, charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed immunoglobulins are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody fused to a cytotoxic polypeptide. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (*e.g.* for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

Where the antibody comprises an Fc region, the carbohydrate attached thereto may be altered. For example, antibodies with a mature carbohydrate structure that lacks fucose attached to an Fc region of the antibody are described in US Pat Appl No US 2003/0157108 (Presta, L.). See also US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Antibodies with a bisecting N-acetylglucosamine (GlcNAc) in the carbohydrate attached to an Fc region of the antibody are referenced in WO 2003/011878, Jean-Mairet *et al.* and US Patent No. 6,602,684, Umana *et al.* Antibodies with at least one galactose residue in the oligosaccharide attached to an Fc region of the antibody are reported in WO 1997/30087, Patel *et al.* See, also, WO 1998/58964 (Raju, S.) and WO 1999/22764 (Raju, S.) concerning antibodies with altered carbohydrate attached to the Fc region thereof. See also US 2005/0123546 (Umana *et al.*) on antigen-binding molecules with modified glycosylation.

The preferred glycosylation variant herein comprises an Fc region, wherein a carbohydrate structure attached to the Fc region lacks fucose. Such variants have improved ADCC function. Optionally, the Fc region further comprises one or more amino acid substitutions therein which further improve ADCC, for example, substitutions at positions 298, 333, and/or 334 of the Fc region (Eu numbering of residues). Examples of publications related to "defucosylated" or "fucose-deficient" antibodies include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US

2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; Okazaki *et al. J. Mol. Biol.* 336:1239-1249 (2004); Yamane-Ohnuki *et al. Biotech. Bioeng.* 87: 614 (2004). Examples of cell lines producing defucosylated antibodies include Lec13 CHO cells deficient in protein fucosylation (Ripka *et al. Arch. Biochem. Biophys.* 249:533-545 (1986); US Pat Appl No US 2003/0157108 A1, Presta, L; and WO 2004/056312 A1, Adams *et al.*, especially at Example 11), and knockout cell lines, such as alpha-1,6-fucosyltransferase gene, *FUT8,*knockout CHO cells (Yamane-Ohnuki *et al. Biotech. Bioeng.* 87: 614 (2004)).

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule replaced by a different residue. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 1

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

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

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

(3) acidic: asp, glu;

(4) basic: his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

One type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants involves affinity maturation using phage display. Briefly, several hypervariable region sites (*e.g.* 6-7 sites) are mutated to generate all possible amino acid substitutions at each site. The antibodies thus generated are displayed from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.* binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or additionally, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

It may be desirable to introduce one or more amino acid modifications in an Fc region of the immunoglobulin polypeptides of the invention, thereby generating a Fc

region variant. The Fc region variant may comprise a human Fc region sequence (*e.g.*, a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (*e.g.* a substitution) at one or more amino acid positions including that of a hinge cysteine.

In accordance with this description and the teachings of the art, it is contemplated that in some embodiments, an antibody used in methods of the invention may comprise one or more alterations as compared to the wild type counterpart antibody, e.g. in the Fc region. These antibodies would nonetheless retain substantially the same characteristics required for therapeutic utility as compared to their wild type counterpart. For example, it is thought that certain alterations can be made in the Fc region that would result in altered (*i.e.*, either improved or diminished) C1q binding and/or Complement Dependent Cytotoxicity (CDC), e.g., as described in WO99/51642. See also Duncan & Winter *Nature* 322:738-40 (1988); US Patent No. 5,648,260; US Patent No. 5,624,821; and WO94/29351 concerning other examples of Fc region variants. WO00/42072 (Presta) and WO 2004/056312 (Lowman) describe antibody variants with improved or diminished binding to FcRs. The content of these patent publications are specifically incorporated herein by reference. See, also, Shields *et al. J. Biol. Chem.* 9(2): 6591-6604 (2001). Antibodies with increased half lives and improved binding to the neonatal Fc receptor (FcRn), which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)), are described in US2005/0014934A1 (Hinton et al.). These antibodies comprise an Fc reg on with one or more substitutions therein which improve binding of the Fc region to FcRn. Polypeptide variants with altered Fc region amino acid sequences and increased or decreased C1q binding capability are described in US patent No. 6,194,551B1, WO99/51642. The contents of those patent publications are specifically incorporated herein by reference. See, also, Idusogie *et al. J. Immunol.* 164: 4178-4184 (2000).

Antibody Derivatives

The antibodies of the present invention can be further modified to contain additional nonproteinaceous moieties that are known in the art and readily available. Preferably, the moieties suitable for derivatization of the antibody are water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl

pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymers are attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody to be improved, whether the antibody derivative will be used in a therapy under defined conditions, etc.

Screening for antibodies with desired properties

The antibodies of the present invention can be characterized for their physical/chemical properties and biological functions by various assays known in the art. In some embodiments, antibodies are characterized for any one or more of binding to DLL4; and/or reduction or blocking of Notch receptor activation; and/or reduction or blocking of Notch receptor downstream molecular signaling; and/or disruption or blocking of Notch receptor binding to DLL4; and/or promotion of endothelial cell proliferation; and/or inhibition of endothelial cell differentiation; and/or inhibition of arterial differentiation; and/or inhibition of tumor vascular perfusion; and/or treatment and/or prevention of a tumor, cell proliferative disorder or a cancer; and/or treatment or prevention of a disorder associated with DLL4 expression and/or activity; and/or treatment or prevention of a disorder associated with Notch receptor expression and/or activity.

The purified antibodies can be further characterized by a series of assays including, but not limited to, N-terminal sequencing, amino acid analysis, non-denaturing size exclusion high pressure liquid chromatography (HPLC), mass spectrometry, ion exchange chromatography and papain digestion.

In certain embodiments of the invention, the antibodies produced herein are analyzed for their biological activity. In some embodiments, the antibodies of the present invention are tested for their antigen binding activity. The antigen binding assays that are known in the art and can be used herein include without limitation any direct or competitive binding assays using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, fluorescent immunoassays, and protein A immunoassays. Illustrative antigen binding assay are provided below in the Examples section.

In still another embodiment, the invention provides anti-DLL4 monoclonal antibodies that compete with a 26.6, 26.14, 26.20, 26.34, and/or 26.82 antibody for binding to DLL4. Such competitor antibodies include antibodies that recognize an DLL4 epitope that is the same as or overlaps with the DLL4 epitope recognized by antibody 26.6, 26.14, 26.20, 26.34, and/or 26.82. Such competitor antibodies can be obtained by screening anti-DLL4 hybridoma supernatants for binding to immobilized DLL4 in competition with labeled 26.6, 26.14, 26.20, 26.34, and/or 26.82 antibody. A hybridoma supernatant containing competitor antibody will reduce the amount of bound, labeled antibody detected in the subject competition binding mixture as compared to the amount of bound, labeled antibody detected in a control binding mixture containing irrelevant (or no) antibody. Any of the competition binding assays described herein are suitable for use in the foregoing procedure.

In another aspect, the invention provides an anti-DLL4 monoclonal antibody that comprises one or more (such as 2, 3, 4, 5, and/or 6) HVRs of an 26.6, 26.14, 26.20, 26.34, and/or 26.82 antibody. An anti-DLL4 monoclonal antibody that comprises one or more HVR(s) of an 26.6, 26.14, 26.20, 26.34, and/or 26.82 antibody can be constructed by grafting one or more HVR(s) of an 26.6, 26.14, 26.20, 26.34, and/or 26.82 antibody onto a template antibody sequence, e.g. a human antibody sequence which is closest to the corresponding murine sequence of the parental antibody, or a consensus sequence of all human antibodies in the particular subgroup of the parental antibody light or heavy chain, and expressing the resulting chimeric light and/or heavy chain variable region sequence(s), with or without accompanying constant region sequence(s), in recombinant host cells as described herein.

Anti-DLL4 antibodies of the invention possessing the unique properties described herein can be obtained by screening anti-DLL4 hybridoma clones for the desired properties by any convenient method. For example, if an anti-DLL4 monoclonal antibody that blocks or does not block the binding of Notch receptors to DLL4 is desired, the candidate antibody can be tested in a binding competition assay, such as a competitive binding ELISA, wherein plate wells are coated with DLL4, and a solution of antibody in an excess of the Notch receptor of interest is layered onto the coated plates, and bound antibody is detected enzymatically, e.g. contacting the bound antibody with HRP-conjugated anti-Ig antibody or biotinylated anti-Ig antibody and developing the HRP color reaction., e.g. by developing plates with streptavidin-HRP and/or hydrogen peroxide and detecting the HRP color reaction by spectrophotometry at 490 nm with an ELISA plate reader.

In one embodiment, the present invention contemplates an altered antibody that possesses some but not all effector functions, which make it a desired candidate for many applications in which the half life of the antibody in vivo is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. In certain embodiments, the Fc activities of the produced immunoglobulin are measured to ensure that only the desired properties are maintained. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). An example of an in vitro assay to assess ADCC activity of a molecule of interest is described in US Patent No. 5,500,362 or 5,821,337. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed *in vivo*, e.g., in a animal model such as that disclosed in Clynes et al. *Proc. Natl. Acad. Sci.A* 95:652-656 (1998). C1q binding assays may also be carried out to confirm that the antibody is unable to bind C1q and hence lacks CDC activity. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed. FcRn binding and *in vivo* clearance/half life determinations can also be performed using methods known in the art, e.g. those described in the Examples section.

Vectors, Host Cells and Recombinant Methods

For recombinant production of an antibody of the invention, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The choice of vector depends in part on the host cell to be used. Generally, preferred host cells are of either prokaryotic or eukaryotic (generally mammalian) origin. It will be appreciated that constant regions of any isotype

can be used for this purpose, including IgG, IgM, IgA, IgD, and IgE constant regions, and that such constant regions can be obtained from any human or animal species.

a. Generating antibodies using prokaryotic host cells:

i. Vector Construction

Polynucleotide sequences encoding polypeptide components of the antibody of the invention can be obtained using standard recombinant techniques. Desired polynucleotide sequences may be isolated and sequenced from antibody producing cells such as hybridoma cells. Alternatively, polynucleotides can be synthesized using nucleotide synthesizer or PCR techniques. Once obtained, sequences encoding the polypeptides are inserted into a recombinant vector capable of replicating and expressing heterologous polynucleotides in prokaryotic hosts. Many vectors that are available and known in the art can be used for the purpose of the present invention. Selection of an appropriate vector will depend mainly on the size of the nucleic acids to be inserted into the vector and the particular host cell to be transformed with the vector. Each vector contains various components, depending on its function (amplification or expression of heterologous polynucleotide, or both) and its compatibility with the particular host cell in which it resides. The vector components generally include, but are not limited to: an origin of replication, a selection marker gene, a promoter, a ribosome binding site (RBS), a signal sequence, the heterologous nucleic acid insert and a transcription termination sequence.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species. pBR322 contains genes encoding ampicillin (Amp) and tetracycline (Tet) resistance and thus provides easy means for identifying transformed cells. pBR322, its derivatives, or other microbial plasmids or bacteriophage may also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of endogenous proteins. Examples of pBR322 derivatives used for expression of particular antibodies are described in detail in Carter et al., U.S. Patent No. 5,648,237.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, bacteriophage such as λGEM™-11 may be utilized in

making a recombinant vector which can be used to transform susceptible host cells such as E. coli LE392.

The expression vector of the invention may comprise two or more promoter-cistron pairs, encoding each of the polypeptide components. A promoter is an untranslated regulatory sequence located upstream (5') to a cistron that modulates its expression. Prokaryotic promoters typically fall into two classes, inducible and constitutive. Inducible promoter is a promoter that initiates increased levels of transcription of the cistron under its control in response to changes in the culture condition, e.g. the presence or absence of a nutrient or a change in temperature.

A large number of promoters recognized by a variety of potential host cells are well known. The selected promoter can be operably linked to cistron DNA encoding the light or heavy chain by removing the promoter from the source DNA via restriction enzyme digestion and inserting the isolated promoter sequence into the vector of the invention. Both the native promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target genes. In some embodiments, heterologous promoters are utilized, as they generally permit greater transcription and higher yields of expressed target gene as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the PhoA promoter, the β-galactamase and lactose promoter systems, a tryptophan (trp) promoter system and hybrid promoters such as the tac or the trc promoter. However, other promoters that are functional in bacteria (such as other known bacterial or phage promoters) are suitable as well. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to cistrons encoding the target light and heavy chains (Siebenlist et al. (1980) Cell 20: 269) using linkers or adaptors to supply any required restriction sites.

In one aspect of the invention, each cistron within the recombinant vector comprises a secretion signal sequence component that directs translocation of the expressed polypeptides across a membrane. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. The signal sequence selected for the purpose of this invention should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the signal sequences native to the heterologous polypeptides, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group consisting of the alkaline phosphatase,

penicillinase, Ipp, or heat-stable enterotoxin II (STII) leaders, LamB, PhoE, PelB, OmpA and MBP. In one embodiment of the invention, the signal sequences used in both cistrons of the expression system are STII signal sequences or variants thereof.

In another aspect, the production of the immunoglobulins according to the invention can occur in the cytoplasm of the host cell, and therefore does not require the presence of secretion signal sequences within each cistron. In that regard, immunoglobulin light and heavy chains are expressed, folded and assembled to form functional immunoglobulins within the cytoplasm. Certain host strains (e.g., the *E. coli* trxB- strains) provide cytoplasm conditions that are favorable for disulfide bond formation, thereby permitting proper folding and assembly of expressed protein subunits. Proba and Pluckthun *Gene*, 159:203 (1995).

Prokaryotic host cells suitable for expressing antibodies of the invention include Archaebacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include Escherichia (e.g., *E. coli*), Bacilli (e.g., *B. subtilis*), Enterobacteria, Pseudomonas species (e.g., *P. aeruginosa*), Salmonella typhimurium, Serratia marcescans, Klebsiella, Proteus, Shigella, Rhizobia, Vitreoscilla, or Paracoccus. In one embodiment, gram-negative cells are used. In one embodiment, E. coli cells are used as hosts for the invention. Examples of E. coli strains include strain W3110 (Bachmann, Cellular and Molecular Biology, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC® Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 ΔfhuA (ΔtonA) ptr3 lac Iq lacL8 ΔompTΔ(nmpc-fepE) degP41 kanR (U.S. Pat. No. 5,639,635). Other strains and derivatives thereof, such as E. coli 294 (ATCC 31,446), *E. coli* B, *E. coli* λ 1776 (ATCC 31,537) and *E. coli* RV308(ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., *Proteins*, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli*, Serratia, or Salmonella species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. Typically the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

ii. Antibody Production

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

Transformation means introducing DNA into the prokaryotic host so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride is generally used for bacterial cells that contain substantial cell-wall barriers. Another method for transformation employs polyethylene glycol/DMSO. Yet another technique used is electroporation.

Prokaryotic cells used to produce the polypeptides of the invention are grown in media known in the art and suitable for culture of the selected host cells. Examples of suitable media include luria broth (LB) plus necessary nutrient supplements. In some embodiments, the media also contains a selection agent, chosen based on the construction of the expression vector, to selectively permit growth of prokaryotic cells containing the expression vector. For example, ampicillin is added to media for growth of cells expressing ampicillin resistant gene.

Any necessary supplements besides carbon, nitrogen, and inorganic phosphate sources may also be included at appropriate concentrations introduced alone or as a mixture with another supplement or medium such as a complex nitrogen source. Optionally the culture medium may contain one or more reducing agents selected from the group consisting of glutathione, cysteine, cystamine, thioglycollate, dithioerythritol and dithiothreitol.

The prokaryotic host cells are cultured at suitable temperatures. For *E. coli* growth, for example, the preferred temperature ranges from about 20°C to about 39°C, more preferably from about 25°C to about 37°C, even more preferably at about 30°C. The pH of the medium may be any pH ranging from about 5 to about 9, depending mainly on the host organism. For E. coli, the pH is preferably from about 6.8 to about 7.4, and more preferably about 7.0.

If an inducible promoter is used in the expression vector of the invention, protein expression is induced under conditions suitable for the activation of the promoter. In one aspect of the invention, PhoA promoters are used for controlling transcription of the polypeptides. Accordingly, the transformed host cells are cultured in a phosphate-limiting

medium for induction. Preferably, the phosphate-limiting medium is the C.R.A.P medium (see, e.g., Simmons et al., *J. Immunol. Methods* (2002), 263:133-147). A variety of other inducers may be used, according to the vector construct employed, as is known in the art.

In one embodiment, the expressed polypeptides of the present invention are secreted into and recovered from the periplasm of the host cells. Protein recovery typically involves disrupting the microorganism, generally by such means as osmotic shock, sonication or lysis. Once cells are disrupted, cell debris or whole cells may be removed by centrifugation or filtration. The proteins may be further purified, for example, by affinity resin chromatography. Alternatively, proteins can be transported into the culture media and isolated therein. Cells may be removed from the culture and the culture supernatant being filtered and concentrated for further purification of the proteins produced. The expressed polypeptides can be further isolated and identified using commonly known methods such as polyacrylamide gel electrophoresis (PAGE) and Western blot assay.

In one aspect of the invention, antibody production is conducted in large quantity by a fermentation process. Various large-scale fed-batch fermentation procedures are available for production of recombinant proteins. Large-scale fermentations have at least 1000 liters of capacity, preferably about 1,000 to 100,000 liters of capacity. These fermentors use agitator impellers to distribute oxygen and nutrients, especially glucose (the preferred carbon/energy source). Small scale fermentation refers generally to fermentation in a fermentor that is no more than approximately 100 liters in volumetric capacity, and can range from about 1 liter to about 100 liters.

In a fermentation process, induction of protein expression is typically initiated after the cells have been grown under suitable conditions to a desired density, e.g., an OD550 of about 180-220, at which stage the cells are in the early stationary phase. A variety of inducers may be used, according to the vector construct employed, as is known in the art and described above. Cells may be grown for shorter periods prior to induction. Cells are usually induced for about 12-50 hours, although longer or shorter induction time may be used.

To improve the production yield and quality of the polypeptides of the invention, various fermentation conditions can be modified. For example, to improve the proper assembly and folding of the secreted antibody polypeptides, additional vectors overexpressing chaperone proteins, such as Dsb proteins (DsbA, DsbB, DsbC, DsbD and or DsbG) or FkpA (a peptidylprolyl cis,trans-isomerase with chaperone activity) can be used to co-transform the host prokaryotic cells. The chaperone proteins have been

demonstrated to facilitate the proper folding and solubility of heterologous proteins produced in bacterial host cells. Chen et al. (1999) *J Bio Chem* 274:19601-19605; Georgiou et al., U.S. Patent No. 6,083,715; Georgiou et al., U.S. Patent No. 6,027,888; Bothmann and Pluckthun (2000) *J. Biol. Chem.* 275:17100-17105; Ramm and Pluckthun (2000) *J. Biol. Chem.* 275:17106-17113; Arie et al. (2001) *Mol. Microbiol.* 39:199-210.

To minimize proteolysis of expressed heterologous proteins (especially those that are proteolytically sensitive), certain host strains deficient for proteolytic enzymes can be used for the present invention. For example, host cell strains may be modified to effect genetic mutation(s) in the genes encoding known bacterial proteases such as Protease III, OmpT, DegP, Tsp, Protease I, Protease Mi, Protease V, Protease VI and combinations thereof. Some *E. coli* protease-deficient strains are available and described in, for example, Joly et al. (1998), *supra*; Georgiou et al., U.S. Patent No. 5,264,365; Georgiou et al., U.S. Patent No. 5,508,192; Hara et al., *Microbial Drug Resistance*, 2:63-72 (1996).

In one embodiment, *E. coli* strains deficient for proteolytic enzymes and transformed with plasmids overexpressing one or more chaperone proteins are used as host cells in the expression system of the invention.

iii. Antibody Purification

Standard protein purification methods known in the art can be employed. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC, chromatography on silica or on a cation-exchange resin such as DEAE, chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, and gel filtration using, for example, Sephadex G-75.

In one aspect, Protein A immobilized on a solid phase is used for immunoaffinity purification of the full length antibody products of the invention. Protein A is a 41kD cell wall protein from *Staphylococcus aureas* which binds with a high affinity to the Fc region of antibodies. Lindmark et al (1983) *J. Immunol. Meth.* 62:1-13. The solid phase to which Protein A is immobilized is preferably a column comprising a glass or silica surface, more preferably a controlled pore glass column or a silicic acid column. In some applications, the column has been coated with a reagent, such as glycerol, in an attempt to prevent nonspecific adherence of contaminants.

As the first step of purification, the preparation derived from the cell culture as described above is applied onto the Protein A immobilized solid phase to allow specific binding of the antibody of interest to Protein A. The solid phase is then washed to remove

contaminants non-specifically bound to the solid phase. Finally the antibody of interest is recovered from the solid phase by elution.

b. Generating antibodies using eukaryotic host cells:

The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal sequence component

A vector for use in a eukaryotic host cell may also contain a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide of interest. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the antibody.

(ii) Origin of replication

Generally, an origin of replication component is not needed for mammalian expression vectors. For example, the SV40 origin may typically be used only because it contains the early promoter.

(iii) Selection gene component

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

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

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

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity (e.g., ATCC CRL-9096).

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

(iv) Promoter component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody polypeptide nucleic acid. Promoter sequences are known for eukaryotes. Virtually alleukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide (SEQ ID NO: 60). At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence (SEQ ID NO: 61). All of these sequences are suitably inserted into eukaryotic expression vectors.

Antibody polypeptide transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A

modification of this system is described in U.S. Patent No. 4,601,978. Alternatively, the Rous Sarcoma Virus long terminal repeat can be used as the promoter.

(v) Enhancer element component

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

(vi) Transcription termination component

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

(vii) Selection and transformation of host cells

Suitable host cells for cloning or expressing the DNA in the vectors herein include higher eukaryote cells described herein, including vertebrate host cells. Propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 (1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells

(HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

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

(viii) Culturing the host cells

The host cells used to produce an antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

(ix) Purification of antibody

When using recombinant techniques, the antibody can be produced intracellularly, or directly secreted into the medium. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, are removed, for example, by centrifugation or ultrafiltration. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a

commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon® ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., *EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a CH3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g., from about 0-0.25 M salt).

Immunoconjugates

The invention also provides immunoconjugates (interchangeably termed "antibody-drug conjugates" or "ADC"), comprising any of the anti-DLL4 antibodies described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, a drug, a growth inhibitory agent, a toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof), or a radioactive isotope (i.e., a radioconjugate).

The use of antibody-drug conjugates for the local delivery of cytotoxic or cytostatic agents, i.e. drugs to kill or inhibit tumor cells in the treatment of cancer (Syrigos and Epenetos (1999) *Anticancer Research* 19:605-614; Niculescu-Duvaz and Springer (1997) *Adv. Drg Del. Rev.* 26:151-172; U.S. patent 4,975,278) allows targeted delivery of the drug moiety to tumors, and intracellular accumulation therein, where systemic administration of these unconjugated drug agents may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated (Baldwin et al., (1986) *Lancet* pp. (Mar. 15, 1986):603-05; Thorpe, (1985) "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in Monoclonal Antibodies '84: Biological And Clinical Applications, A. Pinchera et al. (ed.s), pp. 475-506). Maximal efficacy with minimal toxicity is sought thereby. Both polyclonal antibodies and monoclonal antibodies have been reported as useful in these strategies (Rowland et al., (1986) *Cancer Immunol. Immunother.*, 21:183-87). Drugs used in these methods include daunomycin, doxorubicin, methotrexate, and vindesine (Rowland et al., (1986) supra). Toxins used in antibody-toxin conjugates include bacterial toxins such as diphtheria toxin, plant toxins such as ricin, small molecule toxins such as geldanamycin (Mandler et al (2000) *Jour. of the Nat. Cancer Inst.* 92(19):1573-1581; Mandler et al (2000) *Bioorganic & Med. Chem. Letters* 10:1025-1028; Mandler et al (2002) *Bioconjugate Chem.* 13:786-791), maytansinoids (EP 1391213; Liu et al., (1996) *Proc. Natl. Acad. Sci. USA* 93:8618-8623), and calicheamicin (Lode et al (1998) *Cancer Res.* 58:2928; Hinman et al (1993) *Cancer Res.* 53:3336-3342). The toxins may effect their cytotoxic and cytostatic effects by mechanisms including tubulin binding, DNA binding, or topoisomerase inhibition. Some cytotoxic drugs tend to be inactive or less active when conjugated to large antibodies or protein receptor ligands.

ZEVALIN® (ibritumomab tiuxetan, Biogen/Idec) is an antibody-radioisotope conjugate composed of a murine IgG1 kappa monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes and ^{111}In or ^{90}Y radioisotope bound by a thiourea linker-chelator (Wiseman et al (2000) *Eur. Jour. Nucl. Med.* 27(7):766-77; Wiseman et al (2002) *Blood* 99(12):4336-42; Witzig et al (2002) *J. Clin. Oncol.* 20(10):2453-63; Witzig et al (2002) *J. Clin. Oncol.* 20(15):3262-69). Although ZEVALIN has activity against B-cell non-Hodgkin's Lymphoma (NHL), administration results in severe and prolonged cytopenias in most patients. MYLOTARG™ (gemtuzumab ozogamicin, Wyeth Pharmaceuticals), an antibody drug conjugate composed of a hu CD33 antibody linked to calicheamicin, was approved in 2000

for the treatment of acute myeloid leukemia by injection (*Drugs of the Future* (2000) 25(7):686; US Patent Nos. 4970198; 5079233; 5585089; 5606040; 5693762; 5739116; 5767285; 5773001). Cantuzumab mertansine (Immunogen, Inc.), an antibody drug conjugate composed of the huC242 antibody linked via the disulfide linker SPP to the maytansinoid drug moiety, DM1, is advancing into Phase II trials for the treatment of cancers that express CanAg, such as colon, pancreatic, gastric, and others. MLN-2704 (Millennium Pharm., BZL Biologics, Immunogen Inc.), an antibody drug conjugate composed of the anti-prostate specific membrane antigen (PSMA) monoclonal antibody linked to the maytansinoid drug moiety, DM1, is under development for the potential treatment of prostate tumors. The auristatin peptides, auristatin E (AE) and monomethylauristatin (MMAE), synthetic analogs of dolastatin, were conjugated to chimeric monoclonal antibodies cBR96 (specific to Lewis Y on carcinomas) and cAC10 (specific to CD30 on hematological malignancies) (Doronina et al (2003) *Nature Biotechnology* 21(7):778-784) and are under therapeutic development.

Chemotherapeutic agents useful in the generation of immunoconjugates are described herein (eg., above). Enzymatically active toxins and fragments thereof that can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes. See, e.g., WO 93/21232 published October 28, 1993. A variety of radionuclides are available for the production of radioconjugated antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y, and ^{186}Re. Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science, 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene

triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

Conjugates of an antibody and one or more small molecule toxins, such as a calicheamicin, maytansinoids, dolastatins, aurostatins, a trichothecene, and CC1065, and the derivatives of these toxins that have toxin activity, are also contemplated herein.

i. Maytansine and maytansinoids

In some embodiments, the immunoconjugate comprises an antibody (full length or fragments) of the invention conjugated to one or more maytansinoid molecules.

Maytansinoids are mitototic inhibitors which act by inhibiting tubulin polymerization. Maytansine was first isolated from the east African shrub Maytenus serrata (U.S. Patent No. 3,896,111). Subsequently, it was discovered that certain microbes also produce maytansinoids, such as maytansinol and C-3 maytansinol esters (U.S. Patent No. 4,151,042). Synthetic maytansinol and derivatives and analogues thereof are disclosed, for example, in U.S. Patent Nos. 4,137,230; 4,248,870; 4,256,746; 4,260,608; 4,265,814; 4,294,757; 4,307,016; 4,308,268; 4,308,269; 4,309,428; 4,313,946; 4,315,929; 4,317,821; 4,322,348; 4,331,598; 4,361,650; 4,364,866; 4,424,219; 4,450,254; 4,362,663; and 4,371,533.

Maytansinoid drug moieties are attractive drug moieties in antibody drug conjugates because they are: (i) relatively accessible to prepare by fermentation or chemical modification, derivatization of fermentation products, (ii) amenable to derivatization with functional groups suitable for conjugation through the non-disulfide linkers to antibodies, (iii) stable in plasma, and (iv) effective against a variety of tumor cell lines.

Immunoconjugates containing maytansinoids, methods of making same, and their therapeutic use are disclosed, for example, in U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1, the disclosures of which are hereby expressly incorporated by reference. Liu et al., *Proc. Natl. Acad. Sci. USA* 93:8618-8623 (1996) described immunoconjugates comprising a maytansinoid designated DM1 linked to the monoclonal antibody C242 directed against human colorectal cancer. The conjugate was found to be highly cytotoxic towards cultured colon cancer cells, and showed antitumor activity in an in vivo tumor growth assay. Chari et al., Cancer Research 52:127-131 (1992) describe immunoconjugates in which a maytansinoid was conjugated via a disulfide linker to the murine antibody A7 binding to an antigen on human colon cancer cell lines, or to another murine monoclonal antibody TA.1 that binds the HER-2/neu oncogene. The

cytotoxicity of the TA.1-maytansinoid conjugate was tested in vitro on the human breast cancer cell line SK-BR-3, which expresses 3 x 10^5 HER-2 surface antigens per cell. The drug conjugate achieved a degree of cytotoxicity similar to the free maytansinoid drug, which could be increased by increasing the number of maytansinoid molecules per antibody molecule. The A7-maytansinoid conjugate showed low systemic cytotoxicity in mice.

Antibody-maytansinoid conjugates are prepared by chemically linking an antibody to a maytansinoid molecule without significantly diminishing the biological activity of either the antibody or the maytansinoid molecule. See, e.g., U.S. Patent No. 5,208,020 (the disclosure of which is hereby expressly incorporated by reference). An average of 3-4 maytansinoid molecules conjugated per antibody molecule has shown efficacy in enhancing cytotoxicity of target cells without negatively affecting the function or solubility of the antibody, although even one molecule of toxin/antibody would be expected to enhance cytotoxicity over the use of naked antibody. Maytansinoids are well known in the art and can be synthesized by known techniques or isolated from natural sources. Suitable maytansinoids are disclosed, for example, in U.S. Patent No. 5,208,020 and in the other patents and nonpatent publications referred to hereinabove. Preferred maytansinoids are maytansinol and maytansinol analogues modified in the aromatic ring or at other positions of the maytansinol molecule, such as various maytansinol esters.

There are many linking groups known in the art for making antibody-maytansinoid conjugates, including, for example, those disclosed in U.S. Patent No. 5,208,020 or EP Patent 0 425 235 B1, Chari et al., *Cancer Research* 52:127-131 (1992), and U.S. Patent Application No. 10/960,602, filed Oct. 8, 2004, the disclosures of which are hereby expressly incorporated by reference. Antibody-maytansinoid conjugates comprising the linker component SMCC may be prepared as disclosed in U.S. Patent Application No. 10/960,602, filed Oct. 8, 2004. The linking groups include disulfide groups, thioether groups, acid labile groups, photolabile groups, peptidase labile groups, or esterase labile groups, as disclosed in the above-identified patents, disulfide and thioether groups being preferred. Additional linking groups are described and exemplified herein.

Conjugates of the antibody and maytansinoid may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde),

bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Particularly preferred coupling agents include N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Carlsson et al., *Biochem. J.* 173:723-737 (1978)) and N-succinimidyl-4-(2-pyridylthio)pentanoate (SPP) to provide for a disulfide linkage.

The linker may be attached to the maytansinoid molecule at various positions, depending on the type of the link. For example, an ester linkage may be formed by reaction with a hydroxyl group using conventional coupling techniques. The reaction may occur at the C-3 position having a hydroxyl group, the C-14 position modified with hydroxymethyl, the C-15 position modified with a hydroxyl group, and the C-20 position having a hydroxyl group. In a preferred embodiment, the linkage is formed at the C-3 position of maytansinol or a maytansinol analogue.

ii. Auristatins and dolastatins

In some embodiments, the immunoconjugate comprises an antibody of the invention conjugated to dolastatins or dolostatin peptidic analogs and derivatives, the auristatins (US Patent Nos. 5635483; 5780588). Dolastatins and auristatins have been shown to interfere with microtubule dynamics, GTP hydrolysis, and nuclear and cellular division (Woyke et al (2001) *Antimicrob. Agents and Chemother.* 45(12):3580-3584) and have anticancer (US 5663149) and antifungal activity (Pettit et al (1998) *Antimicrob. Agents Chemother.* 42:2961-2965). The dolastatin or auristatin drug moiety may be attached to the antibody through the N (amino) terminus or the C (carboxyl) terminus of the peptidic drug moiety (WO 02/088172).

Exemplary auristatin embodiments include the N-terminus linked monomethylauristatin drug moieties DE and DF, disclosed in "Monomethylvaline Compounds Capable of Conjugation to Ligands", US Ser. No. 10/983,340, filed Nov. 5, 2004, the disclosure of which is expressly incorporated by reference in its entirety.

Typically, peptide-based drug moieties can be prepared by forming a peptide bond between two or more amino acids and/or peptide fragments. Such peptide bonds can be prepared, for example, according to the liquid phase synthesis method (see E. Schröder and K. Lübke, "The Peptides", volume 1, pp 76-136, 1965, Academic Press) that is well known in the field of peptide chemistry. The auristatin/dolastatin drug moieties may be prepared according to the methods of: US 5635483; US 5780588; Pettit et al (1989) *J. Am. Chem. Soc.* 111:5463-5465; Pettit et al (1998) *Anti-Cancer Drug Design* 13:243-277; Pettit, G.R.,

et al. *Synthesis*, 1996, 719-725; and Pettit et al (1996) *J. Chem. Soc. Perkin Trans.* 1 5:859-863. See also Doronina (2003) *Nat Biotechnol* 21(7):778-784; "Monomethylvaline Compounds Capable of Conjugation to Ligands", US Ser. No. 10/983,340, filed Nov. 5, 2004, hereby incorporated by reference in its entirety (disclosing, e.g., linkers and methods of preparing monomethylvaline compounds such as MMAE and MMAF conjugated to linkers).

iii. Calicheamicin

In other embodiments, the immunoconjugate comprises an antibody of the invention conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. For the preparation of conjugates of the calicheamicin family, see U.S. patents 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, 5,877,296 (all to American Cyanamid Company). Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and θ^I_1 (Hinman et al., *Cancer Research* 53:3336-3342 (1993), Lode et al., *Cancer Research* 58:2925-2928 (1998) and the aforementioned U.S. patents to American Cyanamid). Another anti-tumor drug that the antibody can be conjugated is QFA which is an antifolate. Both calicheamicin and QFA have intracellular sites of action and do not readily cross the plasma membrane. Therefore, cellular uptake of these agents through antibody mediated internalization greatly enhances their cytotoxic effects.

iv. Other cytotoxic agents

Other antitumor agents that can be conjugated to the antibodies of the invention include BCNU, streptozoicin, vincristine and 5-fluorouracil, the family of agents known collectively LL-E33288 complex described in U.S. patents 5,053,394, 5,770,710, as well as esperamicins (U.S. patent 5,877,296).

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

The present invention further contemplates an immunoconjugate formed between an antibody and a compound with nucleolytic activity (e.g., a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

For selective destruction of the tumor, the antibody may comprise a highly radioactive atom. A variety of radioactive isotopes are available for the production of radioconjugated antibodies. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the conjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example tc^{99m} or I^{123}, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as tc^{99m} or I^{123}, .Re^{186}, Re^{188} and In^{111} can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker et al (1978) *Biochem. Biophys. Res. Commun.* 80: 49-57) can be used to incorporate iodine-123. "Monoclonal Antibodies in Immunoscintigraphy" (Chatal,CRC Press 1989) describes other methods in detail.

Conjugates of the antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., *Science* 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or

disulfide-containing linker (Chari et al., *Cancer Research* 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

The compounds of the invention expressly contemplate, but are not limited to, ADC prepared with cross-linker reagents: BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL., U.S.A). See pages 467-498, 2003-2004 Applications Handbook and Catalog.

v. Preparation of antibody drug conjugates

In the antibody drug conjugates (ADC) of the invention, an antibody (Ab) is conjugated to one or more drug moieties (D), e.g. about 1 to about 20 drug moieties per antibody, through a linker (L). The ADC of Formula I may be prepared by several routes, employing organic chemistry reactions, conditions, and reagents known to those skilled in the art, including: (1) reaction of a nucleophilic group of an antibody with a bivalent linker reagent, to form Ab-L, via a covalent bond, followed by reaction with a drug moiety D; and (2) reaction of a nucleophilic group of a drug moiety with a bivalent linker reagent, to form D-L, via a covalent bond, followed by reaction with the nucleophilic group of an antibody. Additional methods for preparing ADC are described herein.

$$Ab–(L–D)_p \qquad I$$

The linker may be composed of one or more linker components. Exemplary linker components include 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), valine-citrulline ("val-cit"), alanine-phenylalanine ("ala-phe"), p-aminobenzyloxycarbonyl ("PAB"), N-Succinimidyl 4-(2-pyridylthio) pentanoate ("SPP"), N-Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1 carboxylate ("SMCC'), and N-Succinimidyl (4-iodo-acetyl) aminobenzoate ("SIAB"). Additional linker components are known in the art and some are described herein. See also "Monomethylvaline Compounds Capable of Conjugation to Ligands", US Ser. No. 10/983,340, filed Nov. 5, 2004, the contents of which are hereby incorporated by reference in its entirety.

In some embodiments, the linker may comprise amino acid residues. Exemplary amino acid linker components include a dipeptide, a tripeptide, a tetrapeptide or a pentapeptide. Exemplary dipeptides include: valine-citrulline (vc or val-cit), alanine-phenylalanine (af or ala-phe). Exemplary tripeptides include: glycine-valine-citrulline

(gly-val-cit) and glycine-glycine-glycine (gly-gly-gly). Amino acid residues which comprise an amino acid linker component include those occurring naturally, as well as minor amino acids and non-naturally occurring amino acid analogs, such as citrulline. Amino acid linker components can be designed and optimized in their selectivity for enzymatic cleavage by a particular enzymes, for example, a tumor-associated protease, cathepsin B, C and D, or a plasmin protease.

Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, e.g. lysine, (iii) side chain thiol groups, e.g. cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, i.e. cysteine bridges. Antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol). Each cysteine bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through the reaction of lysines with 2-iminothiolane (Traut's reagent) resulting in conversion of an amine into a thiol. Reactive thiol groups may be introduced into the antibody (or fragment thereof) by introducing one, two, three, four, or more cysteine residues (e.g., preparing mutant antibodies comprising one or more non-native cysteine amino acid residues).

Antibody drug conjugates of the invention may also be produced by modification of the antibody to introduce electrophilic moieties, which can react with nucleophilic substituents on the linker reagent or drug. The sugars of glycosylated antibodies may be oxidized, e.g. with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or drug moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, e.g. by borohydride reagents to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either glactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the protein that can react with appropriate groups on the drug (Hermanson, Bioconjugate Techniques). In another embodiment, proteins containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino

acid (Geoghegan & Stroh, (1992) *Bioconjugate Chem.* 3:138-146; US 5362852). Such aldehyde can be reacted with a drug moiety or linker nucleophile.

Likewise, nucleophilic groups on a drug moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

Alternatively, a fusion protein comprising the antibody and cytotoxic agent may be made, e.g., by recombinant techniques or peptide synthesis. The length of DNA may comprise respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

In yet another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pre-targeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (e.g., avidin) which is conjugated to a cytotoxic agent (e.g., a radionucleotide).

Pharmaceutical Formulations

Therapeutic formulations comprising an antibody of the invention are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington: The Science and Practice of Pharmacy* 20th edition (2000)), in the form of aqueous solutions, lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, histidine and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as

EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington: The Science and Practice of Pharmacy* 20th edition (2000).

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

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the immunoglobulin of the invention, which matrices are in the form of shaped articles, *e.g.,* films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated immunoglobulins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying

sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Uses

An antibody of the present invention may be used in, for example, *in vitro, ex vivo* and *in vivo* therapeutic methods.

In one aspect, the invention provides methods for treating or preventing a tumor, a cancer, and/or a cell proliferative disorder associated with increased expression and/or activity of DLL4, the methods comprising administering an effective amount of an anti-DLL4 antibody to a subject in need of such treatment.

In one aspect, the invention provides methods for reducing, inhibiting, blocking, or preventing growth of a tumor or cancer, the methods comprising administering an effective amount of an anti-DLL4 antibody to a subject in need of such treatment.

In one aspect, the invention provides methods for treating a tumor, a cancer, and/or a cell proliferative disorder comprising administering an effective amount of an anti-DLL4 antibody to a subject in need of such treatment.

In one aspect, the invention provides methods for inhibiting angiogenesis comprising administering an effective amount of an anti-DLL4 antibody to a subject in need of such treatment.

In one aspect, the invention provides methods for treating a pathological condition associated with angiogenesis comprising administering an effective amount of an anti-DLL4 antibody to a subject in need of such treatment. In some embodiments, the pathological condition associated with angiogenesis is a tumor, a cancer, and/or a cell proliferative disorder. In some embodiments, the pathological condition associated with angiogenesis is an intraocular neovascular disease.

Moreover, at least some of the antibodies of the invention can bind antigen from other species. Accordingly, the antibodies of the invention can be used to bind specific antigen activity, e.g., in a cell culture containing the antigen, in human subjects or in other mammalian subjects having the antigen with which an antibody of the invention cross-reacts (e.g. chimpanzee, baboon, marmoset, cynomolgus and rhesus, pig or mouse). In one embodiment, the antibody of the invention can be used for inhibiting antigen activities by contacting the antibody with the antigen such that antigen activity is inhibited. Preferably, the antigen is a human protein molecule.

In one embodiment, an antibody of the invention can be used in a method for binding an antigen in a subject suffering from a disorder associated with increased antigen expression and/or activity, comprising administering to the subject an antibody of the invention such that the antigen in the subject is bound. Preferably, the antigen is a human protein molecule and the subject is a human subject. Alternatively, the subject can be a mammal expressing the antigen with which an antibody of the invention binds. Still further the subject can be a mammal into which the antigen has been introduced (e.g., by administration of the antigen or by expression of an antigen transgene). An antibody of the invention can be administered to a human subject for therapeutic purposes. Moreover, an antibody of the invention can be administered to a non-human mammal expressing an antigen with which the immunoglobulin cross-reacts (e.g., a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (e.g., testing of dosages and time courses of administration).

The antibodies of the invention can be used to treat, inhibit, delay progression of, prevent/delay recurrence of, ameliorate, or prevent diseases, disorders or conditions associated with expression and/or activity of one or more antigen molecules.

Exemplary disorders include carcinoma, lymphoma, blastoma, sarcoma, and leukemia or lymphoid malignancies. More particular examples of such cancers include squamous cell cancer (e.g., epithelial squamous cell cancer), lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, cancer of the urinary tract, hepatoma, breast cancer, colon cancer, rectal cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney or renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, anal carcinoma, penile carcinoma, melanoma, multiple myeloma and B-cell lymphoma, brain, as well as head and neck cancer, and associated metastases. In some embodiments, the cancer is selected from the group consisting of small cell lung cancer, neuroblastomas, melanoma, breast carcinoma, gastric cancer, colorectal cancer (CRC), and hepatocellular carcinoma. In some embodiments, the cancer is selected from the group consisting of non-small cell lung cancer, colorectal cancer and breast carcinoma, including metastatic forms of those cancers.

In certain embodiments, an immunoconjugate comprising an antibody conjugated with one or more cytotoxic agent(s) is administered to the patient. In some embodiments, the immunoconjugate and/or antigen to which it is bound is/are internalized by the cell, resulting in increased therapeutic efficacy of the immunoconjugate in killing the target cell to which it binds. In one embodiment, the cytotoxic agent targets or interferes with nucleic acid in the target cell. In one embodiment, the cytotoxic agent targets or interferes with microtubule polymerization. Examples of such cytotoxic agents include any of the chemotherapeutic agents noted herein (such as a maytansinoid, auristatin, dolastatin, or a calicheamicin), a radioactive isotope, or a ribonuclease or a DNA endonuclease.

Antibodies of the invention can be used either alone or in combination with other compositions in a therapy. For instance, an antibody of the invention may be co-administered with another antibody, chemotherapeutic agent(s) (including cocktails of chemotherapeutic agents), other cytotoxic agent(s), anti-angiogenic agent(s), cytokines, and/or growth inhibitory agent(s). Where an antibody of the invention inhibits tumor growth, it may be particularly desirable to combine it with one or more other therapeutic agent(s) which also inhibits tumor growth, e.g. anti-VEGF agents including antibodies to VEGF. Alternatively, or additionally, the patient may receive combined radiation therapy (e.g. external beam irradiation or therapy with a radioactive labeled agent, such as an antibody). Such combined therapies noted above include combined administration (where the two or more agents are included in the same or separate formulations), and separate administration, in which case, administration of the antibody of the invention can occur prior to, and/or following, administration of the adjunct therapy or therapies.

Combination Therapies

As indicated above, the invention provides combined therapies in which an anti-DLL4 antibody is administered with another therapy. For example, anti-DLL4 antibodies are used in combinations with anti-cancer therapeutics or anti-neovascularization therapeutics to treat various neoplastic or non-neoplastic conditions. In one embodiment, the neoplastic or non-neoplastic condition is characterized by pathological disorder associated with aberrant or undesired angiogenesis. The anti-DLL4 antibody can be administered serially or in combination with another agent that is effective for those purposes, either in the same composition or as separate compositions. Alternatively, or additionally, multiple inhibitors of DLL4 can be administered.

The administration of the anti-DLL4 antibody can be done simultaneously, e.g., as a single composition or as two or more distinct compositions using the same or different

administration routes. Alternatively, or additionally, the administration can be done sequentially, in any order. In certain embodiments, intervals ranging from minutes to days, to weeks to months, can be present between the administrations of the two or more compositions. For example, the anti-cancer agent may be administered first, followed by the DLL4 inhibitor. However, simultaneous administration or administration of the anti-DLL4 antibody first is also contemplated.

The effective amounts of therapeutic agents administered in combination with an anti-DLL4 antibody will be at the physician's or veterinarian's discretion. Dosage administration and adjustment is done to achieve maximal management of the conditions to be treated. The dose will additionally depend on such factors as the type of therapeutic agent to be used and the specific patient being treated. Suitable dosages for the anti-cancer agent are those presently used and can be lowered due to the combined action (synergy) of the anti-cancer agent and the anti-DLL4 antibody. In certain embodiments, the combination of the inhibitors potentiates the efficacy of a single inhibitor. The term "potentiate" refers to an improvement in the efficacy of a therapeutic agent at its common or approved dose. See also the section entitled Pharmaceutical Compositions herein.

Typically, the anti-DLL4 antibodies and anti-cancer agents are suitable for the same or similar diseases to block or reduce a pathological disorder such as tumor growth or growth of a cancer cell. In one embodiment the anti-cancer agent is an anti-angiogenesis agent.

Antiangiogenic therapy in relationship to cancer is a cancer treatment strategy aimed at inhibiting the development of tumor blood vessels required for providing nutrients to support tumor growth. Because angiogenesis is involved in both primary tumor growth and metastasis, the antiangiogenic treatment provided by the invention is capable of inhibiting the neoplastic growth of tumor at the primary site as well as preventing metastasis of tumors at the secondary sites, therefore allowing attack of the tumors by other therapeutics.

Many anti-angiogenic agents have been identified and are known in the arts, including those listed herein, e.g., listed under Definitions, and by, e.g., Carmeliet and Jain, *Nature* 407:249-257 (2000); Ferrara et al., *Nature Reviews:Drug Discovery*, 3:391-400 (2004); and Sato *Int. J. Clin. Oncol.*, 8:200-206 (2003). See also, US Patent Application US20030055006. In one embodiment, an anti-DLL4 antibody is used in combination with an anti-VEGF neutralizing antibody (or fragment) and/or another VEGF antagonist or a VEGF receptor antagonist including, but not limited to, for example, soluble VEGF

receptor (e.g., VEGFR-1, VEGFR-2, VEGFR-3, neuropillins (e.g., NRP1, NRP2)) fragments, aptamers capable of blocking VEGF or VEGFR, neutralizing anti-VEGFR antibodies, low molecule weight inhibitors of VEGFR tyrosine kinases (RTK), antisense strategies for VEGF, ribozymes against VEGF or VEGF receptors, antagonist variants of VEGF; and any combinations thereof. Alternatively, or additionally, two or more angiogenesis inhibitors may optionally be co-administered to the patient in addition to VEGF antagonist and other agent. In certain embodiment, one or more additional therapeutic agents, e.g., anti-cancer agents, can be administered in combination with anti-DLL4 antibody, the VEGF antagonist, and an anti-angiogenesis agent.

In certain aspects of the invention, other therapeutic agents useful for combination tumor therapy with a anti-DLL4 antibody include other cancer therapies, (e.g., surgery, radiological treatments (e.g., involving irradiation or administration of radioactive substances), chemotherapy, treatment with anti-cancer agents listed herein and known in the art, or combinations thereof). Alternatively, or additionally, two or more antibodies binding the same or two or more different antigens disclosed herein can be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient.

Chemotherapeutic Agents

In certain aspects, the invention provides a method of blocking or reducing tumor growth or growth of a cancer cell, by administering effective amounts of an antagonist of DLL4 and/or an angiogenesis inhibitor(s) and one or more chemotherapeutic agents to a patient susceptible to, or diagnosed with, cancer. A variety of chemotherapeutic agents may be used in the combined treatment methods of the invention. An exemplary and non-limiting list of chemotherapeutic agents contemplated is provided herein under "Definitions."

As will be understood by those of ordinary skill in the art, the appropriate doses of chemotherapeutic agents will be generally around those already employed in clinical therapies wherein the chemotherapeutics are administered alone or in combination with other chemotherapeutics. Variation in dosage will likely occur depending on the condition being treated. The physician administering treatment will be able to determine the appropriate dose for the individual subject.

The invention also provides methods and compositions for inhibiting or preventing relapse tumor growth or relapse cancer cell growth. Relapse tumor growth or relapse cancer cell growth is used to describe a condition in which patients undergoing or treated

with one or more currently available therapies (e.g., cancer therapies, such as chemotherapy, radiation therapy, surgery, hormonal therapy and/or biological therapy/immunotherapy, anti-VEGF antibody therapy, particularly a standard therapeutic regimen for the particular cancer) is not clinically adequate to treat the patients or the patients are no longer receiving any beneficial effect from the therapy such that these patients need additional effective therapy. As used herein, the phrase can also refer to a condition of the "non-responsive/refractory" patient, e.g., which describe patients who respond to therapy yet suffer from side effects, develop resistance, do not respond to the therapy, do not respond satisfactorily to the therapy, etc. In various embodiments, a cancer is relapse tumor growth or relapse cancer cell growth where the number of cancer cells has not been significantly reduced, or has increased, or tumor size has not been significantly reduced, or has increased, or fails any further reduction in size or in number of cancer cells. The determination of whether the cancer cells are relapse tumor growth or relapse cancer cell growth can be made either *in vivo* or *in vitro* by any method known in the art for assaying the effectiveness of treatment on cancer cells, using the art-accepted meanings of "relapse" or "refractory" or "non-responsive" in such a context. A tumor resistant to anti-VEGF treatment is an example of a relapse tumor growth.

The invention provides methods of blocking or reducing relapse tumor growth or relapse cancer cell growth in a subject by administering one or more anti-DLL4 antibodies to block or reduce the relapse tumor growth or relapse cancer cell growth in subject. In certain embodiments, the antagonist can be administered subsequent to the cancer therapeutic. In certain embodiments, the anti-DLL4 antibodies are administered simultaneously with cancer therapy. Alternatively, or additionally, the anti-DLL4 antibody therapy alternates with another cancer therapy, which can be performed in any order. The invention also encompasses methods for administering one or more inhibitory antibodies to prevent the onset or recurrence of cancer in patients predisposed to having cancer. Generally, the subject was or is concurrently undergoing cancer therapy. In one embodiment, the cancer therapy is treatment with an anti-angiogenesis agent, e.g., a VEGF antagonist. The anti-angiogenesis agent includes those known in the art and those found under the Definitions herein. In one embodiment, the anti-angiogenesis agent is an anti-VEGF neutralizing antibody or fragment (e.g. AVASTIN® (Genentech, South San Francisco, CA) or LUCENTIS® (Genentech, South San Francisco, CA)), Y0317, M4, G6, B20, 2C3, etc.). See, e.g., U.S. Patents 6,582,959, 6,884,879, 6,703,020; WO98/45332; WO 96/30046; WO94/10202; EP 0666868B1; US Patent Applications 20030206899,

20030190317, 20030203409, and 20050112126; Popkov et al., *Journal of Immunological Methods* 288:149-164 (2004); and, WO2005012359. Additional agents can be administered in combination with VEGF antagonist and an anti-DLL4 antibody for blocking or reducing relapse tumor growth or relapse cancer cell growth, e.g., see section entitled Combination Therapies herein.

The antibody of the invention (and adjunct therapeutic agent) is/are administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional or intravitreal administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antibody is suitably administered by pulse infusion, particularly with declining doses of the antibody. Dosing can be by any suitable route, e.g. by injections, such as intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

The antibody composition of the invention will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. The effective amount of such other agents depends on the amount of antibodies of the invention present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

For the prevention or treatment of disease, the appropriate dosage of an antibody of the invention (when used alone or in combination with other agents such as chemotherapeutic agents) will depend on the type of disease to be treated, the type of antibody, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 μg/kg to 15 mg/kg (*e.g.* 0.1mg/kg-10mg/kg) of antibody is an initial candidate dosage for administration to the patient,

whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 μg/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. One exemplary dosage of the antibody would be in the range from about 0.05mg/kg to about 10mg/kg. Thus, one or more doses of about 0.5mg/kg, 2.0mg/kg, 4.0mg/kg or 10mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, *e.g.* every week or every three weeks (*e.g.* such that the patient receives from about two to about twenty, *e.g.* about six doses of the antibody). An initial higher loading dose, followed by one or more lower doses may be administered. An exemplary dosing regimen comprises administering an initial loading dose of about 4 mg/kg, followed by a weekly maintenance dose of about 2 mg/kg of the antibody. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

The anti-DLL4 antibodies of the invention are useful in assays detecting DLL4 expression (such as diagnostic or prognostic assays) in specific cells or tissues wherein the antibodies are labeled as described below and/or are immobilized on an insoluble matrix.

In another aspect, the invention provides methods for detection of DLL4, the methods comprising detecting DLL4-anti-DLL4 antibody complex in the sample. The term "detection" as used herein includes qualitative and/or quantitative detection (measuring levels) with or without reference to a control.

In another aspect, the invention provides methods for diagnosing a disorder associated with DLL4 expression and/or activity, the methods comprising detecting DLL4-anti-DLL4 antibody complex in a biological sample from a patient having or suspected of having the disorder. In some embodiments, the DLL4 expression is increased expression or abnormal (undesired) expression. In some embodiments, the disorder is a tumor, cancer, and/or a cell proliferative disorder.

In another aspect, the invention provides any of the anti-DLL4 antibodies described herein, wherein the anti-DLL4 antibody comprises a detectable label.

In another aspect, the invention provides a complex of any of the anti-DLL4 antibodies described herein and DLL4. In some embodiments, the complex is *in vivo* or *in vitro*. In some embodiments, the complex comprises a cancer cell. In some embodiments, the anti-DLL4 antibody is detectably labeled.

Anti-DLL4 antibodies can be used for the detection of DLL4 in any one of a number of well known detection assay methods. For example, a biological sample may be assayed for DLL4 by obtaining the sample from a desired source, admixing the sample with anti-DLL4 antibody to allow the antibody to form antibody/ DLL4 complex with any DLL4 present in the mixture, and detecting any antibody/ DLL4 complex present in the mixture. The biological sample may be prepared for assay by methods known in the art which are suitable for the particular sample. The methods of admixing the sample with antibodies and the methods of detecting antibody/ DLL4 complex are chosen according to the type of assay used. Such assays include immunohistochemistry, competitive and sandwich assays, and steric inhibition assays.

Analytical methods for DLL4 all use one or more of the following reagents: labeled DLL4 analogue, immobilized DLL4 analogue, labeled anti-DLL4 antibody, immobilized anti-DLL4 antibody and steric conjugates. The labeled reagents also are known as "tracers."

The label used is any detectable functionality that does not interfere with the binding of DLL4 and anti-DLL4 antibody. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include:

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Conventional methods are available to bind these labels covalently to proteins or polypeptides. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. Nos. 3,940,475 (fluorimetry) and 3,645,090 (enzymes); Hunter *et al.*, *Nature*, **144**: 945 (1962); David *et al.*, *Biochemistry,* **13**: 1014-1021 (1974); Pain *et al.*, *J. Immunol. Methods*, **40**: 219-230 (1981); and Nygren, *J. Histochem. and Cytochem.,* **30**: 407-412 (1982). Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase. The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for example, O'Sullivan *et al.*, "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in *Methods in Enzymology*, ed. J.J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, New York, 1981), pp. 147-166.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the anti-DLL4 antibody from any DLL4 that remains free in solution. This conventionally is accomplished by either insolubilizing the anti-DLL4 antibody or DLL4 analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich *et al..*, U.S. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the anti-DLL4 antibody or DLL4 analogue afterward, e.g., by immunoprecipitation.

The expression of proteins in a sample may be examined using immunohistochemistry and staining protocols. Immunohistochemical staining of tissue sections has been shown to be a reliable method of assessing or detecting presence of proteins in a sample. Immunohistochemistry ("IHC") techniques utilize an antibody to probe and visualize cellular antigens *in situ*, generally by chromogenic or fluorescent methods. For sample preparation, a tissue or cell sample from a mammal (typically a human patient) may be used. Examples of samples include, but are not limited to, cancer cells such as colon, breast, prostate, ovary, lung, stomach, pancreas, lymphoma, and leukemia cancer cells. The sample can be obtained by a variety of procedures known in the art including, but not limited to surgical excision, aspiration or biopsy. The tissue may be fresh or frozen. In one embodiment, the sample is fixed and embedded in paraffin or the like. The tissue sample may be fixed (*i.e.* preserved) by conventional methodology. One of ordinary skill in the art will appreciate that the choice of a fixative is determined by

the purpose for which the sample is to be histologically stained or otherwise analyzed. One of ordinary skill in the art will also appreciate that the length of fixation depends upon the size of the tissue sample and the fixative used.

IHC may be performed in combination with additional techniques such as morphological staining and/or fluorescence *in-situ* hybridization. Two general methods of IHC are available; direct and indirect assays. According to the first assay, binding of antibody to the target antigen (e.g., DLL4) is determined directly. This direct assay uses a labeled reagent, such as a fluorescent tag or an enzyme-labeled primary antibody, which can be visualized without further antibody interaction. In a typical indirect assay, unconjugated primary antibody binds to the antigen and then a labeled secondary antibody binds to the primary antibody. Where the secondary antibody is conjugated to an enzymatic label, a chromogenic or fluorogenic substrate is added to provide visualization of the antigen. Signal amplification occurs because several secondary antibodies may react with different epitopes on the primary antibody.

The primary and/or secondary antibody used for immunohistochemistry typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

Aside from the sample preparation procedures discussed above, further treatment of the tissue section prior to, during or following IHC may be desired, For example, epitope retrieval methods, such as heating the tissue sample in citrate buffer may be carried out (see, *e.g.*, Leong *et al. Appl. Immunohistochem.* 4(3):201 (1996)).

Following an optional blocking step, the tissue section is exposed to primary antibody for a sufficient period of time and under suitable conditions such that the primary antibody binds to the target protein antigen in the tissue sample. Appropriate conditions for achieving this can be determined by routine experimentation. The extent of binding of antibody to the sample is determined by using any one of the detectable labels discussed above. Preferably, the label is an enzymatic label (*e.g.* HRPO) which catalyzes a chemical alteration of the chromogenic substrate such as 3,3'-diaminobenzidine chromogen. Preferably the enzymatic label is conjugated to antibody which binds specifically to the primary antibody (*e.g.* the primary antibody is rabbit polyclonal antibody and secondary antibody is goat anti-rabbit antibody).

Specimens thus prepared may be mounted and coverslipped. Slide evaluation is then determined, e.g. using a microscope, and staining intensity criteria, routinely used in the art, may be employed. Staining intensity criteria may be evaluated as follows:

TABLE 2

Staining Pattern	Score
No staining is observed in cells.	0
Faint/barely perceptible staining is detected in more than 10% of the cells.	1+
Weak to moderate staining is observed in more than 10% of the cells.	2+
Moderate to strong staining is observed in more than 10% of the cells.	3+

Typically, a staining pattern score of about 2+ or higher in an IHC assay is diagnostic and/or prognostic. In some embodiments, a staining pattern score of about 1+ or higher is diagnostic and/or prognostic. In other embodiments, a staining pattern score of about 3 of higher is diagnostic and/or prognostic. It is understood that when cells and/or tissue from a tumor or colon adenoma are examined using IHC, staining is generally determined or assessed in tumor cell and/or tissue (as opposed to stromal or surrounding tissue that may be present in the sample).

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

Competitive assays rely on the ability of a tracer DLL4 analogue to compete with the test sample DLL4 for a limited number of anti-DLL4 antibody antigen-binding sites. The anti-DLL4 antibody generally is insolubilized before or after the competition and then the tracer and DLL4 bound to the anti-DLL4 antibody are separated from the unbound tracer and DLL4. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample DLL4 is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts of DLL4 are prepared and compared with the test results to quantitatively determine the amount of DLL4 present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the DLL4 is prepared and used such that when anti-DLL4 antibody binds to the DLL4 the presence of the anti-DLL4 antibody modifies the enzyme activity. In this case, the DLL4 or its immunologically

active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with anti-DLL4 antibody so that binding of the anti-DLL4 antibody inhibits or potentiates the enzyme activity of the label. This method *per se* is widely practiced under the name of EMIT.

Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small DLL4 fragment so that antibody to hapten is substantially unable to bind the conjugate at the same time as anti-DLL4 antibody. Under this assay procedure the DLL4 present in the test sample will bind anti-DLL4 antibody, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g., a change in fluorescence when the hapten is a fluorophore.

Sandwich assays particularly are useful for the determination of DLL4 or anti-DLL4 antibodies. In sequential sandwich assays an immobilized anti-DLL4 antibody is used to adsorb test sample DLL4, the test sample is removed as by washing, the bound DLL4 is used to adsorb a second, labeled anti-DLL4 antibody and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample DLL4. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled anti-DLL4. A sequential sandwich assay using an anti-DLL4 monoclonal antibody as one antibody and a polyclonal anti-DLL4 antibody as the other is useful in testing samples for DLL4.

The foregoing are merely exemplary detection assays for DLL4. Other methods now or hereafter developed that use anti-DLL4 antibody for the determination of DLL4 are included within the scope hereof, including the bioassays described herein.

Articles of Manufacture

In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or when combined with another composition(s) effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody of the invention. The label or

package insert indicates that the composition is used for treating the condition of choice, such as cancer. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises an antibody of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further therapeutic agent, including, e.g. a chemotherapeutic agent or an anti-angiogenesis agent, including, e.g., an anti-VEGF antibody (e.g. bevacizumab). The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the first and second antibody compositions can be used to treat a particular condition, e.g. cancer. Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

The following are examples of the methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

EXAMPLES

Commercially available reagents referred to in the Examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following Examples, and throughout the specification, by ATCC® accession numbers is the American Type Culture Collection, Manassas, VA 20108. References cited in the Examples are listed following the examples. All references cited herein are hereby incorporated by reference.

Example 1: Materials and methods

The following materials and methods were used in the Examples.

HUVEC fibrin gel bead assay. Details of the HUVEC fibrin gel bead assay have been described (Nakatsu, M. N. et al. *Microvasc Res* 66, 102-12 (2003)). Briefly, Cytodex™ 3 beads (Amersham Pharmacia Biotech) were coated with 350-400 HUVECs per bead. About 200 HUVEC-coated beads were imbedded in fibrin clot in one well of 12-well tissue culture plate. 8X 10^4 SF cells were plated on top of the clot. Assays were terminated between day 7 and day 9 for immunostaining and imaging. In some

experiments, HUVEC sprouts were visualized by staining with Biotin-anti-CD31 (clone WM59, eBioscience) and strepavidin-Cy3. For HUVEC nuclei staining, fibrin gels were fixed overnight in 2% paraformaldehyde (PFA), and stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma). For Ki67 staining, fibrin gels were treated with 10X trypsin-EDTA for 5 min to remove the top layer SF, neutralized with 10% FBS in PBS, and fixed overnight in 4% PFA. Fibrin gels were then blocked with 10% goat serum in PBST for 4 hr, incubated overnight with rabbit anti-mouse Ki67 (Ready-To-Use, clone Sp6, LabVision), followed by secondary detection with anti-rabbit IgG-Cy3 (Jackson ImmunoResearch). All overnight incubations were done at 4°C.

Mouse neonatal retina study. Neonatal CD1 mice from the same litters were injected i.p. with PBS or YW26.82 (10 mg/kg) on P1 and P3. Eyes were collected on P5, and fixed with 4% PFA in PBS overnight. The dissected retinas were blocked with 10% goat serum in PBST for 3 hrs, then incubated overnight with primary antibodies. The primary cocktail included biotinylated isolectin B4 (25 μg/ml, *Bandeiraea simplicifolia*; Sigma), and one of the following: rabbit anti-mouse Ki67 (1:1, ready-to-use, clone Sp6, Lab Vision), or mouse Cy3-conjugated anti-alpha SMA (1:2000, Sigma-Aldrich), with 10% serum in PBLEC (1% Triton X-100, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM $MnCl_2$, in PBS pH6.8). Retinas were then washed in PBST, and incubated overnight with secondary antibody combination of Alexa Fluor® 488 streptavidin (1:200; Molecular Probes) and Cy3-anti-rabbit IgG (1:200; Jackson ImmunoResearch). After staining was completed, retinas were post fixed with 4% PFA in PBS. All overnight incubations were done at 4°C. Images of flat mounted retinas were captured by confocal fluorescence microscopy.

Tumor models. Beige nude female mice of 8- to 10-week-old were used. To obtain subcutaneous tumors, mice were injected with 0.1 ml cell suspension containing 50% matrigel (BD Bioscience) into the right posterior flank. $5X10^6$ human colon cancer HM7 cells, $10X10^6$ human colon carcinoma Colo205 cells, $10X10^6$ human lung carcinoma Calu6 cells, $10X10^6$ human lung carcinoma MV-522 cells, $10X10^6$ mouse leukemia WEHI-3 cells, $10X10^6$ mouse lymphoma EL4 cells, $10X10^6$ human ovarian cancer SK-OV-3 X1 cells, $10X10^6$ mouse lung cancer LL2 cells, $10X10^6$ leukemia/lymphoma EL4 cells, or $10X10^6$ non-small lung cancer H1299 cells were injected into each mouse. For human melanoma MDA-MB-435 model, mice were injected into the mammary fat pad with 0.1 ml cell ($5X10^6$) suspension containing 50% matrigel. Anti-DLL4 antibody YW26.82 was administered via i.p. (10 mg/kg body weight, twice weekly). For the

following tumor models, each test mouse received a subcutaneous tumor fragment (1 mm^3) implanted in the right flank: non-small lung cancer SKMES-1, human breast cancer MX-1, human colorectal cancer SW620 and human adenocarcinoma LS174T. The tumor growth was quantitated by caliper measurements. Tumor volume (mm^3) was determined by measuring the length (l) and width (w) and calculating the volume ($V = lw2/2$). 10 to 15 animals were included in each group. Statistical comparison of treatment groups was performed using two-tailed Student's t-test.

Tumor vascular labeling and immunohistochemistry. Mice were anesthetized with Isoflurane. FITC-labeled *Lycopersicon esculentum* Lectin (150 μg in 150 μl of 0.9% NaCl; Vector Laboratories) was injected i.v. and allowed to circulate for 5 min before systemic perfusion. The vasculature was perfused transcardially with 1% PFA in PBS for 3 min. Tumors were removed and post fixed by immersion in the same fixative for 2 hr, followed by an incubation in 30% sucrose overnight for cryoprotection, then embedded in OCT. Sections (4μm thickness) were stained with anti-mouse CD31 (1:50, BD Pharmingen), followed by Alexa 594 goat anti-rat IgG (1:800, Molecular Probes).

Histology and immunohistochemistry of mouse intestines. Formalin-fixed and paraffin-embedded mouse small intestine tissues were sectioned at 3 μm thickness. Histochemical identification of intestinal cell types was performed with Alcian blue as recommended by the manufacturer (PolyScientific). For anti-Ki67 staining, sections were pretreated with Target Retrieval Solution (S1700, DAKO), and incubated with rabbit anti-Ki67 (1:200, clone SP6, Neomarkers). Secondary goat anti-rabbit at 7.5g/ml (Vector labs) was detected with the Vectastain ABC Elite Kit (Vector labs). All Ki67 stained sections were counterstained with Mayer's hematoxylin. For HES-1 staining, anti-rat HES-1 (clone NM1, MBL, International), followed by TSA-HRP, was used.

RNA interference. SMARTpool small interfering RNA (siRNA) duplexes targeting human DLL4 and Si Control Non-Target siRNA #2 were purchased from Dharmacon. Transfection of siRNR duplexes (50 nM) was done with HUVECs at 40% confluency using OptiMEM-1® and Lipfectamine™ 2000 (Invitrogen). FACS analysis was done 48 hr after siRNA transfection. The sequences of the 4 anti-DLL4 SMART pool siRNA were as follows:

CAACTGCCCTTATGGCTTTTT (SEQ ID NO: 62) (Oligo 1, sense),
AAAGCCATAAGGGCAGTTGTT (SEQ ID NO: 63) (Oligo 1, antisense),
CAACTGCCCTTCAATTTCATT (SEQ ID NO: 64) (Oligo 2, sense),
TGAAATTGAAGGGCAGTTGTT (SEQ ID NO: 65) (Oligo 2, antisense),

TGACCAAGATCTCAACTACTT (SEQ ID NO: 66) (Oligo 3, sense),
GTAGTTGAGATCTTGGTCATT (SEQ ID NO: 67) (Oligo 3, antisense),
GGCCAACTATGCTTGTGAATT (SEQ ID NO: 68) (Oligo 4, sense),
TTCACAAGCATAGTTGGCCTT (SEQ ID NO: 69) (Oligo 4, antisense).

Notch ligand : Notch blocking ELISA. 96-well microtiter plates were coated with recombinant rat Notch1-Fc (rrNotch1-Fc, R&D Systems) at 0.5 μg/ml. Conditioned medium containing DLL4-AP (amino acid 1-404 of DLL4 fused to human placenta alkaline phosphatase) was used in the assay. To prepare conditioned medium, 293 cells were transiently transfected with plasmid expressing DLL4-AP with Fugen6 reagent (Roche Molecular Biochemicals). Five days posttransfection, the conditioned medium was harvested, filtered and stored at 4°C. Purified antibodies titrated from 0.15 to 25 μg/ml were preincubated for 1hr at room temperature with DLL4-AP conditioned medium at a dilution that conferred 50% maximally achievable binding to coated rrNotch1-Fc. The antibody/DLL4-AP mixture was then added to rrNotch1-Fc coated plate for 1hr at room temperature, after which plates were washed several times in PBS. The bound DLL4-AP was detected using 1-Step PNPP (Pierce) as substrate and OD 405 nm absorbance measurement. Identical assay was performed with DLL1-AP (human DLL1, amino acid 1-445). Similar assays were carried out with purified DLL4-His (C-terminal His-tagged human DLL4, amino acid 1-404) and Jag1-His (R& D system). The bound His-tagged ligands was detected with mouse anti-His mAb (1 μg/ml, Roche Molecular Biochemicals), biotinylated goat-anti-mouse (Jackson ImmunoResearch) and Streptavidin-AP (Jackson ImmunoResearch).

RNA extraction and Real-time quantitative RT-PCR. Extraction of total RNA from HUVECs in 2-D culture was done using RNeasy® Mini Kit (Qiagen) as per instructions of the manufacturer. To extract total RNA from HUVECs growing in fibrin gels, fibrin gels were treated with 10X trypsin-EDTA (Gibco) for 5 min to remove the top layer fibroblasts, followed by neutralization with 10% FBS in PBS. The gel clots were then removed from tissue culture wells and subjected to centrifugation (10K for 5 min) in microtubes to remove excessive fluid. The resulting gel "pellets" were lysed with lysis buffer (RNeasy® Mini Kit), and further processed as with HUVECs in 2-D culture. The quality of RNA was assessed using RNA 6000 Nano Chips and the Agilent 2100 Bioanalyzer (Agilent Technologies). Real-time quantitative RT-PCR reactions were done in triplicate using 7500 Real Time PCR System (Applied Biosystems). Human GAPDH was used as reference gene for normalization. The expression levels are expressed as the

mean (±SEM) fold mRNA changes relative to control from 3 separate determinations. The forward and reverse primer and probe sequences for VEGFR2, TGFβ2 and GAPDH were as follows.

TGFb2

Forward: GTA AAG TCT TGC AAA TGC AGC TA (SEQ ID NO: 70)

Reverse: CAT CAT CAT TAT CAT CAT CAT TGT C (SEQ ID NO: 71)

Probe: AAT TCT TGG AAA AGT GGC AAG ACC AAA AT (SEQ ID NO: 72)

VEGFR2

Forward: CTT TCC ACC AGC AGG AAG TAG (SEQ ID NO: 73)

Reverse: TGC AGT CCG AGG TCC TTT (SEQ ID NO: 74)

Probe: CGC ATT TGA TTT TCA TTT CGA CAA CAG A (SEQ ID NO: 75)

GAPDH

Forward: GAA GAT GGT GAT GGG ATT TC (SEQ ID NO: 76)

Reverse: GAA GGT GAA GGT CGG AGT C (SEQ ID NO: 77)

Probe: CAA GCT TCC CGT TCT CAG CC (SEQ ID NO: 78)

Example 2: Generation of phage anti-DLL4 antibodies

Synthetic phage antibody libraries were built on a single framework (humanized anti-ErbB2 antibody, 4D5) by introducing diversity within the complementarity-determining regions (CDRs) of heavy and light chains (Lee, C. V. et al.*J Mol Biol* 340, 1073-93 (2004); Liang, W. C. et al. *J Biol Chem* 281, 951-61 (2006)). Plate panning with naïve libraries was performed against His-tagged human DLL4 (amino acid 1-404) immobilized on MaxiSorp™ immunoplates. After four rounds of enrichment, clones were randomly picked and specific binders were identified using phage ELISA. The resulting hDLL4 binding clones were further screened with His-tagged murine DLL4 protein to identify cross-species clones. For each positive phage clone, variable regions of heavy and light chains were subcloned into pRK expression vectors that were engineered to express full-length IgG chains. Heavy chain and light chain constructs were co-transfected into 293 or CHO cells, and the expressed antibodies were purified from serum-free medium using protein A affinity column. Purified antibodies were tested by ELISA for blocking the interaction between DLL4 and rat Notch1-Fc, and by FACS for binding to stable cell lines expressing either full-length human DLL4 or murine DLL4. For affinity maturation, phage libraries with three different combination of CDR loops (CDR-L3, -H1, and –H2) derived from the initial clone of interest were constructed by soft randomization strategy so that each selected position was mutated to a non-wild type residue or maintained as wild type at

about 50:50 frequency (Liang et al., 2006, above). High affinity clones were then identified through four rounds of solution phase panning against both human and murine His-tagged DLL4 proteins with progressively increased stringency.

Example 3: Characterization of anti-DLL4 antibodies

To determine the binding affinity of the anti-DLL4 Mabs, surface plasmon resonance (SRP) measurement with a BIAcore™-3000 was used (BIAcore, Inc., Piscataway, NJ). Briefly, carboxymethylated dextran biosensor chips (CM5, BIAcore Inc.) were activated with N-ethyl-N'- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Anti-DLL4 antibody was diluted with 10mM sodium acetate, pH 4.8, into 5μg/ml before injection at a flow rate of 5μl/minute to achieve approximately 500 response units (RU) of coupled antibody. Next, 1M ethanolamine was injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of either human or murine DLL4-His molecules (0.7nM to 500nM) were injected in PBS with 0.05% Tween™ 20 at 25°C at a flow rate of 25μl/min. Association rates (kon) and dissociation rates (koff) were calculated using a simple one-to-one Langmuir binding model (BIAcore Evaluation Software version 3.2). The equilibrium dissociation constant (Kd) was calculated as the ratio koff/kon. The results of this experiment are shown in Table 3. Antibody YW26.81 exhibited similar Kd values against human and murine DLL4 (Kd values of 0.1 nM5 and 0.09 nM, respectively), enabling its evaluation in mouse models.

TABLE 3: Binding affinity and kinetics of anti-DLL4 antibodies to human and mouse DLL4. "NA" signifies that the measurement was not performed.

Clone	murine Delta4 (ECD5.5)			human Delta4 (ECD5.5)		
	kon/10^5	koff10^{-4}	Kd(nM)	kon/10^5	koff/10^{-4}	Kd(nM)
YW26	0.57	16	28	0.3	7.4	25
YW26.6	4.1	0.61	0.15	2	0.49	0.25
YW26.14	3.3	0.73	0.22	1.8	0.66	0.37
YW26.20	3.3	1	0.3	1.7	0.79	0.46
YW26.34	3.6	0.65	0.18	1.9	0.67	0.35
YW26.82	5.4	0.46	0.09	2.4	0.37	0.15

Example 4: Further characterization of anti-DLL4 antibody

Epitope mapping of anti-DLL4 Mab YW26.82: Anti-DLL4 Mab 26.82 recognized a binding determinant present in EGF-like repeat number 2 (EGL2) of the human DLL4 extracellular domain (ECD). EGL2 comprises amino acids 252-282 of the human DLL4 ECD. Briefly, DLL4 ECD mutants were expressed as alkaline phosphatase fusion proteins and binding to antibody as assessed. Fig 11a depicts a schematic representation of a set of DLL4 mutants expressed as C-terminal human placental alkaline phosphatase (AP) fusion proteins. Parentheses indicate DLL4 sequences included in the fusion proteins. 293T cell conditioned media containing the fusion proteins were tested on 96-well microtiter plates coated with purified anti-DLL4 Mab (YW26.82, 0.5 μg/ml). The bound DLL4.AP was detected using 1-Step PNPP (Pierce) as substrate and OD 405 nm absorbance measurement. Mab YW26.82 bound constructs comprising the DLL4 EGL2 domain and did not bind a construct lacking the DLL4 EGL2 domain. This demonstrated that anti-DLL4 Mab YW 26.82 recognized an epitope in the EGL2 domain of the human DLL4 ECD.

Mab YW26.82 selectively binds to mouse and human DLL4. 96-well Nunc Maxisorp plates were coated with purified recombinant proteins as indicated (1 μg/ml). The binding of YW26.82 at indicated concentrations was measured by ELISA assay. Bound antibodies were detected with anti-human antibody HRP conjugate using TMB as substrate and OD 450 nm absorbance measurement. Anti-HER2 and recombinant ErbB2-ECD were used as assay control (Figure 11b). The results of this experiment are shown in Figure 11b. The Mab YW26.82 bound human and mouse DLL4, and did not detectably

bind to human DLL1 and human JAG1. These results demonstrated that Mab YW26.82 selectively binds to DLL4.

FACS analysis of 293 cells transiently transfected with vector, full length DLL4, Jag1 or DLL1 also demonstrated that Mab YW26.82 selectively bound to DLL4. As shown in Figure 11c, significant binding of YW26.82 was only detected on DLL4 transfected cells (top panel). Significant binding was not detected on DLL1 or Jag1 transfected cells. Expression of Jag1 and DLL1 was confirmed by the binding of recombinant rat Notch1-Fc (rrNotch1-Fc, middle panel) and recombinant rat Notch2-Fc (rrNotch2-Fc, bottom panel), respectively. YW26.82, rrNotch1-Fc or rrNotch2-Fc (R& D system) were used at 2 μg/ml followed by goat anti-human IgG-PE (1:500, Jackson ImmunoResearch).

Competition experiments demonstrated that Mab YW26.82 effectively and selectively blocked the interaction of Notch with DLL4, but not other Notch ligands. As shown in Figure 11d, anti-DLL4 Mab blocked the binding of DLL4-AP, but not DLL1-AP, to coated rNotch1, with a calculated IC50 of ~12 nM (left panel). Anti-DLL4 Mab blocked the binding of DLL4-His, but not Jag1-His, to coated rNotch1, with a calculated IC50 of ~8 nM (right panel).

Anti-DLL4 Mab YW26.82 specifically bound to endogenously expressed DLL4 in Human umbilical vein endothelial cells (HUVECs). FACS analysis of HUVECs transfected with control or DLL4-specific siRNA. YW26.82 was used at 2 μg/ml, followed by goat anti-human IgG-PE (1:500, Jackson ImmunoResearch). The results of this experiment are shown in Figure 11e. Binding was observed to untransfected HUVECs (control) and to HUVECs transfected with a control siRNA. By contrast, binding was significantly reduced in HUVECs transfected with DLL4 siRNA. These experiments demonstrated that anti-DLL4 Mab YW26.82 specifically binds to endogenously expressed DLL4 in HUVEC.

Example 5: Treatment with anti-DLL4 antibody increased endothelial cell proliferation *in vitro*

Human umbilical vein endothelial cells (HUVECs) growing in fibrin gels in the presence of co-cultured human skin fibroblast (SF) cells generate sprouts with a distinct lumen-like structure (Nakatsu, M. N. et al. *Microvasc Res* 66, 102-12 (2003)). Addition of anti-DLL4 antibody YW26.82 markedly increased the length and number of sprouts (Fig. 7a). Proteolytic processing of Notch, catalyzed by the γ-secretase activity of a protein complex, is an essential step during Notch activation (Baron, M. *Semin Cell Dev Biol* 14,

113-9 (2003)). Interestingly, the γ-secretase inhibitor dibenzazepine (DBZ) (van Es, J. H. et al. *Nature* 435, 959-63 (2005); Milano, J. et al. *Toxicol Sci* 82, 341-58 (2004)) had the same effect on HUVEC sprouting. Given the distinct mechanisms of these two treatments, the enhanced sprouting was clearly attributable to the attenuation of Notch signaling. Ki67 staining revealed that the enhanced EC sprouting was due to elevated cell proliferation (Fig. 7b). In the original fibrin gel assay, HUVEC sprouting and subsequent lumen formation are supported by cocultured SF cells. By replacing SF cells with conditioned medium, both anti-DLL4 Mab and DBZ were still able to enhance HUVEC sprouting (Fig. 1c), supporting an EC autonomous role of DLL4/Notch signaling. In the converse experiment, activation of Notch by immobilized DLL4 protein resulted in significant growth inhibition (Fig. 7e). These findings suggest that the activation status of DLL4/Notch signaling is closely associated with EC proliferation.

Example 6: Treatment with anti-DLL4 antibody increased endothelial cell proliferation *in vivo*

Early postnatal mouse retina develops a stereotypic vascular pattern in a well-defined sequence of events (Stone, J. & Dreher, Z. *J Comp Neurol* 255, 35-49 (1987); Gerhardt, H. et al. *J Cell Biol* 161, 1163-77 (2003); Fruttiger, M. *Invest Ophthalmol Vis Sci* 43, 522-7 (2002)). Prominent and dynamic expression of DLL4 in growing ECs in the neonatal retinas suggests a possible role for DLL4 to regulate retinal vascular development (Claxton, S. & Fruttiger, M. *Gene Expr Patterns* 5, 123-7 (2004)). Systemic delivery of YW26.82 caused a profound alteration of retinal vasculature. A massive accumulation of ECs occurred in the retina, generating a sheet-like structure with primitive vascular morphology (Fig. 7d). A significant increase of Ki67 labeling in ECs was observed, indicating elevated EC proliferation (Fig. 7h). Therefore, this hyperproliferative phenotype of retinal ECs upon DLL4 blockade in neonatal mice corroborated the in vitro findings.

Example 7: Essential role of DLL4/Notch in regulating epithelial cell proliferation

VEGF controls several fundamental aspects of ECs (Ferrara, *N. Exs*, 209-31 (2005); Coultas, L. et al. *Nature* 438, 937-45 (2005)). It's less understood, however, how VEGF signaling is integrated into the complex vascular processes, such as arteriovenous (AV) differentiation and hierarchical vascular organization, events evidently demanding additional highly coordinated signaling pathways. Genetic studies in zebra fish suggest that VEGF acts upstream of the Notch pathway during arterial endothelial differentiation (Lawson, N. D. et al. *Development* 128, 3675-83 (2001)). We found that VEGF stimulation

of HUVEC caused increased surface expression of DLL4 (data not shown), consistent with a recent report on the upregulation of DLL4 mRNA by VEGF stimulation (Patel, N. S. et al. *Cancer Res* 65, 8690-7 (2005)). Intriguingly, DLL4 itself is upregulated following Notch activation (Figure 12), suggesting a positive-feedback mechanism by which DLL4 effectively relays VEGF signaling to Notch pathway. Briefly, HUVECs were stimulated by immobilized C-terminal His-tagged human DLL4 (amino acids 1-404) in the absence or presence of DBZ (0.08 μM). 36 hr after stimulation, endogenous DLL4 expression was examined by FACS analysis with anti-DLL4 antibody.

Notably, the hyperproliferation of ECs resulting from blocking Notch signaling was still dependent on VEGF. In the 3-D fibrin gel culture, treatment with anti-VEGF Mab abolished most of the EC sprouting, either in the presence or absence of DBZ (Fig. 7f), raising the possibility that the hyperproliferative behavior could be in part due to enhanced VEGF signaling. Indeed, blocking of Notch by YW26.82 or DBZ resulted in upregulation of VEGFR2 (Fig. 7g). Conversely, activation of Notch by immobilized DLL4 suppressed the expression of VEGFR2 (Fig. 7g). Therefore, while VEGF can act upstream of DLL4/Notch pathway, DLL4/Notch is capable of fine-tuning the response through negatively regulating VEGFR2 expression.

Example 8: Treatment with anti-DLL4 antibody blocked endothelial cell differentiation and blocked arterial development

Besides the increase in EC proliferation, antagonizing DLL4/Notch caused a dramatic morphological change of EC sprouts in fibrin gel. The multicellular lumen-like structures were mostly absent (Fig. 8a), suggesting defective EC differentiation. In the Mab YW26.82-treated retinas, the characteristic pattern of radially alternating arteries and veins was severely disrupted. Anti-α smooth muscle actin (ASMA) staining, which is associated with the retinal arteries, was completely absent (Fig. 8c). This observation was remarkably similar to the defective arterial development in DLL4+/- embryos. These findings, from different angles, highlighted the essential role of DLL4/Notch in regulating EC differentiation.

Example 9: TFGβ expression was linked to activation status of Notch

Similar to Notch pathway, TGFβ signaling is context dependent and has diverse and often opposing effects on cell differentiation, proliferation and growth inhibition. Moreover, the TGFβ pathway has been implicated in vascular processes (Urness, L. D. et al, *Nat Genet* 26, 328-31 (2000); Oshima, M. et al, *Dev Biol* 179, 297-302 (1996); Larsson, J. et al. *Embo J* 20, 1663-73 (2001)). For instance, deficiency of Activin receptor-like

kinase 1 (ALK1), an EC specific type I TGFβ receptor, resulted in a primitive EC network in the yolk sacs and arteriovenous malfunction (AVM), a phenotype shared by mice with defective Notch signaling (Urness, L. D. et al, *Nat Genet* 26, 328-31 (2000); Iso, T. et al, *Arterioscler Thromb Vasc Biol* 23, 543-53 (2003)). This led us to investigate the possible connection between these two pathways. We found that the expression of TGFβ2 (Fig. 8b) was tightly linked to the activation status of Notch, suggesting that TGFβ pathway could act downstream of Notch pathway. Together, our findings support a model wherein the DLL4/Notch axis, serving as a "signaling router", integrates VEGF signaling through regulating DLL4 expression and engages TGFβ pathway to promote EC differentiation.

Example 10: Treatment with anti-DLL4 antibody inhibited tumor growth *in vivo*

To directly address the possible role of DLL4/Notch signaling during tumor angiogenesis, we investigated the impact of blocking DLL4 on tumor growth in preclinical tumor models (Figs. 9a-d). In HM7, Colo205 and Calu6 xenograft tumor models (Figs. 9a-c), YW26.82 treatment was initiated after tumor establishment (≥250 mm^3 in tumor size). In all three models, a separation in growth rates between the control and treatment groups became evident three days after dosing. The tumor volume of the treatment group remained static over two weeks of treatment. In addition to subcutaneous tumors, anti-DLL4 Mab also inhibited tumors growing in mouse mammary fat pads. In the MDA-MB-435 tumor model, treatment was started 14 days post tumor cell injection. A difference in tumor growth curves between the control and treatment groups was evident within six days after dosing and became increasingly significant as treatment continued (Fig. 9d).

We also investigated the impact of blocking DLL4 and/or VEGF on numerous tumor growth in preclinical tumor models (Figs. 9e-f; i-p). In MV-522 and WEHI3 xenograft tumor models, YW26.82 treatment and/or anti-VEGF treatment was initiated after tumor establishment (≥250 mm^3 in tumor size). In the MV-522 model, both YW26.82 and anti-VEGF treatment inhibited tumor growth individually, but the combination of the two treatments was most effective. In the WEHI3 model, anti-VEGF treatment showed no effect on tumor growth whereas treatment with YW26.82 showed significant inhibition of tumor growth. In the SK-OV-3X1, LL2, EL4, H1299, SKMES-1, MX-1, SW620 and LS174T models, YW26.82 treatment (5 mg/kg, IP, twice weekly) and/or anti-VEGF treatment (5 mg/kg, IP, twice weekly) was administered after establishment of the tumors. In each of these models, YW26.82 treatment inhibited tumor growth alone. Furthermore,

in all of these models where the combination was tested, YW26.82 exhibited enhanced efficacy in combination with anti-VEGF.

Example 11: Treatment with anti-DLL4 antibody increased tumor endothelial cell proliferation

In light of the tumor growth inhibition, we used the EL4 mouse lymphoma tumor model for vascular histology studies. We found that anti-DLL4 Mab treatment resulted in a dramatic increase in endothelial cell density (Fig. 9g). In contrast, anti-VEGF had a completely opposite effect (Fig. 9g), although both treatments exhibited similar efficacy in this model.

Example 12: Treatment with anti-DLL4 antibody inhibited tumor vascular perfusion

Since *in vitro* blocking of DLL4/Notch pathway impaired the formation of lumen-like structure by ECs (Fig. 8a), it was investigated whether treatment with anti-DLL4 Mab caused similar defect in the tumor vasculature and affected efficient blood flow. Systemic perfusion with FITC-letin revealed that anti-DLL4 Mab treatment resulted in marked reduction of lectin labeling of tumor vessels (Fig. 9h). Notably, it has been shown that arteriovenous malfunction in ALK1-deficient mice causes anomalous blood circulation (Urness, L. D. et al, *Nat Genet* 26, 328-31 (2000)). Given the critical role of DLL4/Notch signaling in AV differentiation, both in embryos and early postnatal retinas, anti-DLL4 Mab could impact cell fate specification of tumor ECs and cause defective directional blood flow. Indeed, in anti-DLL4 Mab treated Colo205 tumors, there were regions where high EC density was associated with low viable tumor cell content, implicating poor vascular function. Further studies utilizing vascular imaging techniques are needed to gain insights into the precise vascular defects.

Example 13: DLL4/Notch is dispensible in homeostasis of the mouse intestine

A major concern about global inhibition of Notch is that it may be deleterious, given the pleiotropic roles of Notch signaling in regulating the homeostasis of postnatal self-renewal systems. For instance, Notch signaling is required to maintain undifferentiated crypt progenitor cells in intestines (van Es, J. H. et al. *Nature* 435, 959-63 (2005); Fre, S. et al. *Nature* 435, 964-8 (2005)). Indeed, γ-secretase inhibitors, which would indiscriminately block all Notch activities, cause unwanted side effects in rodents duo to a massive increase in goblet cells within the crypt compartment (Milano, J. et al. *Toxicol Sci* 82, 341-58 (2004); Wong, G. T. et al. *J Biol Chem* 279, 12876-82 (2004)). We examined the small intestines of mice treated with anti-DLL4 Mab by immunohistochemistry analyses. In

contrast to DBZ treatment, no differences in epithelial crypt cell differentiation or proliferation profiles were identified between anti-DLL4 Mab and control groups after six weeks of treatment (Fig. 10). Furthermore, anti-DLL4 Mab did not alter the expression of the Notch target gene HES-1 in the rapidly dividing transit amplifying (TA) population (Fig. 10). These results support the notion that DLL4/Notch signaling is largely restricted to the vascular system.

Example 14: Treatment with anti-DLL4 antibody does not impact adult retinal vasculature

While blocking of DLL4 had a profound impact on the retinal vascular development in neonatal mouse, administration of anti-DLL4 antibody has no visible impact on adult retinal vasculature (Fig. 8d). Thus, DLL4/Notch signaling is critical during active angiogenesis, but plays a less important role in normal vessel maintenance. In agreement with this notion, during the course of anti-DLL4 Mab treatment, no apparent weight loss or animal death was observed in tumor-bearing mice when dosed at 10 mg/kg twice per week for up to 8 weeks. In tumor models, anti-DLL4 Mab and anti-VEGF exhibit opposite effects on tumor vasculature, suggesting non-overlapping mechanisms of action.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention.

WHAT IS CLAIMED IS:

1. An isolated anti-DLL4 antibody comprising:

(a) at least one, two, three, four or five hypervariable region (HVR) sequences selected from the group consisting of:

(i) HVR-L1 comprising sequence A1-A11, wherein A1-A11 is RASQDVSTAVA (SEQ ID NO: 10)

(ii) HVR-L2 comprising sequence B1-B7, wherein B1-B7 is SASFLYS (SEQ ID NO:11)

(iii) HVR-L3 comprising sequence C1-C9, wherein C1-C9 is QQSYTGTVT (SEQ ID NO:18)

(iv) HVR-H1 comprising sequence D1-D10, wherein D1-D10 is GFTFTDNWIS (SEQ ID NO:1);

(v) HVR-H2 comprising sequence E1-E18, wherein E1-E18 is GYISPNSGFTYYADSVKG (SEQ ID NO:8) and

(vi) HVR-H3 comprising sequence F1-F15, wherein F1-F15 is VYYCARDNFGGYFDY (SEQ ID NO:9) ; and

(b) at least one variant HVR, wherein the variant HVR sequence comprises modification of at least one residue of the sequence depicted in SEQ ID NOs:1-18.

2. An isolated anti-DLL4 antibody comprising:

(a) at least one, two, three, four or five hypervariable region (HVR) sequences selected from the group consisting of:

(i) HVR-L1 comprising sequence A1-A11, wherein A1-A11 is RASQDVSTAVA (SEQ ID NO: 10)

(ii) HVR-L2 comprising sequence B1-B7, wherein B1-B7 is SASFLYS (SEQ ID NO:11)

(iii) HVR-L3 comprising sequence C1-C9, wherein C1-C9 is QQSYNGPST (SEQ ID NO:15)

(iv) HVR-H1 comprising sequence D1-D10, wherein D1-D10 is GFTFTDNWIS (SEQ ID NO:1)

(v) HVR-H2 comprising sequence E1-E18, wherein E1-E18 is GVINPNSGATEYADSVKG (SEQ ID NO:5) and

(vi) HVR-H3 comprising sequence F1-F15, wherein F1-F15 is VYYCARDNFGGYFDY (SEQ ID NO:9) ; and

(b) at least one variant HVR, wherein the variant HVR sequence comprises modification of at least one residue of the sequence depicted in SEQ ID NOs:1-18.

3. The antibody of claim 1 or 2, wherein a HVR-L3 variant comprises 1-6 (1, 2, 3, 4, 5, or 6) substitutions in any combination of the following positions: 91 (S or W), 92 (Y or F), 93 (T, N or S), 94 (T or G), 95 (P, Q, A or T),and/or 96 (P, S, A, or V).

4. The antibody of claim 1 or 2, wherein a HVR- H2 variant comprises 1-4 (1, 2, 3, or 4) substitutions in any combination of the following positions: 50 (V, L or Y), 52 (N or S), 52a (P or S), or 53 (N, Q, T, or I).

5. An isolated anti-DLL4 antibody comprising one, two, three, four, five or six HVRs, wherein each HVR comprises, consists or consists essentially of a sequence selected from the group consisting of SEQ ID NOs: 1-18, and wherein SEQ ID NO:10 corresponds to an HVR-L1, SEQ ID NO:11 corresponds to an HVR-L2, SEQ ID NO:12, 13, 14, 15, 16, 17, or 18 correspond to an HVR-L3, SEQ ID NO:1 or 2 correspond to an HVR-H1, SEQ ID NO:3, 4, 5, 6, 7 or 8 correspond to an HVR-H2, and SEQ ID NO:9 corresponds to an HVR-H3.

6. The antibody of claim 5, wherein the antibody comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 14, 2, 3, and 9.

7. The antibody of claim 5, wherein the antibody comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 15, 1, 5, and 9.

8. The antibody of claim 5, wherein the antibody comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 16, 1, 6, and 9.

9. The antibody of claim 5, wherein the antibody comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 17, 1, 7, and 9.

10. The antibody of claim 5, wherein the antibody comprises HVR-L1, HVR-L2, HVR-L3, HVR-H1, HVR-H2, and HVR-H3, wherein each, in order, comprises SEQ ID NO:10, 11, 18, 1, 8, and 9.

11. The antibody of any of claims 1-10, wherein at least a portion of the framework sequence is a human consensus framework sequence.

12. The antibody of claim 1 or 2, wherein the modification is substitution, insertion or deletion.

13. The antibody of any of claims 1-12, wherein the antibody comprises human κ subgroup consensus framework sequence.

14. The antibody of any of claims 1-12, wherein the antibody comprises heavy chain human subgroup III consensus framework sequence.

15. The antibody of claim 14, wherein the antibody comprises a substitution at one or more of position 71, 73 or 78.

16. The antibody of claim 15, wherein the substitution is one or more of R71A, N73T, or N78A.

17. A polynucleotide encoding an antibody of any of claims 1-16.

18. A vector comprising the polynucleotide of claim 17.

19. The vector of claim 18, wherein the vector is an expression vector.

20. A host cell comprising a vector of claim 18 or 19.

21. The host cell of claim 20, wherein the host cell is prokaryotic.

22. The host cell of claim 20, wherein the host cell is eukaryotic.

23. The host cell of claim 20, wherein the host cell is mammalian.

24. A method for making an anti-DLL4 antibody, said method comprising (a) expressing a vector of claim 19 in a suitable host cell, and (b) recovering the antibody.

25. A method for making an anti-DLL4 immunoconjugate, said method comprising (a) expressing a vector of claim 19 in a suitable host cell, and (b) recovering the antibody.

26. The method of claim 24 or 25, wherein the host cell is prokaryotic.

27. The method of claim 24 or 25, wherein the host cell is eukaryotic.

28. A method for detection of DLL4, the method comprising detecting DLL4-anti-DLL4 antibody complex in a biological sample.

29. A method for diagnosing a disorder associated with DLL4 expression, the method comprising detecting DLL4-anti-DLL4 antibody complex in a biological sample from a patient having or suspected of having the disorder.

30. The method of any of claims 28-29, wherein the anti-DLL4 antibody is detectably labeled.

31. A composition comprising an anti-DLL4 antibody of any of claims 1-16.

32. A composition comprising a polynucleotide of any of claims 17-19.

33. The composition of claim 31 or 32, wherein the composition further comprises a carrier.

34. A method for treating a tumor, cancer, or cell proliferative disorder comprising administering an effective amount of an antibody of any of claims 1-16 to a subject in need of such treatment, whereby the tumor, cancer or cell proliferative disorder is treated.

35. The method of claim 34, wherein the tumor, cancer or cell proliferative disorder is colon cancer, lung cancer or breast cancer.

36. The method of claim 34 or 35, further comprising administering an effective amount of an anti-angiogenic agent.

37. The method of claim 36, wherein the anti-angiogenic agent is an antagonist of vascular endothelial growth factor (VEGF).

38. The method of claim 37, wherein the VEGF antagonist is an anti-VEGF antibody.

39. The method of claim 38, wherein the anti-VEGF antibody is bevacizumab.

40. The method of any of claims 34-39 further comprising administering an effective amount of a chemotherapeutic agent.

41. A method of enhancing efficacy of an anti-angiogenic agent in a subject having a pathological condition associated with angiogenesis, comprising administering to the subject an effective amount of an antibody of any of claims 1-16 in addition to the anti-antigenic agent, thereby enhancing said anti-angiogenic agent's efficacy.

42. The method of claim 41, wherein the pathological condition associated with angiogenesis is a tumor, a cancer, and/or a cell proliferative disorder.

43. The method of claim 41, wherein the pathological condition associated with angiogenesis is an intraocular neovascular disease.