COMPOSITIONS AND METHODS FOR MODULATING VASCULAR DEVELOPMENT

FIELD OF THE INVENTION

The present invention relates generally to compositions and methods that are useful for modulating vascular development. In part, the present invention relates to the use of Delta-like 4 (DLL4) antagonists for the diagnosis and treatment of disorders associated with angiogenesis.

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, D. *Science* 277:48-50 (1997); Hogan, B. L. & Kolodziej, P. A. *Nature Reviews Genetics*. 3:513-23 (2002); Lubarsky, B. & Krasnow, M. A. *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.*, 267:10931-10934 (1992); Klagsbrun *et al., Annu. Rev. Physiol.* 53:217-239 (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* 340:1120-1124 (1992); Macchiarini *et al., Lancet* 340:145-146 (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, 1995, *Nat Med* 1(1):27-31).

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.* 91:153-159 (1993); Brown *et al., Human Pathol.* 26:86-91 (1995); Brown *et al., Cancer Res.* 53:4727-4735 (1993); Mattern *et al., Brit. J. Cancer* 73:931-934 (1996); Dvorak *et al., Am. J. Pathol.* 146:1029-1039 (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.* 331:1480-1487 (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.* 37:855-868 (1996).

Anti-VEGF neutralizing antibodies suppress the growth of a variety of human tumor cell lines in nude mice (Kim *et al., Nature* 362:841-844 (1993); Warren *et al., J. Clin. Invest.* 95:1789-1797 (1995); Borgström *et al., Cancer Res.* 56:4032-4039 (1996); Melnyk *et al., Cancer Res.* 56:921-924 (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 WO98/45331 and WO98/45332, both published October 15, 1998. One of the anti-VEGF antibodies, bevacizumab, has been approved by the FDA for use in combination with a chemotherapy regimen to treat metastatic colorectal cancer (CRC). And bevacizumab is being investigated in many ongoing clinical trials for treating various cancer indications.

In view of the role of angiogenesis in many diseases and disorders, it is desirable to have a means of modulating one or more of the biological effects causing these processes. 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 present invention is based in part on the discovery that vascular development is inhibited by treatment with an agent that modulates Delta-like 4 (interchangeably termed "DLL4") activation of the Notch receptor pathway. Treatment with a DLL4 antagonist resulted in increased endothelial cell (EC) proliferation, improper endothelial cell differentiation and improper arterial development in vasculature, including tumor vasculature. Strikingly, treatment with an anti-DLL4 antibody resulted in inhibition of

tumor growth in several different cancers. Accordingly, the invention provides methods, compositions, kits and articles of manufacture for modulating (e.g., promoting or inhibiting) processes involved in angiogenesis and for use in targeting pathological conditions associated with angiogenesis.

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 a DLL4 antagonist 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 antagonist to a subject in need of such treatment.

In one aspect, the invention provides methods for inhibiting angiogenesis comprising administering an effective amount of a DLL4 antagonist (such as 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 a DLL4 antagonist (such as 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.

In one aspect, the invention provides methods for stimulating endothelial cell proliferation comprising administering an effective amount of a DLL4 antagonist to a subject in need of such treatment. In some embodiments, the subject has a pathological condition associated with angiogenesis (such as a tumor, a cancer and/or a cell proliferative disorder).

In one aspect, the invention provides methods for inhibiting endothelial cell differentiation comprising administering an effective amount of a DLL4 antagonist to a subject in need of such treatment. In some embodiments, the subject has a pathological condition associated with angiogenesis (such as a tumor, a cancer and/or a cell proliferative disorder).

In one aspect, the invention provides methods for inhibiting arterial development comprising administering an effective amount of DLL4 antagonist to a subject in need of such treatment. In some embodiments, the subject has a pathological condition associated with angiogenesis (such as a tumor, a cancer and/or a cell proliferative disorder).

In one aspect, the invention provides methods for inhibiting vascular perfusion comprising administering an effective amount of a DLL4 antagonist to a subject in need of such treatment. In some embodiments, the subject has a pathological condition associated with angiogenesis (such as a tumor, a cancer and/or a cell proliferative disorder).

In another aspect, the invention provides a method of enhancing the efficacy of an anti-angiogenic agent treatment in a subject having a pathological condition associated with angiogenesis, comprising administering to the subject an effective amount of DLL4 antagonist in combination with the anti-angiogenic agent. Such a method will be useful in treating disorders, for example cancers or intraocular neovascular diseases, especially those diseases or stages of the disorders that responded poorly to a treatment with the anti-angiogenic agent alone. The anti-angiogenic agent can be any agent capable of reducing or inhibiting angiogenesis, including VEGF antagonists such as anti-VEGF antibody.

In one aspect, the invention provides methods comprising administration of an effective amount of a DLL4 antagonist (such as an anti-DLL4 antibody) in combination with and effective amount of another therapeutic agent (such as an anti-angiogenesis agent). For example, DLL4 antagonists are used in combinations with anti-cancer agent or an anti-angiogenic agent to treat various neoplastic or non-neoplastic conditions. In one embodiment, the neoplastic or non-neoplastic condition is a pathological condition associated with angiogenesis. In some embodiments, the other therapeutic agent is an anti-angiogenic agent, an anti-neoplastic agent, and/or a chemotherapeutic agent.

The DLL4 antagonist can be administered serially or in combination with the other therapeutic agent that is effective for those purposes, either in the same composition or as separate compositions. The administration of the DLL4 antagonist and the other therapeutic agent (e.g., anti-cancer agent, anti-angiogenic agent) 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. Alternatively, or additionally, the steps can be performed as a combination of both sequentially and simultaneously, 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 antagonist. However, simultaneous administration or administration of the DLL4 antagonist first is also contemplated. Accordingly, in one aspect, the invention provides methods comprising administration of a DLL4 antagonist (such as an anti-DLL4 antibody), followed by administration of an anti-

angiogenic agent (such as an anti-VEGF antibody, such as bevacizumab). 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.

In certain aspects, the invention provides a method of treating a disorder (such as a tumor, a cancer, and/or a cell proliferative disorder) by administering effective amounts of an antagonist of DLL4 and/or an angiogenesis inhibitor(s) and one or more chemotherapeutic agents. 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." The administration of the DLL4 antagonist and the chemotherapeutic agent 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. Alternatively, or additionally, the steps can be performed as a combination of both sequentially and simultaneously, 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 chemotherapeutic agent may be administered first, followed by the DLL4 antagonist. However, simultaneous administration or administration of the DLL4 antagonist first is also contemplated. Accordingly, in one aspect, the invention provides methods comprising administration of a DLL4 antagonist (such as an anti-DLL4 antibody), followed by administration of a chemotherapeutic agent. 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.

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

In one aspect, the invention provides methods for treating a disorder comprising administering an effective amount of a DLL4 agonist to a subject in need of such treatment. In some embodiments, the disorder is associated with expression and/or activity of the DLL4-Notch receptor pathways (such as increased activity of the DLL4-Notch receptor pathway). In some embodiments, the disorder is a disorder wherein angiogenesis, neovascularization and/or hypertrophy is desired, e.g. vascular trauma, wounds, lacerations, incisions, burns, ulcers (e.g., diabetic ulcers, pressure ulcers, haemophiliac ulcers, varicose

ulcers), tissue growth, weight gain, peripheral arterial disease, induction of labor, hair growth, epidermolysis bullosa, retinal atrophy, bone fractures, bone spinal fusions, meniscal tears, etc. In some embodiments, the disorder is a disorder wherein inhibition of angiogenesis is desired. In some embodiments, the DLL4 agonist is DBZ.

DLL4 antagonists and agonists are known in the art and some are described and exemplified herein. In some embodiments, the DLL4 antagonist is a molecule which binds to DLL4 and neutralizes, blocks, inhibits, abrogates, reduces or interferes with one or more aspects of DLL4-associated effect. In some embodiments, the DLL4 antagonist is a molecule which binds to Notch receptor (such as Notch1, Notch2, Notch3 and/or Notch4) and neutralizes, blocks, inhibits, abrogates, reduces or interferes with one or more aspects of DLL4-associated effects. In some embodiments, the DLL4 antagonist is capable of promoting endothelial cell proliferation, inhibiting endothelial cell differentiation, inhibiting arterial development and/or reducing vascular perfusion. As is well-established in the art, endothelial cell proliferation, endothelial cell differentiation, arterial development and vascular function (such as vascular perfusion) can be assessed using any of a variety of assays (some of which are described and exemplified herein), and expressed in terms of a variety of quantitative values. In some embodiments, the ability of a DLL4 antagonist to promote endothelial cell proliferation, inhibit endothelial cell differentiation, inhibit arterial development and/or reduce vascular function (such as reduced vascular perfusion) is assessed relative to level of endothelial cell proliferation, endothelial cell differentiation, arterial development and/or vascular function (such as vascular perfusion) in the absence of treatment with the DLL4 antagonist. In some embodiments, ability to promote endothelial cell proliferation, inhibit endothelial cell differentiation, inhibit arterial development and/or reduce vascular function (such as reduced vascular perfusion) is determined in an in vitro assay (such as the HUVEC assay described herein). In some embodiments, ability to promote endothelial cell proliferation, inhibit endothelial cell differentiation, inhibit arterial development and/or reduce vascular function (such as reduced vascular perfusion) is determined in an in vivo assay (such as the mouse retinal development assay described herein).

The DLL4 antagonist may be an anti-DLL4 antibody. In some embodiments, the anti-DLL4 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 some embodiments, the antibody comprises the heavy and light chain variable regions shown in Table 1.

Table 1

VH

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDNWISWVRQAPGKGLEWVGYI SPNSGFTYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARDNFGGYFD YWGQGTLVT (SEQ ID NO: 1)

VL

DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSAS FLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATTYYCQQSYTGTVTFGQGTKVEIKR (SEQ ID NO: 2)

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 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 embodiment, the DLL4 antagonist is a DLL4 immunoadhesin.

In one aspect, the invention provides compositions comprising one or more DLL4 antagonist and a carrier. In one embodiment, the carrier is pharmaceutically acceptable. In some embodiments, the DLL4 antagonist is an anti-DLL4 antibody.

In one aspect, the invention provides a composition for use in treating a tumor, a cancer and/or a cell proliferative disorder comprising an effective amount of a DLL4 antagonist and a pharmaceutically acceptable carrier, wherein said use comprises simultaneous or sequential administration of an anti-angiogenesis agent. In some embodiments, the DLL4 antagonist is an anti-DLL4 antibody. In some embodiments, the anti-angiogenesis agent is an anti-VEGF antibody (such as bevacizumab).

In one aspect, the invention provides a composition for use in treating a tumor, a cancer and/or a cell proliferative disorder comprising an effective amount of a DLL4 antagonist and a pharmaceutically acceptable carrier, wherein said use comprises simultaneous or sequential administration of an anti-cancer agent. In some embodiments, the DLL4 antagonist is an anti-DLL4 antibody. In some embodiments, the anti-cancer agent is a chemotherapeutic agent. In some embodiments, the use further comprises simultaneous or sequential administration of an anti-angiogenesis agent. In some embodiments, the DLL4 antagonist is an anti-DLL4 antibody. In some embodiments, the anti-angiogenesis agent is an anti-VEGF antibody (such as bevacizumab).

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 DLL4 antagonists or DLL4 agonists.

In one aspect, the invention provides a kit comprising a first container comprising a composition comprising one or more DLL4 antagonists or DLL4 agonists; and a second container comprising a buffer. In one embodiment, the buffer is pharmaceutically acceptable. In one embodiment, the DLL4 antagonist is an anti-DLL4 antibody.

In another aspect, the present invention provides a method for preparing a composition comprising admixing a therapeutically effective amount of a DLL4 antagonist or DLL4 agonist with a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: DLL4-mediated Notch signaling regulates 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 2: DLL4-mediated Notch signaling regulates 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 3: 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 4: DLL4/Notch is 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 5: Characterization of anti-DLL4 antibody. a, Epitope mapping of anti-DLL4 antibody (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 6: 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

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

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.

"DLL4 nucleic acid" is RNA or DNA that encodes a DLL4 polypeptide, as defined above, or which hybridizes to such DNA or RNA and remains stably bound to it under

stringent hybridization conditions and is greater than about 10 nucleotides in length. Stringent conditions are those which (1) employ low ionic strength and high temperature for washing, for example, 0.15 M NaCl/0.015 M sodium citrate/0.1% $NaDodSO_4$ at 50°C, or (2) use during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinlypyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM $NaCl_2$, 75 mM sodium citrate at 42°C.

A "chimeric DLL4" molecule is a polypeptide comprising full-length DLL4 or one or more domains thereof fused or bonded to heterologous polypeptide. The chimeric DLL4 molecule will generally share at least one biological property in common with naturally occurring DLL4. An example of a chimeric DLL4 molecule is one that is epitope tagged for purification purposes. Another chimeric DLL4 molecule is a DLL4 immunoadhesin.

The term "DLL4 immunoadhesin" is used interchangeably with the term "DLL4-immunoglobulin chimera", and refers to a chimeric molecule that combines at least a portion of a DLL4 molecule (native or variant) with an immunoglobulin sequence. The immunoglobulin sequence preferably, but not necessarily, is an immunoglobulin constant domain (Fc region). Immunoadhesins can possess many of the valuable chemical and biological properties of human antibodies. Since immunoadhesins can be constructed from a human protein sequence with a desired specificity linked to an appropriate human immunoglobulin hinge and constant domain (Fc) sequence, the binding specificity of interest can be achieved using entirely human components. Such immunoadhesins are minimally immunogenic to the patient, and are safe for chronic or repeated use. In some embodiments, the Fc region is a native sequence Fc region. In some embodiments, the Fc region is a variant Fc region. In some embodiments, the Fc region is a functional Fc region.

Examples of homomultimeric immunoadhesins which have been described for therapeutic use include the CD4-IgG immunoadhesin for blocking the binding of HIV to cell-surface CD4. Data obtained from Phase I clinical trials, in which CD4-IgG was administered to pregnant women just before delivery, suggests that this immunoadhesin may be useful in the prevention of maternal-fetal transfer of HIV (Ashkenazi *et al., Intern. Rev. Immunol.* 10:219-227 (1993)). An immunoadhesin which binds tumor necrosis factor (TNF) has also been developed. TNF is a proinflammatory cytokine which has been shown to be a major mediator of septic shock. Based on a mouse model of septic shock, a TNF receptor immunoadhesin has shown promise as a candidate for clinical use in treating septic shock (Ashkenazi, A. *et al. PNAS USA* 88:10535-10539 (1991)). ENBREL® (etanercept),

an immunoadhesin comprising a TNF receptor sequence fused to an IgG Fc region, was approved by the U.S. Food and Drug Administration (FDA), on November 2, 1998, for the treatment of rheumatoid arthritis. The new expanded use of ENBREL® in the treatment of rheumatoid arthritis was approved by FDA on June 6, 2000. For recent information on TNF blockers, including ENBREL®, see Lovell *et al., N. Engl. J. Med.* 342:763-169 (2000), and accompanying editorial on p810-811; and Weinblatt *et al., N. Engl. J. Med.* 340:253-259 (1999); reviewed in Maini and Taylor, *Annu. Rev. Med.* 51:207-229 (2000).

If the two arms of the immunoadhesin structure have different specificities, the immunoadhesin is called a "bispecific immunoadhesin" by analogy to bispecific antibodies. Dietsch *et al., J. Immunol. Methods* 162:123 (1993) describe such a bispecific immunoadhesin combining the extracellular domains of the adhesion molecules, E-selectin and P-selectin, each of which selectins is expressed in a different cell type in nature. Binding studies indicated that the bispecific immunoglobulin fusion protein so formed had an enhanced ability to bind to a myeloid cell line compared to the monospecific immunoadhesins from which it was derived.

The term "heteroadhesin" is used interchangeably with the expression "chimeric heteromultimer adhesin" and refers to a complex of chimeric molecules (amino acid sequences) in which each chimeric molecule combines a biologically active portion, such as the extracellular domain of each of the heteromultimeric receptor monomers, with a multimerization domain. The "multimerization domain" promotes stable interaction of the chimeric molecules within the heteromultimer complex. The multimerization domains may interact via an immunoglobulin sequence, leucine zipper, a hydrophobic region, a hydrophilic region, or a free thiol that forms an intermolecular disulfide bond between the chimeric molecules of the chimeric heteromultimer. The multimerization domain may comprise an immunoglobulin constant region. In addition a multimerization region may be engineered such that steric interactions not only promote stable interaction, but further promote the formation of heterodimers over homodimers from a mixture of monomers. "Protuberances" are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the protuberances are optionally created on the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). The immunoglobulin sequence preferably, but not necessarily, is an immunoglobulin constant domain. The immunoglobulin moiety in the

chimeras of the present invention may be obtained from IgG_1, IgG_2, IgG_3 or IgG_4 subtypes, IgA, IgE, IgD or IgM, but preferably IgG_1 or IgG_3.

The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue.

Unless indicated otherwise, herein the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991), expressly incorporated herein by reference. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

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

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification, preferably one or more amino acid substitution(s). Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a

parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will preferably possess at least about 80% homology with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and most preferably at least about 90% homology therewith, more preferably at least about 95% homology therewith.

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. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The terms "antibody" and "immunoglobulin" are used interchangeably in the broadest sense and include monoclonal antibodies (for 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 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., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. 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. PNAS (USA) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and 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.

"Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time. "Intermittent" administration is treatment that is not consecutively done without interruption, but rather is cyclic in nature.

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 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 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 omega1I (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.

An "intraocular neovascular disease" is a disease characterized by ocular neovascularization. Examples of intraocular neovascular diseases include, but are not limited to, proliferative retinopathies, choroidal neovascularization (CNV), age-related macular degeneration (AMD), diabetic and other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, retinal vein occlusions, including Central Retinal Vein Occlusion (CRVO), corneal neovascularization, retinal neovascularization, etc.

The "pathology" of a disease includes all phenomena that compromise the well-being of the patient. For cancer, this includes, without limitation, abnormal or uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, etc.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as a DLL4 polypeptide or antibody thereto) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The terms "VEGF" and "VEGF-A" are used interchangeably to refer to the 165-amino acid vascular endothelial cell growth factor and related 121-, 145-, 183-, 189-, and 206- amino acid vascular endothelial cell growth factors, as described by Leung et al. Science, 246:1306 (1989), Houck et al. Mol. Endocrin., 5:1806 (1991), and, Robinson & Stringer, Journal of Cell Science, 144(5):853-865 (2001), together with the naturally occurring allelic and processed forms thereof.

A "VEGF antagonist" refers to a molecule capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with VEGF activities including its binding to one or more VEGF receptors. VEGF antagonists include anti-VEGF antibodies and antigen-binding fragments thereof, receptor molecules and derivatives which bind specifically to VEGF thereby sequestering its binding to one or more receptors, anti-VEGF receptor antibodies and VEGF receptor antagonists such as small molecule inhibitors of the VEGFR tyrosine kinases, and fusions proteins, e.g., VEGF-Trap (Regeneron), VEGF121-gelonin (Peregrine). VEGF antagonists also include antagonist variants of VEGF, antisense molecules directed to VEGF, RNA aptamers, and ribozymes against VEGF or VEGF receptors.

An "anti-VEGF antibody" is an antibody that binds to VEGF with sufficient affinity and specificity. The anti-VEGF antibody can be used as a therapeutic agent in targeting and

interfering with diseases or conditions wherein the VEGF activity is involved. See, e.g., U.S. Patents 6,582,959, 6,703,020; WO98/45332; WO 96/30046; WO94/10202, WO2005/044853; ; EP 0666868B1; US Patent Applications 20030206899, 20030190317, 20030203409, 20050112126, 20050186208, and 20050112126; Popkov et al., Journal of Immunological Methods 288:149-164 (2004); and WO2005012359. An anti-VEGF antibody will usually not bind to other VEGF homologues such as VEGF-B or VEGF-C, nor other growth factors such as PlGF, PDGF or bFGF. The anti-VEGF antibody "Bevacizumab (BV)", also known as "rhuMAb VEGF" or "Avastin®", is a recombinant humanized anti-VEGF monoclonal antibody generated according to Presta et al. Cancer Res. 57:4593-4599 (1997). It comprises mutated human IgG1 framework regions and antigen-binding complementarity-determining regions from the murine anti-hVEGF monoclonal antibody A.4.6.1 that blocks binding of human VEGF to its receptors. Approximately 93% of the amino acid sequence of Bevacizumab, including most of the framework regions, is derived from human IgG1, and about 7% of the sequence is derived from the murine antibody A4.6.1. Bevacizumab has a molecular mass of about 149,000 daltons and is glycosylated. Bevacizumab and other humanized anti-VEGF antibodies, including the anti-VEGF antibody fragment "ranibizumab", also known as "Lucentis®", are further described in U.S. Pat. No. 6,884,879 issued February 26, 2005.

The term "biological activity" and "biologically active" with regard to a DLL4 polypeptide refer to physical/chemical properties and biological functions associated with DLL4. In some embodiments, DLL4 "biological activity" includes one or more of: binding a Notch receptor (eg, Notch1, Notch2, Notch3, Notch4), activating a Notch receptor, and activating a Notch receptor downstream molecular signaling. In this context, the term "modulate" includes both promotion and inhibition.

A "DLL4 antagonist" refers to a molecule capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with the activities of a DLL4 including, for example, 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. DLL4 antagonists include antibodies and antigen-binding fragments thereof, proteins, peptides, glycoproteins, glycopeptides, glycolipids, polysaccharides, oligosaccharides, nucleic acids, bioorganic molecules, peptidomimetics, pharmacological agents and their metabolites, transcriptional and translation control sequences, and the like. Antagonists also include small molecule

inhibitors of a protein, and fusions proteins, receptor molecules and derivatives which bind specifically to protein thereby sequestering its binding to its target, antagonist variants of the protein, siRNA molecules directed to a protein, antisense molecules directed to a protein, RNA aptamers, and ribozymes against a protein. In some embodiments, the DLL4 antagonist is a molecule which binds to DLL4 and neutralizes, blocks, inhibits, abrogates, reduces or interferes with a biological activity of DLL4. In some embodiments, the DLL4 antagonist is a molecule which binds to Notch receptor (such as Notch1, Notch2, Notch3 and/or Notch4) and neutralizes, blocks, inhibits, abrogates, reduces or interferes with a biological activity of DLL4. In some embodiments, the DLL4 antagonist modulates 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.

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 "angiogenic factor or agent" is a growth factor which stimulates the development of blood vessels, e.g., promotes angiogenesis, endothelial cell growth, stability of blood vessels, and/or vasculogenesis, etc. For example, angiogenic factors, include, but are not limited to, e.g., VEGF and members of the VEGF family, PlGF, PDGF family, fibroblast growth factor family (FGFs), TIE ligands (Angiopoietins), ephrins, ANGPTL3, DLL4, etc. It would also include factors that accelerate wound healing, such as growth hormone, insulin-like growth factor-I (IGF-I), VIGF, epidermal growth factor (EGF), CTGF and members of its family, and TGF-α and TGF-β. See, e.g., Klagsbrun and D'Amore, Annu. Rev. Physiol., 53:217-39 (1991); Streit and Detmar, Oncogene, 22:3172-3179 (2003); Ferrara & Alitalo, Nature Medicine 5(12):1359-1364 (1999); Tonini et al., Oncogene, 22:6549-6556 (2003) (e.g., Table 1 listing angiogenic factors); and, Sato Int. J. Clin. Oncol., 8:200-206 (2003).

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, 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).

Methods and compositions of the invention

The present invention is based in part on the discovery that vascular development is inhibited by treatment with an agent that modulates Delta-like 4 (interchangeably termed "DLL4") activation of the Notch receptor pathway. Treatment with a DLL4 antagonist resulted in increased endothelial cell (EC) proliferation, improper endothelial cell differentiation and improper arterial development in vasculature, including tumor vasculature. Strikingly, treatment with an anti-DLL4 antibody resulted in inhibition of tumor growth in several different cancers. Without being bound by theory, it is believed that increased EC proliferation and impaired EC differentiation results in improper tumor vascular function, leading to inhibition of tumor growth. Accordingly, DLL4 antagonists are believed to demonstrate a broadly efficacious approach for the treatment of cancer.

Accordingly, the invention provides methods, compositions, kits and articles of manufacture for modulating (e.g., promoting or inhibiting) processes involved in angiogenesis and for use in targeting pathological conditions associated with angiogenesis, such as cancer.

It is contemplated that, according to the present invention, the DLL4 modulators and/or combinations of DLL4 modulators and other therapeutic agents can be used to treat various disorders.

Accordingly, the invention encompasses methods for inhibiting angiogenesis using an effective amount of a DLL4 antagonist (such as an anti-DLL4 antibody or a DLL4 immunoadhesin) to inhibit DLL4 activation of Notch receptors (such as Notch1, Notch2, Notch3, and/or Notch4). In another aspect, the invention provides methods for inhibiting angiogenesis comprising administering an effective amount of a DLL4 antagonist to a subject in need of such treatment. In some embodiments, the DLL4 antagonist is capable of promoting endothelial cell proliferation, inhibits endothelial cell differentiation, inhibits arterial development and/or reduces vascular perfusion. In another aspect, the invention provides methods for stimulating endothelial cell proliferation, inhibiting endothelial cell

differentiation, inhibiting arterial development and/or inhibiting tumor vascular perfusion comprising administering an effective amount of a DLL4 antagonist to a subject in need of such treatment.

Examples of neoplastic disorders to be treated with a DLL4 antagonist (such as an anti-DLL4 antibody) include, but are not limited to, those described herein under the terms "cancer" and "cancerous." Non-neoplastic conditions that are amenable to treatment with antagonists useful in the invention, but are not limited to, e.g., undesired or aberrant hypertrophy, arthritis, rheumatoid arthritis (RA), psoriasis, psoriatic plaques, sarcoidosis, atherosclerosis, atherosclerotic plaques, edema from myocardial infarction, 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), obesity, adipose tissue mass growth, 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. Further examples of disorders to be treated with a DLL4 antagonist (such as an anti-DLL4 antibody) include an epithelial or cardiac disorder.

Modulators of DLL4, e.g., agonists or activators of DLL4, can be utilized for treatment of pathological disorders. In some embodiments, modulators of DLL4, e.g., agonists of DLL4, can be utilized in the treatment of pathological disorders where inhibition of angiogenesis is desired. Modulators of DLL4, e.g. DLL agonists, can also be used for

treatment of pathological disorders where angiogenesis or neovascularization and/or hypertrophy is desired, which include, but are not limited to, e.g., vascular trauma, wounds, lacerations, incisions, burns, ulcers (e.g., diabetic ulcers, pressure ulcers, haemophiliac ulcers, varicose ulcers), tissue growth, weight gain, peripheral arterial disease, induction of labor, hair growth, epidermolysis bullosa, retinal atrophy, bone fractures, bone spinal fusions, meniscal tears, etc.

Combination Therapies

As indicated above, the invention provides combined therapies in which a DLL4 antagonist (such as an anti-DLL4 antibody) or a DLL4 agonist is administered with another therapy. For example, DLL4 antagonists are used in combinations with anti-cancer agent or an anti-angiogenic agent 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 DLL4 antagonist 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 DLL4 antagonist (or DLL4 agonist) and the other therapeutic agent (e.g., anti-cancer agent, anti-angiogenic agent) 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. Alternatively, or additionally, the steps can be performed as a combination of both sequentially and simultaneously, 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 antagonist. However, simultaneous administration or administration of the DLL4 antagonist first is also contemplated. Accordingly, in one aspect, the invention provides methods comprising administration of a DLL4 antagonist (such as an anti-DLL4 antibody), followed by administration of an anti-angiogenic agent (such as anti-VEGF). 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.

The effective amounts of therapeutic agents administered in combination with a DLL4 antagonist (or DLL4 agonist) 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 DLL4 antagonist. 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 DLL4 antagonists and anti-cancer agents are suitable for the same or similar diseases to block or reduce a pathological disorder such as a tumor, a cancer or a cell proliferative disorder. 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, a DLL4 antagonist 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 DLL4 antagonist, the VEGF antagonist, and an anti-angiogenesis agent.

In certain aspects of the invention, other therapeutic agents useful for combination tumor therapy with a DLL4 antagonist (or DLL4 agonist) 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 one aspect, the invention provides a method of treating a disorder (such as a tumor, a cancer, or a cell proliferative disorder) by administering effective amounts of an antagonist of DLL4 (or DLL4 agonist) and/or an angiogenesis inhibitor(s) and one or more chemotherapeutic agents. 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." The administration of the DLL4 antagonist and the chemotherapeutic agent 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. Alternatively, or additionally, the steps can be performed as a combination of both sequentially and simultaneously, 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 chemotherapeutic agent may be administered first, followed by the DLL4 antagonist. However, simultaneous administration or administration of the DLL4 antagonist first is also contemplated. Accordingly, in one aspect, the invention provides methods comprising administration of a DLL4 antagonist (such as an anti-DLL4 antibody), followed by administration of a chemotherapeutic agent. 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.

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.

Relapse Tumor Growth

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 DLL4 antagonist (or DLL4 agonist) 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 DLL4 antagonists are administered simultaneously with cancer therapy. Alternatively, or additionally, the DLL4 antagonist 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., humanized A4.6.1, AVASTIN ® (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 a DLL4 antagonist for blocking or reducing relapse tumor growth or relapse cancer cell growth, e.g., see section entitled Combination Therapies herein.

DLL4

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 acids529-551); cytoplasmic domain (amino acids 552-685). DLL4 nucleic acid and amino acid sequences are known in the art and are further discussed herein. 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 DLL4 can be used. The accession number of human DLL4 is NM_019074, and the accession number of mouse DLL4 is NM_019454.

DLL4 binds the Notch receptors. The evolutionarily conserved Notch pathway is a key regulator of many developmental processes as well as postnatal self-renewing organ systems. From invertebrates to mammals, Notch signaling guides cells through a myriad of cell fate decisions and influences proliferation, differentiation and apoptosis (Miele and Osborne, 1999). The Notch family consists of structurally conserved cell surface receptors that are activated by membrane-bound ligands of the DSL gene family (named for Delta and Serrate from Drosophila and Lag-2 from C. elegans). Mammals have four receptors (Notch1, Notch2, Notch3, Notch4) and five ligands (Jag1, Jag2, DLL1, Dll3 and DLL4).

Upon activation by ligands presented on neighboring cells, Notch receptors undergo successive proteolytic cleavages. This leads to the release of the Notch Intra-Cellular Domain (NICD), which translocates into the nucleus and forms a transcriptional complex with the DNA binding protein, RBP-Jk also know as CSL [for CBF1/Su(H)/Lag-1] and other transcriptional cofactors. The primary target genes of Notch activation include the HES (Hairy/Enhancer of Split) gene family and HES-related genes (Hey, CHF, HRT, HESR), which in turn regulate the downstream transcriptional effectors in a tissue and cell-type specific manner (Iso et al., 2003; Li and Harris, 2005).

DLL4 Modulators

Modulators of DLL4 are molecules that modulate the activity of DLL4, e.g., agonists and antagonists. The term "DLL4 agonist" is used to refer to peptide and non-peptide analogs of DLL4 (such as the multimerized DLL4 described herein), and to other agents provided they have the ability to signal through a native Notch receptor (e.g., Notch1, Notch2, Notch3, Notch4). The term "agonist" is defined in the context of the biological role of a Notch receptor. In certain embodiments, agonists possess the biological activities of a DLL4, as defined above, such as binding a Notch receptor (e.g., Notch1, Notch2, Notch3, Notch4), activating a Notch receptor, and activating a Notch receptor downstream molecular signaling. In some embodiments, DLL4 agonists inhibit endothelial cell proliferation, promote epithelial cell differentiation, and/or promote arterial development. In some embodiments, DLL4 agonists inhibit vascular development.

DLL4 modulators are known in the art, and some are described and exemplified herein. An exemplary and non-limiting list of DLL4 antagonists (such as an anti-DLL4 antibody and a DLL4 immunoadhesin) contemplated is provided herein under "Definitions."

The modulators useful in the present invention can be characterized for their physical/chemical properties and biological functions by various assays known in the art. In some embodiments, DLL4 antagonists are characterized for any one or more of: binding to DLL4, binding to Notch receptor, 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, DLL4 agonists are characterized for any one or more of: binding a Notch receptor (e.g., Notch1, Notch2, Notch3, Notch4), activating a Notch receptor, activating a Notch receptor downstream molecular signaling, inhibiting endothelial cell proliferation, promoting epithelial cell differentiation, and/or promoting arterial development. Methods for characterizing DLL4 antagonists and agonists are known in the art, and some are described and exemplified herein.

Antibodies

DLL4 antibodies are known in the art and some are described and exemplified herein. The anti-DLL4 antibodies 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 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 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 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 (Kd^{-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 (Kd-1 of about 106 to 107 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 and are further discussed herein. 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 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 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 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.

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 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 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 CH3 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 2 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 2, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 2

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, thereby generating a Fc region variant. The Fc region variant may comprise a human Fc region sequence (e.g., a human IgG_1, IgG_2, IgG_3 or IgG_4 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 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 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 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, 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.

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.

Anti-DLL4 antibodies possessing the unique properties described herein can be obtained by screening anti-DLL4 hybridoma clones for the desired properties by any convenient method, some of which are described and exemplified herein. 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 antibody is 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. PNAS (USA) 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, 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 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 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. 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 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. coliλ 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 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, 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, 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.

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. 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: 3). 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: 4). 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 0-0.25M salt).

Immunoconjugates

The invention contemplates immunoconjugates (interchangeably termed "antibody-drug conjugates" or "ADC"), comprising a anti-DLL4 antibodies 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 111In or 90Y 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) 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 105 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 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 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, γ1I, α2I, α3I, N-acetyl-γ1I, PSAG and θI1 (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 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 tc99m or I123, 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 $tc^{99}m$ 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 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), 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 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).

Covalent Modifications to DLL4 polypeptides

Covalent modifications of polypeptide antagonists or agonists (e.g., a polypeptide antagonist fragment, a DLL4 fusion molecule (e.g., a DLL4 imunoadhesin), an anti-DLL4 antibody), are included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the polypeptide, if applicable. Other types of covalent modifications of the polypeptide are introduced into the molecule by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues, or by incorporating a modified amino acid or unnatural amino acid into the growing polypeptide chain, e.g., Ellman et al. *Meth. Enzym.* 202:301-336 (1991); Noren et al. *Science* 244:182 (1989); and, & US Patent application publications 20030108885 and 20030082575.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized

by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is typically performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino-terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification involves chemically or enzymatically coupling glycosides to a polypeptide of the invention. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

Removal of any carbohydrate moieties present on a polypeptide of the invention may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, *et al. Arch. Biochem. Biophys.* 259:52 (1987) and by Edge *et al. Anal. Biochem.*, 118:131 (1981). Enzymatic cleavage of carbohydrate moieties, e.g., on antibodies, can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura *et al. Meth. Enzymol.* 138:350 (1987).

Another type of covalent modification of a polypeptide of the invention comprises linking the polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Pharmaceutical Formulations

Therapeutic formulations comprising an antibody 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, 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.

It is further contemplated that an agent useful in the invention can be introduced to a subject by gene therapy. Gene therapy refers to therapy performed by the administration of a nucleic acid to a subject. In gene therapy applications, genes are introduced into cells in order to achieve in vivo synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy" includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes in vivo. See, e.g., DLL4-SiRNA described in the Examples. It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane. (Zamecnik et al., Proc. Natl. Acad. Sci. USA 83:4143-4146 (1986)). The oligonucleotides can be modified to enhance their uptake, e.g. by substituting their negatively charged phosphodiester groups by uncharged groups. For general reviews of the methods of gene therapy, see, for example, Goldspiel et al. Clinical Pharmacy 12:488-505 (1993); Wu and Wu Biotherapy 3:87-95 (1991); Tolstoshev Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan Science 260:926-932 (1993); Morgan and Anderson Ann. Rev. Biochem. 62:191-217 (1993); and May TIBTECH 11:155-215 (1993). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al.

eds. (1993) Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler (1990) Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

Dosage and Administration

The molecules are administered to a human patient, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes, and/or subcutaneous administration.

In certain embodiments, the treatment of the invention involves the combined administration of a DLL4 antagonist and one or more anti-cancer agents, e.g., anti-angiogenesis agents. In one embodiment, additional anti-cancer agents are present, e.g., one or more different anti-angiogenesis agents, one or more chemotherapeutic agents, etc. The invention also contemplates administration of multiple inhibitors, e.g., multiple antibodies to the same antigen or multiple antibodies to different cancer active molecules. In one embodiment, a cocktail of different chemotherapeutic agents is administered with the DLL4 antagonist and/or one or more anti-angiogenesis agents. The combined administration includes coadministration, using separate formulations or a single pharmaceutical formulation, and/or consecutive administration in either order. For example, a DLL4 antagonist may precede, follow, alternate with administration of the anti-cancer agents, or may be given simultaneously therewith. In one embodiment, there is a time period while both (or all) active agents simultaneously exert their biological activities.

For the prevention or treatment of disease, the appropriate dosage of DLL4 antagonist will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the inhibitor is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the inhibitor, and the discretion of the attending physician. The inhibitor is suitably administered to the patient at one time or over a series of treatments. In a combination therapy regimen, the compositions of the invention are administered in a therapeutically effective amount or a therapeutically synergistic amount. As used herein, a therapeutically effective amount is such that administration of a composition of the invention and/or co-administration of DLL4 antagonist and one or more other therapeutic agents, results in reduction or inhibition of the targeting disease or condition. The effect of the administration of a combination of agents can be additive. In one embodiment, the result of the administration is a synergistic effect. A therapeutically synergistic amount is that amount of DLL4 antagonist and one or

more other therapeutic agents, e.g., an angiogenesis inhibitor, necessary to synergistically or significantly reduce or eliminate conditions or symptoms associated with a particular disease.

Depending on the type and severity of the disease, about 1 μg/kg to 50 mg/kg (e.g. 0.1-20mg/kg) of DLL4 antagonist or angiogenesis inhibitor is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μg/kg to about 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. Typically, the clinician will administered a molecule(s) until a dosage(s) is reached that provides the required biological effect. The progress of the therapy of the invention is easily monitored by conventional techniques and assays.

For example, preparation and dosing schedules for angiogenesis inhibitors, e.g., anti-VEGF antibodies, such as AVASTIN® (Genentech), may be used according to manufacturers' instructions or determined empirically by the skilled practitioner. In another example, preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992).

Efficacy of the Treatment

The efficacy of the treatment of the invention can be measured by various endpoints commonly used in evaluating neoplastic or non-neoplastic disorders. For example, cancer treatments can be evaluated by, e.g., but not limited to, tumor regression, tumor weight or size shrinkage, time to progression, duration of survival, progression free survival, overall response rate, duration of response, and quality of life. Because the anti-angiogenic agents described herein target the tumor vasculature and not necessarily the neoplastic cells themselves, they represent a unique class of anticancer drugs, and therefore can require unique measures and definitions of clinical responses to drugs. For example, tumor shrinkage of greater than 50% in a 2-dimensional analysis is the standard cut-off for declaring a response. However, the inhibitors may cause inhibition of metastatic spread without shrinkage of the primary tumor, or may simply exert a tumouristatic effect. Accordingly, approaches to determining efficacy of the therapy can be employed, including

for example, measurement of plasma or urinary markers of angiogenesis and measurement of response through radiological imaging.

The following Examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

The disclosures of all patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the Examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following Examples, and throughout the specification, by 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 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: 5) (Oligo 1, sense),
AAAGCCATAAGGGCAGTTGTT (SEQ ID NO: 6) (Oligo 1, antisense),
CAACTGCCCTTCAATTTCATT (SEQ ID NO: 7) (Oligo 2, sense),
TGAAATTGAAGGGCAGTTGTT (SEQ ID NO: 8) (Oligo 2, antisense),
TGACCAAGATCTCAACTACTT (SEQ ID NO: 9) (Oligo 3, sense),
GTAGTTGAGATCTTGGTCATT (SEQ ID NO: 10) (Oligo 3, antisense),
GGCCAACTATGCTTGTGAATT (SEQ ID NO: 11) (Oligo 4, sense),
TTCACAAGCATAGTTGGCCTT (SEQ ID NO: 12) (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 ImmunoRescarch) 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: 13)

Reverse: CAT CAT CAT TAT CAT CAT CAT TGT C (SEQ ID NO: 14)

Probe: AAT TCT TGG AAA AGT GGC AAG ACC AAA AT (SEQ ID NO: 15)

VEGFR2

Forward: CTT TCC ACC AGC AGG AAG TAG (SEQ ID NO: 16)

Reverse: TGC AGT CCG AGG TCC TTT (SEQ ID NO: 17)

Probe: CGC ATT TGA TTT TCA TTT CGA CAA CAG A (SEQ ID NO: 18)

GAPDH

Forward: GAA GAT GGT GAT GGG ATT TC (SEQ ID NO: 19)

Reverse: GAA GGT GAA GGT CGG AGT C (SEQ ID NO: 20)

Probe: CAA GCT TCC CGT TCT CAG CC (SEQ ID NO: 21)

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 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 5a 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 5b). The results of this experiment are shown in Figure 5b. 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 5c, 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 5d, 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 was performed. 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 5e. 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 4: 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. 1a). 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. 1b). 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. 1e). These findings suggest that the activation status of DLL4/Notch signaling is closely associated with EC proliferation.

Example 5: 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. 1d). A significant increase of Ki67 labeling in ECs was observed,

indicating elevated EC proliferation (Fig. 1h). Therefore, this hyperproliferative phenotype of retinal ECs upon DLL4 blockade in neonatal mice corroborated the in vitro findings.

Example 6: 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 6), 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. 1f), 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. 1g). Conversely, activation of Notch by immobilized DLL4 suppressed the expression of VEGFR2 (Fig. 1g). 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 7: 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. 2a), 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. 2c). 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 8: 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. 2b) 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 9: 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. 3a-d). In HM7, Colo205 and Calu6 xenograft tumor models (Figs. 3a-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.3d).

We also investigated the impact of blocking DLL4 and/or VEGF on numerous tumor growth in preclinical tumor models (Figs. 3e-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 10: 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.3g). In contrast, anti-VEGF had a completely opposite effect (Fig.3g), although both treatments exhibited similar efficacy in this model.

Example 11: 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. 2a), 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. 3h). 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 12: DLL4/Notch is dispensable 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.4). 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.4). These results support the notion that DLL4/Notch signaling is largely restricted to the vascular system.

Example 13: 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. 2d). 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.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. However, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for treating a tumor, cancer, or cell proliferative disorder comprising administering an effective amount of a DLL4 antagonist to a subject in need of such treatment, whereby the tumor, cancer or cell proliferative disorder is treated.

2. The method of claim 1, wherein the tumor, cancer or cell proliferative disorder is colon cancer, lung cancer, melanoma or lymphoma.

3. A method for treating a pathological condition associated with angiogenesis comprising administering an effective amount of a DLL4 antagonist to a subject in need of such treatment, whereby the pathological condition associated with angiogenesis is treated, wherein the DLL4 antagonist is capable of stimulating endothelial cell proliferation, inhibiting endothelial cell differentiation, inhibiting arterial development, or inhibiting vascular perfusion.

4. The method of claim 3, wherein the pathological condition associated with angiogenesis is a tumor, a cancer, and/or a cell proliferative disorder.

5. The method of claim 3, wherein the pathological condition associated with angiogenesis is an intraocular neovascular disease.

6. A method for stimulating endothelial cell proliferation in a subject in need of such treatment comprising administering an effective amount of a DLL4 agonist to the subject, whereby endothelial cell proliferation is stimulated.

7. A method for reducing or inhibiting endothelial cell differentiation in a subject in need of such treatment comprising administering an effective amount of a DLL4 antagonist to the subject, whereby endothelial cell differentiation is inhibited.

8. A method of reducing or inhibiting arterial development in a subject in need of such treatment comprising administering an effective amount of a DLL4 antagonist to the subject, whereby arterial development is inhibited.

9. A method of reducing or inhibiting tumor vascular perfusion in a subject in need of such treatment comprising administering an effective amount of a DLL4 antagonist to the subject, whereby tumor vascular perfusion is inhibited.

10. The method of any of claims 1-9, further comprising administering to the subject an effective amount of an anti-angiogenic agent.

11. The method of claim 10, wherein the anti-angiogenic agent is administered prior to or subsequent to the administration of the DLL4 antagonist.

12. The method of claim 10, wherein the ~~anti-~~angiogenic agent is administered concurrently with the DLL4 antagonist.

13. The method of any of claims 10-12, wherein the anti-angiogenic agent is an antagonist of vascular endothelial cell growth factor (VEGF).

14. The method of claim 13, wherein the VEGF antagonist is an anti-VEGF antibody.

15. The method of claim 14, wherein the anti-VEGF antibody is bevacizumab.

16. The method of any of claims 1-15 further comprising administering an effective amount of a chemotherapeutic agent.

17. 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 a DLL4 antagonist in combination with the anti-angiogenic agent, thereby enhancing said anti-angiogenic agent's inhibitory activity.

18. The method of claim 17, wherein the pathological condition associated with angiogenesis is a tumor, a cancer, and/or a cell proliferative disorder.

19. The method of claim 17, wherein the pathological condition associated with angiogenesis is an intraocular neovascular disease.

20. The method of any of claims 1-19 wherein the DLL4 antagonist is an anti-DLL4 antibody.

21. The method of any of claims 1-19 wherein the DLL4 antagonist is a DLL4 immunoadhesin.

22. The method of claim 20, wherein the DLL4 antibody is a monoclonal antibody.

23. The method of claim 20, wherein the DLL4 antibody is a human antibody, a humanized antibody, or a chimeric antibody.

24. The method of claim 20, wherein the DLL4 antibody is an antibody fragment.

25. The method of claim 24, wherein the antibody fragment is a Fab, Fab', Fab'-SH, $F(ab')_2$, or scFv.

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