



(86) Date de dépôt PCT/PCT Filing Date: 2010/06/23
(87) Date publication PCT/PCT Publication Date: 2011/01/13
(85) Entrée phase nationale/National Entry: 2011/12/20
(86) N° demande PCT/PCT Application No.: US 2010/039686
(87) N° publication PCT/PCT Publication No.: 2011/005581
(30) Priorité/Priority: 2009/06/24 (US61/220,077)

(51) Cl.Int./Int.Cl. *A61K 39/395* (2006.01)

(71) Demandeur/Applicant:
LPATH, INC., US

(72) Inventeurs/Inventors:
PEBAY, ALICE MARIE, US;
TURNLEY, ANN MAREE, US

(74) Agent: MARKS & CLERK

(54) Titre : PROCEDES D'AUGMENTATION DE LA DIFFERENCIATION NEURONALE A L'AIDE D'ANTICORPS
DIRIGES CONTRE L'ACIDE LYSOPHOSPHATIDIQUE
(54) Title: METHODS OF INCREASING NEURONAL DIFFERENTIATION USING ANTIBODIES TO
LYSOPHOSPHATIDIC ACID

(57) **Abrégé/Abstract:**

Methods are provided for increasing neuronal differentiation of neuronal stem cells using antibodies that bind lysophosphatidic acid (LPA). Particularly preferred antibodies to LPA are monoclonal antibodies, including humanized monoclonal antibodies to LPA. Such antibodies, and derivatives and variants thereof, can be used in increasing neuronal differentiation, and in treatment and/or prevention of injuries, diseases, or conditions associated with insufficient neuronal differentiation and/or with elevated LPA levels in neural tissues.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 January 2011 (13.01.2011)



(10) International Publication Number
WO 2011/005581 A3

(51) International Patent Classification:
C12N 5/02 (2006.01)

(21) International Application Number:
PCT/US2010/039686

(22) International Filing Date:
23 June 2010 (23.06.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/220,077 24 June 2009 (24.06.2009) US

(71) Applicant (for all designated States except US): **LPATH, INC.** [US/US]; 6335 Ferris Square Suite A, San Diego, CA 92121-3249 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **PEBAY, Alice, Marie** [FR/AU]; 6335 Ferris Square Suite A, San Diego, CA 92121-3249 (US). **TURNLEY, Ann, Maree** [AU/AU]; 6335 Ferris Square Suite A, San Diego, CA 92121-3249 (US).

(74) Agents: **CHAMBERS, Daniel, M.** et al.; Bio Technology Law Group, 12707 High Bluff Drive, Suite 200, San Diego, CA 92130-2037 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

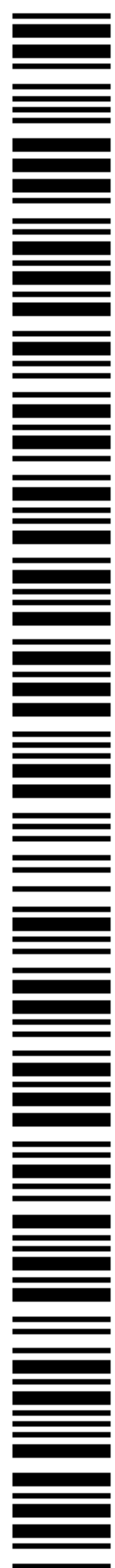
Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(88) Date of publication of the international search report:
3 March 2011

(54) Title: METHODS OF INCREASING NEURONAL DIFFERENTIATION USING ANTIBODIES TO LYSOPHOSPHATIDIC ACID

(57) Abstract: Methods are provided for increasing neuronal differentiation of neuronal stem cells using antibodies that bind lysophosphatidic acid (LPA). Particularly preferred antibodies to LPA are monoclonal antibodies, including humanized monoclonal antibodies to LPA. Such antibodies, and derivatives and variants thereof, can be used in increasing neuronal differentiation, and in treatment and/or prevention of injuries, diseases, or conditions associated with insufficient neuronal differentiation and/or with elevated LPA levels in neural tissues.



WO 2011/005581 A3

METHODS OF INCREASING NEURONAL DIFFERENTIATION USING ANTIBODIES TO LYSOPHOSPHATIDIC ACID

TECHNICAL FIELD

The present invention relates to methods for increasing neuronal differentiation of neuronal stem cells using antibodies that bind lysophosphatidic acid (LPA). Particularly preferred antibodies to LPA are monoclonal antibodies, preferably humanized monoclonal antibodies to LPA. Such antibodies, and derivatives and variants thereof, can be used in increasing neuronal differentiation, and in treatment and/or prevention of injuries, diseases or conditions associated with insufficient neuronal differentiation.

BACKGROUND OF THE INVENTION

1. Introduction.

The following description includes information that may be useful in understanding the present invention. It is not an admission that any such information is prior art, or relevant, to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art or even particularly relevant to the presently claimed invention.

2. Background.

A. Neuronal differentiation and the role of LPA

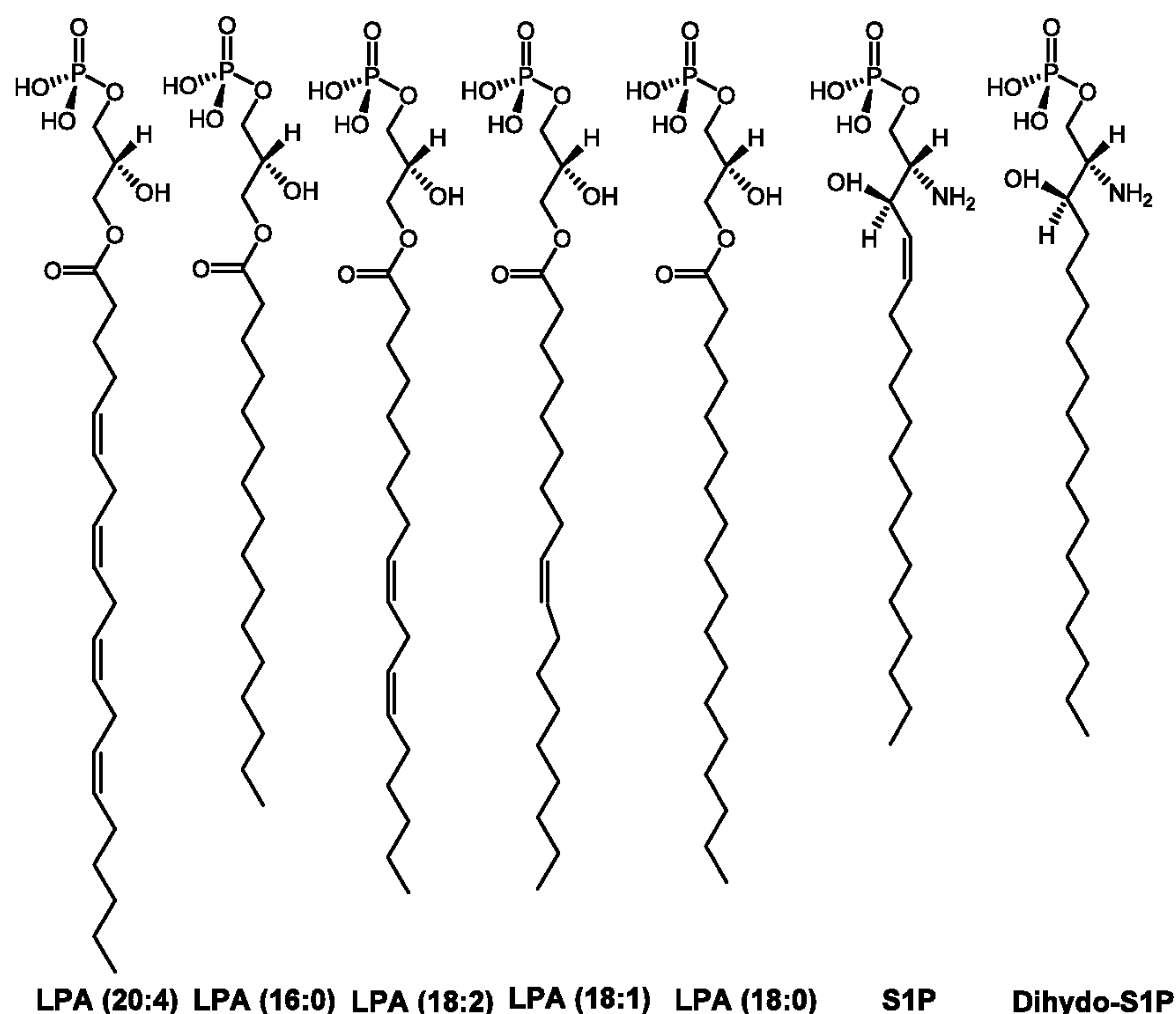
Neural stem cells (NSC) are found in areas of neurogenesis in the central nervous system (CNS) and can migrate to sites of neural injury. Thus NSC are under study with the goal of replacing neurons and restoring connections in a neurodegenerative environment. Dottori, M.. et al. (2008) "Lysophosphatidic Acid Inhibits Neuronal Differentiation of Neural Stem/Progenitor Cells Derived from Human Embryonic Stem Cells." Stem Cells 26: 1146-1154.

Following injury, hemorrhage or trauma to the nervous system, levels of LPA within the nervous system are believed to reach high levels. Dottori et al. (ibid) have shown that LPA levels equivalent to those reached after injury can inhibit neuronal differentiation of human NSC, suggesting that high levels of LPA within the CNS following injury might inhibit differentiation of NSC into neurons, thus inhibiting endogenous neuronal regeneration. Modulating LPA signaling may thus have a significant impact in nervous system injury, allowing new potential therapeutic approaches.

B. LPA and other Lysolipids

Lysolipids are low molecular weight lipids that contain a polar head group and a single hydrocarbon backbone, due to the absence of an acyl group at one or both possible positions of acylation. Relative to the polar head group at sn-3, the hydrocarbon chain can be at the sn-2 and/or sn-1 position(s) (the term "lyso," which originally related to hemolysis, has been redefined by IUPAC to refer to deacylation). See "Nomenclature of Lipids, www.chem.qmul.ac.uk/iupac/lipid/lip1n2.html. These lipids are representative of signaling, bioactive lipids,

and their biologic and medical importance highlight what can be achieved by targeting lipid signaling molecules for therapeutic, diagnostic/prognostic, or research purposes (Gardell, *et al.* (2006), Trends in Molecular Medicine, vol 12: 65-75). Two particular examples of medically important lysolipids are LPA (glycerol backbone) and S1P (sphingoid backbone). Other lysolipids include sphingosine, lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (lysosphingomyelin), ceramide, ceramide-1-phosphate, sphinganine (dihydrosphingosine), dihydrosphingosine-1-phosphate and N-acetyl-ceramide-1-phosphate. In contrast, the plasmalogens, which contain an *O*-alkyl (-O-CH₂-) or *O*-alkenyl ether at the C-1 (*sn*1) and an acyl at C-2, are excluded from the lysolipid genus. The structures of selected LPAs, S1P, and dihydro S1P are presented below.



LPA is not a single molecular entity but a collection of endogenous structural variants with fatty acids of varied lengths and degrees of saturation (Fujiwara, *et al.* (2005), J Biol Chem, vol. 280: 35038-35050). The structural backbone of the LPAs is derived from glycerol-based phospholipids such as phosphatidylcholine (PC) or phosphatidic acid (PA). In the case of lysosphingolipids such as S1P, the fatty acid of the ceramide backbone at *sn*-2 is missing. The structural backbone of S1P, dihydro S1P (DHS1P) and sphingosylphosphorylcholine (SPC) is based on sphingosine, which is derived from sphingomyelin.

LPA and S1P are bioactive lipids (signaling lipids) that regulate various cellular signaling pathways by binding to the same class of multiple transmembrane domain G protein-coupled (GPCR) receptors (Chun J, Rosen H (2006), Current Pharm Des, vol. 12: 161-171, and Moolenaar, WH (1999), Experimental Cell Research, vol. 253: 230-238). The S1P receptors are designated as S1P₁, S1P₂, S1P₃, S1P₄ and S1P₅ (formerly EDG-1, EDG-5/AGR16, EDG-3, EDG-6 and EDG-8) and the LPA receptors designated as LPA₁, LPA₂, LPA₃ (formerly,

EDG-2, EDG-4, and EDG-7). A fourth LPA receptor of this family has been identified for LPA (LPA₄), and other putative receptors for these lysophospholipids have also been reported.

LPA have long been known as precursors of phospholipid biosynthesis in both eukaryotic and prokaryotic cells, but LPA have emerged only recently as signaling molecules that are rapidly produced and released by activated cells, notably platelets, to influence target cells by acting on specific cell-surface receptor (see, e.g., Moolenaar, *et al.* (2004), *BioEssays*, vol. 26: 870-881, and van Leewen *et al.* (2003), *Biochem Soc Trans*, vol 31: 1209-1212). Besides being synthesized and processed to more complex phospholipids in the endoplasmic reticulum, LPA can be generated through the hydrolysis of pre-existing phospholipids following cell activation; for example, the sn-2 position is commonly missing a fatty acid residue due to deacylation, leaving only the sn-1 hydroxyl esterified to a fatty acid. Moreover, a key enzyme in the production of LPA, autotoxin (lysoPLD/NPP2), may be the product of an oncogene, as many tumor types up-regulate autotoxin (Brindley, D. (2004), *J Cell Biochem*, vol. 92: 900-12). The concentrations of LPA in human plasma and serum have been reported, including determinations made using a sensitive and specific LC/MS procedure (Baker, *et al.* (2001), *Anal Biochem*, vol 292: 287-295). For example, in freshly prepared human serum allowed to sit at 25°C for one hour, LPA concentrations have been estimated to be approximately 1.2 mM, with the LPA analogs 16:0, 18:1, 18:2, and 20:4 being the predominant species. Similarly, in freshly prepared human plasma allowed to sit at 25°C for one hour, LPA concentrations have been estimated to be approximately 0.7 mM, with 18:1 and 18:2 LPA being the predominant species.

LPA influences a wide range of biological responses, ranging from induction of cell proliferation, stimulation of cell migration and neurite retraction, gap junction closure, and even slime mold chemotaxis (Goetzi, *et al.* (2002), *Scientific World Journal*, vol. 2: 324-338). The body of knowledge about the biology of LPA continues to grow as more and more cellular systems are tested for LPA responsiveness. For instance, it is now known that, in addition to stimulating cell growth and proliferation, LPA promote cellular tension and cell-surface fibronectin binding, which are important events in wound repair and regeneration (Moolenaar, *et al.* (2004), *BioEssays*, vol. 26: 870-881). Recently, anti-apoptotic activity has also been ascribed to LPA, and it has recently been reported that peroxisome proliferation receptor gamma is a receptor/target for LPA (Simon, *et al.* (2005), *J Biol Chem*, vol. 280: 14656-14662).

LPA has proven to be difficult targets for antibody production, although there has been a report in the scientific literature of the production of polyclonal murine antibodies against LPA (Chen, *et al.* (2000), *Med Chem Lett*, vol 10: 1691-3).

3. Definitions.

Before describing the instant invention in detail, several terms used in the context of the present invention will be defined. In addition to these terms, others are defined elsewhere in the specification, as necessary. Unless otherwise expressly defined herein, terms of art used in this specification will have their art-recognized meanings.

The term “antibody” (“Ab”) or “immunoglobulin” (Ig) refers to any form of a peptide, polypeptide derived from, modeled after or encoded by, an immunoglobulin gene, or fragment thereof, that is capable of binding an antigen or epitope. See, e.g., Immunobiology, Fifth Edition, C. A. Janeway, P. Travers, M., Walport, M.J. Shlomchik, ed. Garland Publishing (2001).

An “antibody derivative” is an immune-derived moiety, i.e., a molecule that is derived from an antibody. This comprehends, for example, antibody variants, antibody fragments, chimeric antibodies, humanized antibodies, multivalent antibodies, antibody conjugates and the like, which retain a desired level of binding activity for antigen.

As used herein, “antibody fragment” refers to a portion of an intact antibody that includes the antigen binding site or variable regions of an intact antibody, wherein the portion can be free of the constant heavy chain domains (e.g., CH2, CH3, and CH4) of the Fc region of the intact antibody. Alternatively, portions of the constant heavy chain domains (e.g., CH2, CH3, and CH4) can be included in the “antibody fragment”. Antibody fragments retain antigen binding ability and include Fab, Fab', F(ab')₂, Fd, and Fv fragments; diabodies; triabodies; single-chain antibody molecules (sc-Fv); minibodies, nanobodies, and multispecific antibodies formed from antibody fragments. 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. By way of example, a Fab fragment also contains the constant domain of a light chain and the first constant domain (CH1) of a heavy chain. “Fv” is the minimum antibody fragment that contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site. “Single-chain Fv” or “sFv” antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the sFv to form the desired structure for antigen binding. For a review of sFv, see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

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 carboxyl terminus of the heavy chain CH1 domain including one or more cysteine(s) 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.

An "antibody variant" refers herein to a molecule which differs in amino acid sequence from a native antibody (e.g., an anti-LPA antibody) amino acid sequence by virtue of addition, deletion and/or substitution of one or more amino acid residue(s) in the antibody sequence and which retains at least one desired activity of the parent anti-binding antibody. Desired activities can include the ability to bind the antigen specifically, the ability to inhibit proliferation in vitro, the ability to inhibit angiogenesis in vivo, and the ability to alter cytokine profile in vitro. The amino acid change(s) in an antibody variant may be within a variable region or a constant region of a light chain and/or a heavy chain, including in the Fc region, the Fab region, the CH₁ domain, the CH₂ domain, the CH₃ domain, and the hinge region. In one embodiment, the variant comprises one or more amino acid substitution(s) in one or more hypervariable region(s) of the parent antibody. For example, the variant may comprise at least one, e.g. from about one to about ten, and preferably from about two to about five, substitutions in one or more hypervariable regions of the parent antibody. Ordinarily, the variant will have an amino acid sequence having at least 75% amino acid sequence identity with the parent antibody heavy or light chain variable domain sequences, more preferably at least 65%, more preferably at 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the parent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology. The variant retains the ability to bind LPA and preferably has desired activities which are superior to those of the parent antibody. For example, the variant may have a stronger binding affinity, enhanced ability to reduce angiogenesis and/or halt tumor progression. To analyze such desired properties (for example less immunogenic, longer half-life, enhanced stability, enhanced potency), one should compare a Fab form of the variant to a Fab form of the parent antibody or a full length form of the variant to a full length form of the parent antibody, for example, since it has been found that the format of the anti-sphingolipid antibody impacts its activity in the biological activity assays disclosed herein. The variant antibody of particular interest herein can be one which displays at least about 10 fold, preferably at least about % 5, 25, 59, or more of at least one desired activity. The preferred variant is one that has superior biophysical properties as measured in vitro or superior activities biological as measured in vitro or in vivo when compared to the parent antibody.

The term "antigen" refers to a molecule that is recognized and bound by an antibody molecule or immune-derived moiety that binds to the antigen. The specific portion of an antigen that is bound by an antibody is termed the "epitope."

An "anti-LPA antibody" refers to any antibody or antibody-derived molecule that binds lysophosphatidic acid. The terms "anti-LPA antibody," "antibody that binds LPA" and "antibody reactive with LPA" are interchangeable.

A "bioactive lipid" refers to a lipid signaling molecule. Bioactive lipids are distinguished from structural lipids (e.g., membrane-bound phospholipids) in that they mediate extracellular and/or intracellular signaling and

thus are involved in controlling the function of many types of cells by modulating differentiation, migration, proliferation, secretion, survival, and other processes.

The term “biologically active,” in the context of an antibody or antibody fragment or variant, refers to an antibody or antibody fragment or antibody variant that is capable of binding the desired epitope and in some ways exerting a biologic effect. Biological effects include, but are not limited to, the modulation of a growth signal, the modulation of an anti-apoptotic signal, the modulation of an apoptotic signal, the modulation of the effector function cascade, and modulation of other ligand interactions.

A “biomarker” is a specific biochemical in the body which has a particular molecular feature that makes it useful for measuring the progress of disease or the effects of treatment.

A “carrier” refers to a moiety adapted for conjugation to a hapten, thereby rendering the hapten immunogenic. A representative, non-limiting class of carriers is proteins, examples of which include albumin, keyhole limpet hemocyanin, hemagglutinin, tetanus, and diphtheria toxoid. Other classes and examples of carriers suitable for use in accordance with the invention are known in the art. These, as well as later discovered or invented naturally occurring or synthetic carriers, can be adapted for application in accordance with the invention.

The term “chemotherapeutic agent” means anti-cancer and other anti-hyperproliferative agents. Thus chemotherapeutic agents are a subset of therapeutic agents in general. Chemotherapeutic agents include, but are not limited to: DNA damaging agents and agents that inhibit DNA synthesis: anthracyclines (doxorubicin, daunorubicin, epirubicin), alkylating agents (bendamustine, busulfan, carboplatin, carmustine, chlorambucil, cyclophosphamide, dacarbazine, hexamethylmelamine, ifosfamide, lomustine, mechlorethamine, melphalan, mitotane, mytomicin, pipobroman, procarbazine, streptozocin, thiotepa, and triethylenemelamine), platinum derivatives (cisplatin, carboplatin, cis diammine-dichloroplatinum), and topoisomerase inhibitors (Camptosar); anti-metabolites such as capecitabine, chlorodeoxyadenosine, cytarabine (and its activated form, ara-CMP), cytosine arabinoside, dacarbazine, floxuridine, fludarabine, 5-fluorouracil, 5-DFUR, gemcitabine, hydroxyurea, 6-mercaptopurine, methotrexate, pentostatin, trimetrexate, 6-thioguanine); anti-angiogenics (bevacizumab, thalidomide, sunitinib, lenalidomide, TNP-470, 2-methoxyestradiol, ranibizumab, sorafenib, erlotinib, bortezomib, pegaptanib, endostatin); vascular disrupting agents (flavonoids/flavones, DMXAA, combretastatin derivatives such as CA4DP, ZD6126, AVE8062A, etc.); biologics such as antibodies (Herceptin, Avastin, Panorex, Rituxin, Zevalin, Mylotarg, Campath, Bexxar, Erbitux); endocrine therapy: aromatase inhibitors (4-hydroandrostendione, exemestane, aminoglutethimide, anastrozole, letrozole), anti-estrogens (Tamoxifen, Toremifene, Raloxifene, Faslodex), steroids such as dexamethasone; immuno-modulators: cytokines such as IFN-beta and IL2), inhibitors to integrins, other adhesion proteins and matrix metalloproteinases); histone deacetylase inhibitors like suberoylanilide hydroxamic acid; inhibitors of signal transduction such as inhibitors of tyrosine kinases like imatinib (Gleevec); inhibitors of heat shock proteins like 17-N-allylamino-17-demethoxygeldanamycin; retinoids such as all trans retinoic acid; inhibitors of growth factor receptors or the growth factors themselves; anti-mitotic compounds and/or tubulin-depolymerizing agents such as the taxoids (paclitaxel, docetaxel, taxotere, BAY 59-8862), navelbine, vinblastine, vincristine, vindesine and vinorelbine; anti-inflammatories such as COX inhibitors and cell cycle regulators, e.g., check point regulators and telomerase inhibitors.

The term “chimeric” antibody (or immunoglobulin) refers to a molecule comprising a heavy and/or light chain which is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly, *et al.*, *infra*; Morrison *et al.*, Proc. Natl. Acad. Sci. U.S.A., vol. 81:6851 (1984)).

The term “combination therapy” refers to a therapeutic regimen that involves the provision of at least two distinct therapies to achieve an indicated therapeutic effect. For example, a combination therapy may involve the administration of two or more chemically distinct active ingredients, for example, a fast-acting chemotherapeutic agent and an anti-lipid antibody. Alternatively, a combination therapy may involve the administration of an anti-lipid antibody and/or one or more chemotherapeutic agents, alone or together with the delivery of another treatment, such as radiation therapy and/or surgery. In the context of the administration of two or more chemically distinct active ingredients, it is understood that the active ingredients may be administered as part of the same composition or as different compositions. When administered as separate compositions, the compositions comprising the different active ingredients may be administered at the same or different times, by the same or different routes, using the same or different dosing regimens, all as the particular context requires and as determined by the attending physician. Similarly, when one or more anti-lipid antibody species, for example, an anti-LPA antibody, alone or in conjunction with one or more chemotherapeutic agents are combined with, for example, radiation and/or surgery, the drug(s) may be delivered before or after surgery or radiation treatment.

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

“Effective concentration” refers to the absolute, relative, and/or available concentration and/or activity, for example of certain undesired bioactive lipids. In other words, the effective concentration of a bioactive lipid is the amount of lipid available, and able, to perform its biological function. In the present invention, an immune-derived moiety such as, for example, a monoclonal antibody directed to a bioactive lipid (such as, for example, C1P) is able to reduce the effective concentration of the lipid by binding to the lipid and rendering it unable to perform its biological function. In this example, the lipid itself is still present (it is not degraded by the antibody, in other words) but can no longer bind its receptor or other targets to cause a downstream effect, so “effective concentration” rather than absolute concentration is the appropriate measurement. Methods and assays exist for directly and/or indirectly measuring the effective concentration of bioactive lipids.

An “epitope” or “antigenic determinant” refers to that portion of an antigen that reacts with an antibody antigen-binding portion derived from an antibody.

A "fully human antibody" can refer to an antibody produced in a genetically engineered (i.e., transgenic) mouse (e.g., from Medarex) that, when presented with an immunogen, can produce a human antibody that does not necessarily require CDR grafting. These antibodies are fully human (100% human protein sequences) from animals such as mice in which the non-human antibody genes are suppressed and replaced with human antibody gene expression. The applicants believe that antibodies could be generated against bioactive lipids when presented to these genetically engineered mice or other animals that might be able to produce human frameworks for the relevant CDRs.

A "hapten" is a substance that is non-immunogenic but can react with an antibody or antigen-binding portion derived from an antibody. In other words, haptens have the property of antigenicity but not immunogenicity. A hapten is generally a small molecule that can, under most circumstances, elicit an immune response (i.e., act as an antigen) only when attached to a carrier, for example, a protein, polyethylene glycol (PEG), colloidal gold, silicone beads, or the like. The carrier may be one that also does not elicit an immune response by itself.

The term "heteroconjugate antibody" can refer to two covalently joined antibodies. Such antibodies can be prepared using known methods in synthetic protein chemistry, including using crosslinking agents. As used herein, the term "conjugate" refers to molecules formed by the covalent attachment of one or more antibody fragment(s) or binding moieties to one or more polymer molecule(s).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. Or, looked at another way, a humanized antibody is a human antibody that also contains selected sequences from non-human (e.g., murine) antibodies in place of the human sequences. A humanized antibody can include conservative amino acid substitutions or non-natural residues from the same or different species that do not significantly alter its binding and/or biologic activity. Such antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulins. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, camel, bovine, goat, or rabbit having the desired properties. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. The CDRs can be placed into any of a variety of frameworks as long as a desired level of antigen binding is retained.

Furthermore, humanized antibodies can comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. Thus, in general, a humanized antibody will comprise all of at least one, and in one aspect two, variable domains, in which all or all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), or that of a human immunoglobulin. See, e.g., Cabilly, *et al.*, U.S. Pat. No. 4,816,567; Cabilly, *et al.*, European Patent No. 0,125,023 B1; Boss, *et al.*, U.S. Pat. No. 4,816,397; Boss, *et al.*, European

Patent No. 0,120,694 B1; Neuberger, *et al.*, WO 86/01533; Neuberger, *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Pat. No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Padlan, *et al.*, European Patent Application No. 0,519,596 A1; Queen, *et al.* (1989), Proc. Nat'l Acad. Sci. USA, vol. 86:10029-10033). For further details, see Jones *et al.*, Nature 321:522-525 (1986); Reichmann *et al.*, Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992) and Hansen, WO2006105062.

The term "hyperproliferative disorder" refers to diseases and disorders associated with, the uncontrolled proliferation of cells, including but not limited to uncontrolled growth of organ and tissue cells resulting in cancers and benign tumors. Hyperproliferative disorders associated with endothelial cells can result in diseases of angiogenesis such as angiomas, endometriosis, obesity, age-related macular degeneration and various retinopathies, as well as the proliferation of endothelial cells and smooth muscle cells that cause restenosis as a consequence of stenting in the treatment of atherosclerosis. Hyperproliferative disorders involving fibroblasts (i.e., fibrogenesis) include but are not limited to disorders of excessive scarring (i.e., fibrosis) such as age-related macular degeneration, cardiac remodeling and failure associated with myocardial infarction, excessive wound healing such as commonly occurs as a consequence of surgery or injury, keloids, and fibroid tumors and stenting.

An "immunogen" is a molecule capable of inducing a specific immune response, particularly an antibody response in an animal to whom the immunogen has been administered. In the instant invention, the immunogen is a derivatized bioactive lipid conjugated to a carrier, i.e., a "derivatized bioactive lipid conjugate". The derivatized bioactive lipid conjugate used as the immunogen may be used as capture material for detection of the antibody generated in response to the immunogen. Thus the immunogen may also be used as a detection reagent. Alternatively, the derivatized bioactive lipid conjugate used as capture material may have a different linker and/or carrier moiety from that in the immunogen.

To "inhibit," particularly in the context of a biological phenomenon, means to decrease, suppress or delay. For example, a treatment yielding "inhibition of tumorigenesis" may mean that tumors do not form at all, or that they form more slowly, or are fewer in number than in the untreated control.

An "isolated" antibody is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would 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 word "label" when used herein refers to a detectable compound or composition, such as one that is conjugated directly or indirectly to the antibody. The label may itself be detectable by itself (e.g., radioisotope

labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition that is detectable.

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata et al. Protein Eng. 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H - C_H1 - V_H - C_H1) that form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

In the context of this invention, a "liquid composition" refers to one that, in its filled and finished form as provided from a manufacturer to an end user (e.g., a doctor or nurse), is a liquid or solution, as opposed to a solid. Here, "solid" refers to compositions that are not liquids or solutions. For example, solids include dried compositions prepared by lyophilization, freeze-drying, precipitation, and similar procedures.

The term "metabolites" refers to compounds from which LPAs are made, as well as those that result from the degradation of LPAs; that is, compounds that are involved in the lysophospholipid metabolic pathways. The term "metabolic precursors" may be used to refer to compounds from which sphingolipids are made.

The term "monoclonal antibody" (mAb) as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, or to said population of antibodies. The individual antibodies comprising the population are essentially identical, except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler, et al., Nature 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson, et al., Nature 352:624-628 (1991) and Marks, et al., J. Mol. Biol. 222:581-597 (1991), for example, or by other methods known in the art. The monoclonal antibodies herein specifically include chimeric antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison, et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984)).

"Monotherapy" refers to a treatment regimen based on the delivery of one therapeutically effective compound, whether administered as a single dose or several doses over time.

The term "multispecific antibody" can refer to an antibody, or a monoclonal antibody, having binding properties for at least two different epitopes. In one embodiment, the epitopes are from the same antigen. In

another embodiment, the epitopes are from two or more different antigens. Methods for making multispecific antibodies are known in the art. Multispecific antibodies include bispecific antibodies (having binding properties for two epitopes), trispecific antibodies (three epitopes) and so on. For example, multispecific antibodies can be produced recombinantly using the co-expression of two or more immunoglobulin heavy chain/light chain pairs. Alternatively, multispecific antibodies can be prepared using chemical linkage. One of skill can produce multispecific antibodies using these or other methods as may be known in the art. Multispecific antibodies include multispecific antibody fragments.

“Neoplasia” or “cancer” refers to abnormal and uncontrolled cell growth. A “neoplasm”, or tumor or cancer, is an abnormal, unregulated, and disorganized proliferation of cell growth, and is generally referred to as cancer. A neoplasm may be benign or malignant. A neoplasm is malignant, or cancerous, if it has properties of destructive growth, invasiveness, and metastasis. Invasiveness refers to the local spread of a neoplasm by infiltration or destruction of surrounding tissue, typically breaking through the basal laminae that define the boundaries of the tissues, thereby often entering the body's circulatory system. Metastasis typically refers to the dissemination of tumor cells by lymphatics or blood vessels. Metastasis also refers to the migration of tumor cells by direct extension through serous cavities, or subarachnoid or other spaces. Through the process of metastasis, tumor cell migration to other areas of the body establishes neoplasms in areas away from the site of initial appearance.

“Neural” means pertaining to nerves. Nerves are bundles of fibers made up of neurons.

“Neural stem cells” (NSCs) are the self-renewing, multipotent cells that differentiate into the main phenotypes of the nervous system. NSCs give rise to glial and neuronal cells. Neuronal stem cells give rise to neuronal cells. Neural progenitor cells (NPCs) are the progeny of stem cell division that normally undergo a limited number of replication cycles in vivo.

“Neuron” refers to an excitable cell type in the nervous system that processes and transmits information by electrochemical signalling. Neurons are the core components of the CNS (brain and spinal cord) and the peripheral nerves. “Neuronal” means “pertaining to neurons.”

“Neuronal differentiation” is the conversion of neural stem cells toward the mature cell types of the nervous system, such as neurons, astrocytes, etc. Such differentiation occurs in vivo but can be caused to occur in vitro in model systems such as neurospheres. Differentiation may be a multistep or multistage process and thus multiple phases or steps of differentiation can be studied in vitro.

The “parent” antibody herein is one that is encoded by an amino acid sequence used for the preparation of the variant. The parent antibody may be a native antibody or may already be a variant, e.g., a chimeric antibody. For example, the parent antibody may be a humanized or human antibody.

A “patentable” composition, process, machine, or article of manufacture according to the invention means that the subject matter satisfies all statutory requirements for patentability at the time the analysis is performed. For example, with regard to novelty, non-obviousness, or the like, if later investigation reveals that one or more claims encompass one or more embodiments that would negate novelty, non-obviousness, *etc.*, the claim(s), being limited by definition to “patentable” embodiments, specifically exclude the non-patentable

embodiment(s). Also, the claims appended hereto are to be interpreted both to provide the broadest reasonable scope, as well as to preserve their validity. Furthermore, the claims are to be interpreted in a way that (1) preserves their validity and (2) provides the broadest reasonable interpretation under the circumstances, if one or more of the statutory requirements for patentability are amended or if the standards change for assessing whether a particular statutory requirement for patentability is satisfied from the time this application is filed or issues as a patent to a time the validity of one or more of the appended claims is questioned.

The term “pharmaceutically acceptable salt” refers to a salt, such as used in formulation, which retains the biological effectiveness and properties of the agents and compounds of this invention and which are is biologically or otherwise undesirable. In many cases, the agents and compounds of this invention are capable of forming acid and/or base salts by virtue of the presence of charged groups, for example, charged amino and/or carboxyl groups or groups similar thereto. Pharmaceutically acceptable acid addition salts may be prepared from inorganic and organic acids, while pharmaceutically acceptable base addition salts can be prepared from inorganic and organic bases. For a review of pharmaceutically acceptable salts (see Berge, *et al.* (1977) J. Pharm. Sci., vol. 66, 1-19).

A “plurality” means more than one.

The terms “separated”, “purified”, “isolated”, and the like mean that one or more components of a sample contained in a sample-holding vessel are or have been physically removed from, or diluted in the presence of, one or more other sample components present in the vessel. Sample components that may be removed or diluted during a separating or purifying step include, chemical reaction products, non-reacted chemicals, proteins, carbohydrates, lipids, and unbound molecules.

By “solid phase” is meant a non-aqueous matrix such as one to which the antibody of the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g. controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g. an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

The term “species” is used herein in various contexts, e.g., a particular species of chemotherapeutic agent. In each context, the term refers to a population of chemically indistinct molecules of the sort referred in the particular context.

The term “specific” or “specificity” in the context of antibody-antigen interactions refers to the selective, non-random interaction between an antibody and its target epitope. Here, the term “antigen” refers to a molecule that is recognized and bound by an antibody molecule or other immune-derived moiety. The specific portion of an antigen that is bound by an antibody is termed the “epitope”. This interaction depends on the presence of structural, hydrophobic/hydrophilic, and/or electrostatic features that allow appropriate chemical or molecular interactions between the molecules. Thus an antibody is commonly said to “bind” (or “specifically bind”) or be “reactive with” (or “specifically reactive with”), or, equivalently, “reactive against” (or

“specifically reactive against”) the epitope of its target antigen. Antibodies are commonly described in the art as being “against” or “to” their antigens as shorthand for antibody binding to the antigen. Thus an “antibody that binds LPA,” an “antibody reactive against LPA,” an “antibody reactive with LPA,” an “antibody to LPA,” and an “anti-LPA antibody” all have the same meaning. Antibody molecules can be tested for specificity of binding by comparing binding to the desired antigen to binding to unrelated antigen or analogue antigen or antigen mixture under a given set of conditions. Preferably, an antibody according to the invention will lack significant binding to unrelated antigens, or even analogs of the target antigen.

A “subject” or “patient” refers to an animal in need of treatment that can be effected by molecules of the invention. Animals that can be treated in accordance with the invention include vertebrates, with mammals such as bovine, canine, equine, feline, ovine, porcine, and primate (including humans and non-human primates) animals being particularly preferred examples.

A “therapeutic agent” refers to a drug or compound that is intended to provide a therapeutic effect including, but not limited to: anti-inflammatory drugs including COX inhibitors and other NSAIDS, anti-angiogenic drugs, chemotherapeutic drugs as defined above, cardiovascular agents, immunomodulatory agents, agents that are used to treat neurodegenerative disorders, ophthalmic drugs, etc.

A “therapeutically effective amount” (or “effective amount”) refers to an amount of an active ingredient, e.g., an agent according to the invention, sufficient to effect treatment when administered to a subject in need of such treatment. Accordingly, what constitutes a therapeutically effective amount of a composition according to the invention may be readily determined by one of ordinary skill in the art. For example, in the context of cancer therapy, a “therapeutically effective amount” is one that produces an objectively measured change in one or more parameters associated with cancer cell survival or metabolism, including an increase or decrease in the expression of one or more genes correlated with the particular cancer, reduction in tumor burden, cancer cell lysis, the detection of one or more cancer cell death markers in a biological sample (e.g., a biopsy and an aliquot of a bodily fluid such as whole blood, plasma, serum, urine, etc.), induction of apoptosis or other cell death pathways, etc. Of course, the therapeutically effective amount will vary depending upon the particular subject and condition being treated, the weight and age of the subject, the severity of the disease condition, the particular compound chosen, the dosing regimen to be followed, timing of administration, the manner of administration and the like, all of which can readily be determined by one of ordinary skill in the art. It will be appreciated that in the context of combination therapy, what constitutes a therapeutically effective amount of a particular active ingredient may differ from what constitutes a therapeutically effective amount of that active ingredient when administered as a monotherapy (*i.e.*, a therapeutic regimen that employs only one chemical entity as the active ingredient).

As used herein, the terms “therapy” and “therapeutic” encompasses the full spectrum of prevention and/or treatments for a disease, disorder or physical trauma. A “therapeutic” agent of the invention may act in a manner that is prophylactic or preventive, including those that incorporate procedures designed to target individuals that can be identified as being at risk (pharmacogenetics); or in a manner that is ameliorative or curative in nature; or may act to slow the rate or extent of the progression of at least one symptom of a disease

or disorder being treated; or may act to minimize the time required, the occurrence or extent of any discomfort or pain, or physical limitations associated with recuperation from a disease, disorder or physical trauma; or may be used as an adjuvant to other therapies and treatments.

The term "treatment" or "treating" means any treatment of a disease or disorder, including preventing or protecting against the disease or disorder (that is, causing the clinical symptoms not to develop); inhibiting the disease or disorder (*i.e.*, arresting, delaying or suppressing the development of clinical symptoms; and/or relieving the disease or disorder (*i.e.*, causing the regression of clinical symptoms). As will be appreciated, it is not always possible to distinguish between "preventing" and "suppressing" a disease or disorder because the ultimate inductive event or events may be unknown or latent. Those "in need of treatment" include those already with the disorder as well as those in which the disorder is to be prevented. Accordingly, the term "prophylaxis" will be understood to constitute a type of "treatment" that encompasses both "preventing" and "suppressing". The term "protection" thus includes "prophylaxis".

The term "therapeutic regimen" means any treatment of a disease or disorder using chemotherapeutic and cytotoxic agents, radiation therapy, surgery, gene therapy, DNA vaccines and therapy, siRNA therapy, anti-angiogenic therapy, immunotherapy, bone marrow transplants, aptamers and other biologics such as antibodies and antibody variants, receptor decoys and other protein-based therapeutics.

The term "variable" region (of an antibody) comprises framework and complementarity regions or CDRs (otherwise known as hypervariable regions) refers to certain portions of the variable domains that differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions (CDRs) both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FRs (FR1, FR2, FR3 and FR4, respectively), largely adopting a β -sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (for example residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (for example residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health,

Bethesda, Md. (1991), pages 647-669). Thus the uniqueness of an antibody for binding its antigen comes from the CDRs (hypervariable regions) and their arrangement in space, rather than the particular framework which holds them there. The CDRs can be placed into any of a variety of frameworks as long as a desired level of antigen binding is retained.

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.

SUMMARY OF THE INVENTION

This invention provides methods for increasing neuronal differentiation of neural stem cells comprising delivering an antibody, or a fragment, variant, or derivative thereof, that binds lysophosphatidic acid, to an environment that contains neural stem cells (e.g., tissue within the central nervous system) so that levels of lysophosphatidic acid are decreased and neuronal differentiation is increased. The increase in neuronal differentiation may occur in vivo or in vitro, including in neurospheres. Such methods use an antibody, or an antibody fragment, variant, or derivative thereof, that binds lysophosphatidic acid. Such an antibody may be a monoclonal antibody, or a fragment, variant or derivative thereof, and may be a humanized antibody.

This invention also provides methods for treating a disease, condition, or injury of the nervous system in an animal, such as a human. The disease, condition, or injury of the nervous system is one that is associated with undesirably high levels of lysophosphatidic acid, or one that is associated with insufficient neuronal differentiation. These methods involve treating the animal with an antibody, or an antibody fragment, variant, or derivative, that binds lysophosphatidic acid. This antibody may be a monoclonal antibody, or a fragment, variant or derivative thereof, and may be a humanized antibody. Examples of diseases or conditions suitable for treatment with these methods include traumatic brain injury, brain or spinal cord hemorrhage, spinal cord injury, stroke or a neurodegenerative disease. Examples of neurodegenerative diseases are Parkinson's disease, Alzheimer's disease, and Huntington's disease.

These and other aspects and embodiments of the invention are discussed in greater detail in the sections that follow.

As those in the art will appreciate, the following description describes certain preferred embodiments of the invention in detail, and is thus only representative and does not depict the actual scope of the invention. Before describing the present invention in detail, it is understood that the invention is not limited to the particular molecules, systems, and methodologies described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the invention defined by the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

This application contains one figure executed in color. Copies of this application with color drawing will be provided upon request and payment of the necessary fee. A brief summary of the figure is provided below.

Figure 1 is a micrograph showing mouse brains after cortical injury. Panel A on the left shows a mouse brain with an area of hemorrhage as typically seen after TBI in the cortical impact model. Panel B on the right shows a mouse brain after TBI in the same model, but treated with anti-LPA antibody. The hemorrhage normally observed in this model is greatly reduced.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for increasing neuronal differentiation in vitro or in vivo using antibodies to lysolipids, particularly lysophosphatidic acid (LPA).

1. Antibodies

Antibody molecules or immunoglobulins are large glycoprotein molecules with a molecular weight of approximately 150 kDa, usually composed of two different kinds of polypeptide chain. One polypeptide chain, termed the heavy chain (H) is approximately 50 kDa. The other polypeptide, termed the light chain (L), is approximately 25 kDa. Each immunoglobulin molecule usually consists of two heavy chains and two light chains. The two heavy chains are linked to each other by disulfide bonds, the number of which varies between the heavy chains of different immunoglobulin isotypes. Each light chain is linked to a heavy chain by one covalent disulfide bond. In any given naturally occurring antibody molecule, the two heavy chains and the two light chains are identical, harboring two identical antigen-binding sites, and are thus said to be divalent, i.e., having the capacity to bind simultaneously to two identical molecules.

The light chains of antibody molecules from any vertebrate species can be assigned to one of two clearly distinct types, kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains. The ratio of the two types of light chain varies from species to species. As a way of example, the average κ to λ ratio is 20:1 in mice, whereas in humans it is 2:1 and in cattle it is 1:20.

The heavy chains of antibody molecules from any vertebrate species can be assigned to one of five clearly distinct types, called isotypes, based on the amino acid sequences of their constant domains. Some isotypes have several subtypes. The five major classes of immunoglobulin are immunoglobulin M (IgM), immunoglobulin D (IgD), immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin E (IgE). IgG is the most abundant isotype and has several subclasses (IgG1, 2, 3, and 4 in humans). The Fc fragment and hinge regions differ in antibodies of different isotypes, thus determining their functional properties. However, the overall organization of the domains is similar in all isotypes.

Of note, variability is not uniformly distributed throughout the variable domains of antibodies, but 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 region (FR). The variable domains of native heavy and light chains each comprise four FR regions connected by three CDRs. 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)). Collectively, the 6 CDRs contribute to the binding properties of the antibody molecule. 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 (see Pluckthun, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore, eds., Springer-Verlag, New York, pp. 269-315 (1994)).

The constant domain refers to the C-terminal region of an antibody heavy or light chain. Generally, the constant domains are not directly involved in the binding properties of an antibody molecule to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity. Here, "effector functions" refer to the different physiological effects of antibodies (e.g., opsonization, cell lysis, mast cell, basophil and eosinophil degranulation, and other processes) mediated by the recruitment of immune cells by the molecular interaction between the Fc domain and proteins of the immune system. The isotype of the heavy chain determines the functional properties of the antibody. Their distinctive functional properties are conferred by the carboxy-terminal portions of the heavy chains, where they are not associated with light chains.

Antibody molecules can be tested for specificity of antigen binding by comparing binding to the desired antigen to binding to unrelated antigen or analogue antigen or antigen mixture under a given set of conditions. Preferably, an antibody according to the invention will lack significant binding to unrelated antigens, or even analogs of the target antigen.

The term "antibody," in the context of this invention, is used in the broadest sense, and encompasses monoclonal, polyclonal, multispecific (e.g., bispecific, wherein each arm of the antibody is reactive with a different epitope of the same or different antigen), minibody, heteroconjugate, diabody, triabody, chimeric, and synthetic antibodies, as well as antibody fragments, derivatives and variants that specifically bind an antigen with a desired binding property and/or biological activity.

Desired activities can include the ability to bind the antigen specifically, the ability to inhibit proliferation in vitro, the ability to inhibit angiogenesis in vivo, and the ability to alter cytokine profile(s) in vitro.

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

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 may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

2. Antibodies to LPA

Although polyclonal antibodies against naturally-occurring LPA have been reported in the literature (Chen JH, *et al.*, Bioorg Med Chem Lett. 2000 Aug 7;10(15):1691-3), monoclonal antibodies to LPA had not been described until Sabbadini, *et al.*, U.S. Patent Application 20080145360, published June 19, 2008, and U.S. Patent Application 20090136483, published May 28, 2009, both of which are herein incorporated by reference in their entirety for all purposes. The former publication describes the production and characterization of a series of murine monoclonal antibodies against LPA and the latter publication describes a humanized monoclonal antibody against LPA. The specificity of each antibody for various LPA isoforms is shown in Table 1, below. IC₅₀: Half maximum inhibition concentration; MI: Maximum inhibition (% of binding in the absence of inhibitor); ---: not estimated because of weak inhibition. A high inhibition result indicates recognition of the competitor lipid by the antibody.

Table 1. Specificity profile of six anti-LPA mAbs [from U.S. Pub. No. 20080145360]

	14:0 LPA		16:0 LPA		18:1 LPA		18:2 LPA		20:4 LPA	
	IC ₅₀	MI	IC ₅₀	MI	IC ₅₀	MI	IC ₅₀	MI	IC ₅₀	MI
	uM	%	uM	%	uM	%	uM	%	uM	%
B3	0.02	72.3	0.05	70.3	0.287	83	0.064	72.5	0.02	67.1
B7	0.105	61.3	0.483	62.9	>2.0	100	1.487	100	0.161	67
B58	0.26	63.9	5.698	>100	1.5	79.3	1.240	92.6	0.304	79.8
B104	0.32	23.1	1.557	26.5	28.648	>100	1.591	36	0.32	20.1
D22	0.164	34.9	0.543	31	1.489	47.7	0.331	31.4	0.164	29.5
A63	1.147	31.9	5.994	45.7	---	---	---	---	0.119	14.5
B3A6	0.108	59.9	1.151	81.1	1.897	87.6	---	---	0.131	44.9

Interestingly, the anti-LPA mAbs were able to discriminate between 12:0 (lauroyl), 14:0 (myristoyl), 16:0 (palmitoyl), 18:1 (oleoyl), 18:2 (linoleoyl), and 20:4 (arachidonoyl) LPAs. A desirable EC₅₀ rank order for ultimate drug development is 18:2> 18:1>20:4 for unsaturated lipids and 14:0>16:0>18:0 for the saturated lipids, along with high specificity. The specificity of the anti-LPA mAbs was assessed for their binding to LPA-related biolipids such as distearoyl-phosphatidic acid, lysophosphatidylcholine, S1P, ceramide, and ceramide-1-phosphate. None of the anti-LPA antibodies demonstrated cross-reactivity to distearoyl PA and LPC, the immediate metabolic precursor of LPA.

Tables 2- 6 show primary amino acid sequences of the heavy and light chain variable domains (V_H and V_L) of five anti-LPA monoclonal antibodies.

Table 2: Clone B3 variable domain amino acid sequences without leader sequence and cut sites

Sequence	SEQ ID NO:
B3 Heavy Chain	
QVKLQQSGPELVRPGTSVKVSCITASGDAFTNYLIEWVKQRPGQGLEWIGLIYPDSGYIN YNENFKGKATLTADRSSSTAYMQLSSLTSEDSAVYFCARRFAYYGSYYFDYWGGQT TLTVSS	1
B3 Light Chain	
DVVMQTPLSLPVS LGDQASISCRSSQSLLKTNGNTYLHWYLQKPGQSPKLLIFKVS NR FSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHFPFTFGTGTKLEIK	2

Table 3: Clone B7 variable domain amino acid sequences without leader sequence and cut sites

Sequence	SEQ ID NO:
B7 Heavy Chain	
QVQLQQSGAELVRPGTSVKVSCKASGYGFINYLIEWIKQRPGQGLEWIGLINPGSDYTN YNENFKGKATLTADKSSSTAYMHLSSLTSEDSAVYFCARRFGYYGSGNYFDYWGGQT TLTVSS	3
B7 Light Chain	
DVVMQTPLSLPVS LGDQASISCTSGQSLVHINGNTYLHWYLQKPGQSPKLLIYKVS NL FSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHFPFTFGTGTKLEIK	4

Table 4: Clone B58 variable domain amino acid sequences without leader sequence and cut sites

Sequence	SEQ ID NO:
B58 Heavy Chain	
QVQLQQSGAELVRPGTSVKVSCKASGDAFTNYLIEWVKQRPGQGLEWIGLIIPGTGYT NYNENFKGKATLTADKSSSTAYMQLSSLTSEDSAVYFCARRFGYYGSSNYFDYWGGQ TTLTVSS	5
B58 Light Chain	
DVVMQTPLSLPVS LGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPKLLIYKVS N RFSGVPDRFSGSGPGTDFTLKISRVEAEDLGIYFCSQSTHFPFTFGTGTKLEIK	6

Table 5: Clone 3A6 variable domain amino acid sequences without leader sequence and cut sites

Sequence	SEQ ID NO:
3A6 Heavy Chain	
QVQLQQSGAELVRPGTSVKLSCKASGDAFTNYLIEWVKQRPGQGLEWIGLIIPGTGYTN YNENFKGKATLTADKSSSTAYMQLSSLTSEDSAVYFCARRFGYYGSYYFDYWGGQT TLTVSS	7
3A6 Light Chain	
DVVMQTPLSLPVS LGDQASISCRSSQSLVHSNGNTYLHWYLQKPGQSPKLLIYKVS N RFSGVPDRFSGSGPGTDFTLKISRVEAEDLGVYFCSQSTHFPFTFGTGTKLEIK	8

Table 6: Clone A63 variable domain amino acid sequences without leader sequence and cut sites

Sequence	SEQ ID NO:
A63 Heavy Chain	
DIQLQESGPGLVKPSQSLSLTCSVTGFSITSGYYWTWIRQFPGNKLEWWAYIGYDGSN DSNPSLKNRISITRDTSKNQFFLKLNSVTTEDTATYYCARAMLRRGFDYWGQGTTLTVS S	9
A63 Light Chain	
QIVLTQSPAIMSASPGEKVTMTCSASSLSYMHWWYQQKPGTSPKRWIYDTSKLAGVP ARFSGSGSGTSYSLTISSMEAEDAATYYCHRRSSYTFGGGTKLEIK	10

Tables 7-11, below, show the amino acid sequences of the CDRs of each of the antibodies represented in Tables 2-6, above.

Table 7: CDR amino acid sequences of V_H and V_L domains for clone B3 of mouse anti-LPA mAb

CLONE	V _H CDR	CDR	SEQ ID NO:
B3	GDAFTNYLIE*	CDRH1	11
B3	LIYPDSGYINYNNENFKG	CDRH2	12
B3	RFAYYGSYYFDY	CDRH3	13
	V _L CDR		
B3	RSSQSLLKTNGNTYLH	CDRL1	14
B3	KVSNRFS	CDRL2	15
B3	SQSTHFPFT	CDRL3	16

* CDRH1 as defined according to Chothia/AbM is the 10-amino acid sequence shown. The bolded five-amino acid portion (**NYLIE**; SEQ ID NO: 17) is the CDRH1 sequence defined according to Kabat.

Table 8: CDR amino acid sequences of V_H and V_L domains for clone B7 of mouse anti-LPA mAb

CLONE	V _H CDR	CDR	SEQ ID NO:
B7	GYGFINYLIE*	CDRH1	18
B7	LINPGSDYTNYNENFKG	CDRH2	19
B7	RFGYYGSGNYFDY	CDRH3	20
	V _L CDR		
B7	TSGQSLVHINGNTYLH	CDRL1	21
B7	KVSNLFS	CDRL2	22
B7	SQSTHFPFT	CDRL3	23

* CDRH1 as defined according to Chothia/AbM is the 10-amino acid sequence shown. The bolded five-amino acid portion (**NYLIE**; SEQ ID NO: 17) is the CDRH1 sequence defined according to Kabat.

Table 9: CDR amino acid sequences of V_H and V_L domains for clone B58 of mouse anti-LPA mAb

CLONE	V _H CDR	CDR	SEQ ID NO:
B58	GDAFTNYLIE*	CDRH1	24

B58	LIIPGTGYTNYNENFKG	CDRH2	25
B58	RFGYYGSSNYFDY	CDRH3	26
	V _L CDR		
B58	RSSQSLVHSNGNTYLH	CDRL1	27
B58	KVSNRFS	CDRL2	28
B58	SQSTHFPFT	CDRL3	29

* CDRH1 as defined according to Chothia/AbM is the 10-amino acid sequence shown. The bolded five-amino acid portion (**NYLIE**; SEQ ID NO: 17) is the CDRH1 sequence defined according to Kabat.

Table 10: CDR amino acid sequences of V_H and V_L domains for clone 3A6 of mouse anti-LPA mAb

CLONE	V _H CDR	CDR	SEQ ID NO:
3A6	GDAFT NYLIE *	CDRH1	30
3A6	LIIPGTGYTNYNENFKG	CDRH2	31
3A6	RFGYYGSGYYFDY	CDRH3	32
	V _L CDR		
3A6	RSSQSLVHSNGNTYLH	CDRL1	33
3A6	KVSNRFS	CDRL2	34
3A6	SQSTHFPFT	CDRL3	35

* CDRH1 as defined according to Chothia/AbM is the 10-amino acid sequence shown. The bolded five-amino acid portion (**NYLIE**; SEQ ID NO: 17) is the CDRH1 sequence defined according to Kabat.

Table 11: CDR amino acid sequences of V_H and V_L domains for clone A63 of mouse anti-LPA mAb

CLONE	V _H CDR	CDR	SEQ ID NO:
A63	GFSIT SGYYWT *	CDRH1	36
A63	YIGYDGSNDSNP SLKN	CDRH2	37
A63	AMLRRGFDY	CDRH3	38
	V _L CDR		
A63	SASSSLSYM H	CDRL1	39
A63	DTSKLAS	CDRL2	40
A63	HRRSSYT	CDRL3	41

* CDRH1 as defined according to Chothia/AbM is the 11-amino acid sequence shown. The bolded six-amino acid portion (**SGYYWT**; SEQ ID NO: 42) is the CDRH1 sequence defined according to Kabat.

Biophysical Properties of Lpathomab/LT3000

Lpathomab/LT3000 (also referred to herein as the “B7” anti-LPA monoclonal antibody) has high affinity for the signaling lipid LPA (K_D of 1-50 pM as demonstrated by surface plasmon resonance in the BiaCore assay, and in a direct binding ELISA assay); in addition, LT3000 demonstrates high specificity for LPA, having shown no binding affinity for over 100 different bioactive lipids and proteins, including over 20 bioactive lipids, some of which are structurally similar to LPA. The murine antibody is a full-length IgG1k isotype antibody composed of two identical light chains and two identical heavy chains with a total molecular weight of 155.5 kDa. The biophysical properties are summarized in Table 12, below.

Table 12: General Properties of Monoclonal antibody B7, also called Lpathomab or LT3000

Identity	LT3000 (B7)
Antibody isotype	Murine IgG1k
Specificity	Lysophosphatidic acid (LPA)
Molecular weight	155.5 kDa
OD of 1 mg/mL	1.35 (solution at 280 nm)
K_D	1-50 pM
Apparent T_m	67°C at pH7.4
Appearance	Clear if dissolved in 1x PBS buffer (6.6 mM phosphate, 154 mM sodium chloride, pH 7.4)
Solubility	>40 mg/mL in 6.6 mM phosphate, 154 mM sodium chloride, pH 7.4

Lpathomab has also shown biological activity in preliminary cell based assays such as cytokine release, migration and invasion; these are summarized below along with data showing specificity of LT3000 for LPA isoforms and other bioactive lipids, and in vitro biological effects of LT3000.

Table 13: Biologic properties of Monoclonal Antibody B7

LT3000 (Lpathomab, B7 antibody)					
A. Competitor Lipid	14:0 LPA	16:0 LPA	18:1 LPA	18:2 LPA	20:4 LPA
IC ₅₀ (mM)	0.105	0.483	>2.0	1.487	0.161
MI (%)	61.3	62.9	100	100	67
B. Competitor Lipid	LPC	S1P	C1P	Cer	DSPA
MI (%)	0	2.7	1.0	1	0
C. Cell based assay	LPA isoform	% Inhibition (over LPA taken as 100)			
<i>Migration</i>	18:1	35*			
<i>Invasion</i>	14:0	95*			
<i>IL-8 Release</i>	18:1	20			
<i>IL-6 Release</i>	18:1	23*			
		% Induction (over LPA+TAXOL taken as 100)			
<i>Apoptosis</i>	18:1	79			
A. Competition ELISA assay was performed with Lpathomab and 5 LPA isoforms. 18:1 LPA was captured on ELISA plates. Each competitor lipid (up to 10mM) was serially diluted in BSA/PBS and incubated with 3nM Lpathomab. Mixtures were then transferred to LPA coated wells and the amount of bound antibody was measured. B. Competition ELISA was performed to assess specificity of Lpathomab. Data were normalized to maximum signal (<i>A</i> ₄₅₀) and were expressed as percent inhibition (n=3). IC ₅₀ : half maximum inhibition concentration; MI%: maximum inhibition (% of binding in the absence of inhibitor). C. <i>Migration assay</i> : Lpathomab (150mg/mL) reduced SKOV3 cell migration triggered by 1mM LPA (n=3); <i>Invasion assay</i> : Lpathomab (15mg/mL) blocked SKOV3 cell invasion triggered by 2mM LPA (n=2); <i>Cytokine release of human IL-8 and IL-6</i> : Lpathomab (300-600 mg/mL, respectively) reduced 1mM LPA-induced release of pro-angiogenic and metastatic IL-8 and IL-6 in SKOV3 conditioned media (n=3). <i>Apoptosis</i> : SKOV3 cells were treated with 1mM Taxol; 1mM LPA blocked Taxol induced caspase-3 activation. The addition to Lpathomab (150mg/mL) blocked LPA-induced protection from apoptosis (n=1). Data Analysis: Student-t test, * denotes p<0.05.					

The potent and specific binding of Lpathomab/LT3000 to LPA results in reduced availability of extracellular LPA with potentially therapeutic effects against cancer-, angiogenic- and fibrotic-related disorders.

A second murine anti-LPA antibody, B3, was also subjected to binding analysis as shown in Table 14, below.

Table 14: Biochemical characteristics of Monoclonal Antibody B3

Biochemical characteristics of B3 antibody		
A. BIACORE	High density surface	Low density surface
Lipid Chip	12:0 LPA	18:0 LPA
K _D (pM), site 1 (site2)	61(32)	1.6 (0.3)
B. Competition Lipid Cocktail (C ₁₆ :C ₁₈ :C _{18:1} :C _{18:2} :C _{20:4} , ratio 3:2:5:11:2)	(μM)	
IC ₅₀	0.263	
C. Neutralization Assay		
B3 antibody (nmol)	LPA (nmol)	
0	0.16	
0.5	0.0428	
1	0.0148	
2	under limit of detection	
A. Biacore analysis for B3 antibody. 12:0 and 18:0 isoforms of LPA were immobilized onto GLC sensor chips; solutions of B3 were passed over the chips and sensograms were obtained for both 12:0 and 18:0 LPA chips. Resulted sensograms showed complex binding kinetics of the antibody due to monovalent and bivalent antibody binding capacities. K_D values were calculated approximately for both LPA 12 and LPA 18. B. Competition ELISA assay was performed with B3 and a cocktail of LPA isoforms (C₁₆:C₁₈:C_{18:1}:C_{18:2}:C_{20:4} in ratio 3:2:5:11:2). Competitor/Cocktail lipid (up to 10μM) was serially diluted in BSA/PBS and incubated with 0.5 μg/mL B3. Mixtures were then transferred to a LPA coated well plate and the amount of bound antibody was measured. Data were normalized to maximum signal (A₄₅₀) and were expressed as IC₅₀ (half maximum inhibition concentration). C. Neutralization assay: Increasing concentrations of B3 were conjugated to a gel. Mouse plasma was then activated to increase endogenous levels of LPA. Activated plasma samples were then incubated with the increasing concentrations of the antibody-gel complex. LPA leftover which did not complex to the antibody was then determined by ELISA. LPA was sponged up by B3 in an antibody concentration dependent way.		

Humanization of LT3000

The variable domains of the B7 murine anti-LPA monoclonal antibody (LT3000, Lpathomab), were humanized by grafting the murine CDRs into human framework regions (FR). See United States Provisional Patent Application 61/170,595, filed April 17, 2009, the contents of which are herein incorporated by reference in their entirety for all purposes. For descriptions of CDR grafting techniques, see, for example, Lefranc, M.P, (2003). Nucleic Acids Res, 31: 307-10; Martin and Thornton (1996), J Mol Biol, 1996. 263: 800-15; Morea, et al. (2000), Methods, 20: 267-79; Foote and Winter (1992), J Mol Biol, 224: 487-99; Chothia, et al., (1985). J Mol Biol, 186:651-63.

Suitable acceptor human FR sequences were selected from the IMGT and Kabat databases based on a homology to LT3000 using a sequence alignment and analysis program (SR v7.6). Lefranc (2003), supra; Kabat, et al. (1991), Sequences of Proteins of Immunological Interest, NIH National Techn. Inform. Service, pp. 1-3242. Sequences with high identity at FR, vernier, canonical and VH-VL interface residues (VCI) were initially selected. From this subset, sequences with the most non-conservative VCI substitutions, unusual proline or cysteine residues and somatic mutations were excluded. AJ002773 was thus selected as the human framework on which

to base the humanized version of LT3000 heavy chain variable domain and DQ187679 was thus selected as the human framework on which to base the humanized version of LT3000 light chain variable domain.

A three-dimensional (3D) model containing the humanized VL and VH sequences was constructed to identify FR residues juxtaposed to residues that form the CDRs. These FR residues potentially influence the CDR loop structure and the ability of the antibody to retain high affinity and specificity for the antigen. Based on this analysis, 6 residues in AJ002773 and 3 residues in DQ187679 were identified, deemed significantly different from LT3000, and considered for mutation back to the murine sequence.

The sequence of the murine anti-LPA mAb LT3000 was humanized with the goal of producing an antibody that retains high affinity, specificity and binding capacity for LPA. Further, seven humanized variants were transiently expressed in HEK 293 cells in serum-free conditions, purified and then characterized in a panel of assays. Plasmids containing sequences of each light chain and heavy chain were transfected into mammalian cells for production. After 5 days of culture, the mAb titer was determined using quantitative ELISA. All combinations of the heavy and light chains yielded between 2-12 ug of antibody per ml of cell culture.

Characterization and activity of the humanized variants

All the humanized anti-LPA mAb variants exhibited binding affinity in the low picomolar range similar to a chimeric anti-LPA antibody (also known as LT3010) and the murine antibody LT3000. All of the humanized variants exhibited a T_M similar to or higher than that of LT3000. With regard to specificity, the humanized variants demonstrated similar specificity profiles to that of LT3000. For example, LT3000 demonstrated no cross-reactivity to lysophosphatidyl choline (LPC), phosphatidic acid (PA), various isoforms of lysophosphatidic acid (14:0 and 18:1 LPA, cyclic phosphatidic acid (cPA), and phosphatidylcholine (PC).

Five humanized variants were further assessed in *in vitro* cell assays. LPA is important in eliciting release of interleukin-8 (IL-8) from cancer cells. LT3000 reduced IL-8 release from ovarian cancer cells in a concentration-dependent manner. The humanized variants exhibited a similar reduction of IL-8 release compared to LT3000.

Two humanized variants were also tested for their effect on microvessel density (MVD) in a Matrigel tube formation assay for neovascularization. Both were shown to decrease MVD formation.

Humanized anti-LPA variable region sequences

The humanized variant sequences are shown in Tables 15 and 17. Backmutations are shown in **bold**. CDR sequences are shown in gray. Canonical residues are numbered according to which CDR (1, 2, or 3) with which they are associated.

Table 15: Sequences of the variable domains of anti-LPA light chain humanized variants. CDRs are shaded, backmutations are in bold.

[illegible]

Table 16: LPA humanized antibody light chain variant variable domain sequences and vectors containing them.

Vector name	Description	Number of backmutations	Identity of backmutations
pATH500LC	pCONkappa (Lonza vector alone)		
pATH501	B7 humanized light chain RKA in vector pATH500LC, no back mutations	0	-
pATH502	B7 humanized light chain RKB in vector pATH500, 3 back mutations	3	I2V, Q45K, Y87F
pATH503	B7 humanized light chain RKC in vector pATH500, 2 back mutations	2	Q45K, Y87F
pATH504	B7 humanized light chain RKD in vector pATH500, 2 back mutations	2	I2V, Y87F
pATH505	B7 humanized light chain RKE in vector pATH500, 2 back mutations	2	I2V, Q45K
pATH506	B7 humanized light chain RKF in vector pATH500, 1 back mutation	1	I2V

Table 17: Sequences of the variable domains of anti-LPA heavy chain humanized variants. CDRs are shaded, backmutations are in bold

Kabat #	1	2	3	4	5	6	7	8	9	10	11	SEQ ID NO:
	123456789012345678901234567890123456789012A34567890123456789012345678901234567890123											
Canonical	1	11	1	1	2	22	2		1			
Vernier	*		****		***		* * * *	*	*	**	*	
Interface			I I I	I I					I I I	I	I	
B7 VH murine	QVQLVQSGAELVRPGTSVKVSCKASGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTTLTVSS									50
B7RH0	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								51
B7RH1	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								52
B7RH2	EVQLVQSGAEVKKPGESLKISCCQ	S FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								53
B7RH3	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								54
B7RH4	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								55
B7RH5	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								56
B7RH6	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								57
B7RH7	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								58
B7RH8	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								59
B7RH9	EVQLVQSGAEVKKPGESLKISCCQ	S FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								60
B7HX	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								61
B7HY	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								62
B7HZ	EVQLVQSGAEVKKPGESLKISCCQ	A FGYGF	INLVKPGDYLINENPKQATLSADKSSSTAYLQWSSLKASDTAM	YFCARRFVYGGSNFDYWGGTMTVSS								63

Table 18: LPA humanized antibody heavy chain variant variable domain sequences and vectors containing them.

Vector name	Description	Number of backmutations	Identity of backmutations
pATH600HC	pCONgamma (Lonza vector alone)		
pATH601	B7 humanized heavy chain RH0 in vector pATH600	0	-
pATH602	B7 humanized heavy chain RH1 in vector pATH600	6	S24A, I28G, V37I, M48I, V67A, I69L
pATH603	B7 humanized heavy chain RH8 in vector pATH600	3	S24A, I28G, M48I
pATH604	B7 humanized heavy chain RH9 in vector pATH600	4	I28G, M48I, V67A, I69L
pATH605	B7 humanized heavy chain HX in vector pATH600	2	I28G and M48I
pATH606	B7 humanized heavy chain HY in vector pATH600	2	S24A and M48I
pATH607	B7 humanized heavy chain HZ in vector pATH600	4	S24A, I28G, V37I, M48I

LT3015

LT3015 was selected as a preferred humanized anti-LPA monoclonal antibody. LT3015 is a recombinant, humanized, monoclonal antibody that binds with high affinity to the bioactive lipid lysophosphatidic acid (LPA). LT3015 is a full-length IgG1k isotype antibody composed of two identical light chains and two identical heavy chains with a total molecular weight of 150 kDa. The heavy chain contains an N-linked glycosylation site. The two heavy chains are covalently coupled to each other through two intermolecular disulfide bonds, consistent with the structure of a human IgG1.

LT3015 was originally derived from a murine monoclonal antibody which was produced using hybridomas generated from mice immunized with LPA. The humanization of the murine antibody involved the insertion of the six murine complementarity determining regions (CDRs) in place of those of a human antibody framework selected for its structure similarity to the murine parent antibody. A series of substitutions were made in the framework to engineer the humanized antibody. These substitutions are called back mutations and replace human with murine residues that are involved in the interaction with the antigen. The final humanized version contains six murine back mutation in the human framework of variable domain of the heavy chain (pATH602) and three murine back mutations in the human framework of the variable domain of the light chain (pATH502), shown in Tables 15-18, above.

3. Neuronal differentiation and the role of LPA

Neural stem cells (NSC) are found in areas of neurogenesis in the central nervous system (CNS) and can migrate to sites of neural injury. Thus NSC are under study with the goal of replacing neurons and restoring connections in a neurodegenerative environment. Dottori, M.. et al. (2008) "Lysophosphatidic Acid Inhibits

Neuronal Differentiation of Neural Stem/Progenitor Cells Derived from Human Embryonic Stem Cells.” *Stem Cells* 26: 1146-1154. NSC can be maintained in vitro as floating neurospheres and can differentiate in vitro into neurons. This can be assayed by visualizing and quantitating neuronal outgrowth from the neurospheres, which is visible under a microscope.

Neuronal stem cells have the option of proceeding into neuronal differentiation or into glial differentiation (gliogenesis), the formation of non-neuronal glial cells. Macroglial cells (glia) include astrocytes and oligodendrocytes. Thus, in general, as neuronal differentiation increases, glial differentiation decreases and vice versa. Thus an increase in neuronal differentiation may be determined by an increase in neuron formation, or by a decrease in glial differentiation.

Following injury, hemorrhage, or trauma to the nervous system, levels of LPA within the nervous system are believed to increase to 10 μ M. Dottori, et al. (ibid) have shown that 10 μ M LPA can inhibit neuronal differentiation of human NSC, while lower concentrations do not, suggesting that high levels of LPA within the CNS following injury might inhibit differentiation of NSC toward neurons, thus inhibiting endogenous neuronal regeneration. Modulating LPA signaling may thus have a significant impact in nervous system injury, allowing new potential therapeutic approaches.

4. Applications

The instant invention is drawn to methods for increasing neuronal differentiation of neural stem cells (NSCs). These methods use antibodies to LPA to achieve this desired result. While not wanting to be bound by theory, it is generally believed that antibodies to LPA bind to LPA and sponge up LPA molecules, thus lowering the effective concentration of LPA. High concentrations of LPA are known to inhibit neuronal differentiation of NSCs.

The invention is drawn to methods for increasing neuronal differentiation of neural stem cells, including by decreasing gliogenesis, and methods for treating or preventing diseases or conditions associated with insufficient neuronal differentiation. These methods use antibodies to LPA to achieve the desired result.

Without wishing to be bound by any particular theory, it is believed that undesirably high concentrations of lipids such as LPA and/or its metabolites, which are sufficient to block neuronal differentiation of NSCs, may contribute to the development or symptomology of various neurologic diseases and disorders that are associated with insufficient neuronal differentiation. Such diseases are believed to include neurodegenerative diseases (including Parkinson's, Alzheimer's, and Huntington's diseases), in which there is a net loss of neurons, stroke and other conditions such as hemorrhage in which blood contacts the CNS, and brain cancers. Reactive astrocytes and glioma can produce high levels of LPA. LPA does not stop glial differentiation from NSCs. Dottori M, Leung J, Turnley AM, Pébay A., (2008) *Stem Cells*. May; 26(5):1146-54. Epub 2008 Feb 28. Thus it is believed that blocking LPA using anti-LPA antibodies would have an impact on tumor growth by reducing its effect on astrocyte (and thus glioma) proliferation. It is also believed that blocking LPA using anti-LPA antibodies might also reduce the bias of NSC differentiation toward more glial cells. Increasing neuronal differentiation is particularly useful following brain/spinal cord injury, when many lost neurons need to be replaced. A net loss of

neurons may occur even though there may be some, stem cell response to the disease or injury, if this is insufficient to overcome the loss.

EXAMPLES

The invention will be further described by reference to the following detailed examples. These Examples are in no way to be considered to limit the scope of the invention in any manner.

EXAMPLE 1: Neurosphere formation, treatment, and differentiation

Neurospheres were formed and cultured as described in Dottori, M. et al. (2008), supra. Briefly, human embryonic stem cells (HES-2, HES-3, and HES-4, WiCell Research Institute, Madison WI) were cultured according to previously published methods. Neuronal induction using noggin was performed according to published methods and after growth and subculture, cells were grown as neurospheres in the presence of growth factors. Neurospheres could be plated on dishes coated with laminin or fibronectin. When plated onto laminin and cultured with neural basal medium (NBM, R&D Systems, Minneapolis MN), neurospheres typically differentiate into neurons. Quantitation of neuron-forming spheres (a measure of neuronal differentiation) was done by counting the number of neurospheres from which neuronal outgrowth was visible. Neurospheres that failed to attach to the plate were not counted.

Plated neurospheres were incubated in the presence or absence of LPA (Sigma Aldrich, St. Louis, MO) and/or antibody (concentrations shown) for 5 days. Dilutions of LPA were made in 0.1% fatty acid-free bovine serum albumin (final concentration 0.01% BSA).

EXAMPLE 2: LPA Inhibits Neurosphere Formation and Neuronal Differentiation

As shown by Dottori et al., LPA inhibits the ability of NSC to form neurospheres, even in the presence of bFGF and EGF. Briefly, noggin-treated cells were incubated in the presence or absence of LPA while being subcultured in suspension in NBM with bFGF and EGF (20 ng/ml each) for 11-14 days. The number of neurospheres formed was counted and it was found that in the presence of 10 μ M LPA, 13.47% $\pm$ 6.94% of cultures formed neurospheres, compared to 48.60% $\pm$ 8.15% for control cultures untreated with LPA. Dottori, M. et al. (2008), supra.

The effect of LPA on an additional differentiation step, the differentiation of NSC toward mature cells, was also measured. When plated on laminin in NBM, neurospheres typically differentiate into neurons, as assayed by visible neurons, elongated cell shape and/or positive staining for β -tubulin. Dottori, et al. observed the formation of elongated cells positive for β -tubulin in the untreated control cells, the NSC incubated in LPA did not differentiate into elongated cells, and there were few if any β -tubulin positive cells in the neurospheres. In general, neurospheres plated in the presence of 10 μ M LPA did not give rise to neuronal cells.

EXAMPLE 3: Anti-LPA antibodies block LPA inhibition of neurosphere formation

Using the conditions used in Example 2 for LPA treatment alone, noggin-treated cells were incubated in the presence or absence of LPA while being subcultured in suspension in NBM with bFGF and EGF (20 ng/ml each) for 5-7 days. The number of neurospheres formed was counted and it was found that in the presence of 10 μ M LPA, as before, neurosphere formation was decreased ($n \geq 3$). Whereas control cells yielded $90.482 \pm 5.346\%$ neurosphere formation, cells treated with 10 μ M LPA yielded only $13.500 \pm 5.590\%$ neurosphere formation. Cells treated with LPA at 1 μ M, in contrast, yielded $50 \pm 12.50\%$ neurosphere formation. Anti-LPA antibody B3 alone gave neurosphere formation comparable to control ($91.667 \pm 8.333\%$ for 0.1 mg/ml B3 and $91.667 \pm 4.167\%$ at 1.0 mg/ml B3). Notably, the combination of 1 mg/ml B3 and 10 μ M LPA also gave neurosphere formation comparable to control ($95.833 \pm 4.167\%$), indicating that the antibody to LPA had blocked inhibition of neurosphere formation that normally occurs in the presence of LPA.

The size of the neurospheres was also measured after LPA +/- B3 antibody treatment ($n=3$ for each) under the same conditions as above. The neurosphere area after treatment with B3 antibody alone was $93.94\% \pm 3.61\%$ of untreated control; neurosphere area after treatment with LPA + B3 was $75.18\% \pm 9.89\%$ of control. Measurements after treatment with LPA alone were not possible because neurospheres do not form. Statistics indicate the variation in size between the treatment groups is not significant.

The data show that blocking LPA (from endogenous production by NSCs) does not significantly increase neurosphere size, and more importantly, that the effect of LPA on the growth of neurospheres is totally abolished by B3 (ie the size is normal and comparable to control): this reveals the potency of B3 in blocking LPA activity.

EXAMPLE 4: Humanized and murine anti-LPA antibodies block LPA inhibition of neuronal differentiation

Using the same conditions used in Example 2 for LPA treatment alone, plated neurospheres were treated with 10 μ M LPA alone, or with anti-LPA antibody B3 or B7 (1 mg/ml) alone, or with 10 μ M LPA in combination with 1 mg/ml of antibody B3 or B7. Similarly, cells were treated with 10 μ M LPA alone, humanized anti-LPA antibody LT3015 alone (1 mg/ml) or with 10 μ M LPA in combination with 1 mg/ml LT3015. The percent of neuron-forming neurospheres was quantitated as in Example 2 (beta-tubulin staining and quantification of neuron-forming spheres, as described in Dottori, et al (2008)). LPA alone reduced neuron-forming neurospheres to approximately $25.00 \pm 6.45\%$ of untreated control. Neurosphere samples treated with B3 antibody alone had neuron-forming neurospheres equivalent to control (100%). Neurospheres treated with the combination of LPA and B3 antibody had neuron forming neurospheres equal to $86.66 \pm 5.65\%$ of control, indicating that the antibody had blocked the inhibition of neuron formation that normally occurs in the presence of LPA. Cells treated with the combination of LPA and LT3015 humanized antibody showed nearly identical neuron formation to B3-treated cells ($87.5\% \pm 12.50\%$ of control). The antibody B7, under similar conditions, had little to no effect in this experiment ($37.00 \pm 5.31\%$ of control).

EXAMPLE 5: Humanized and murine anti-LPA antibodies block LPA inhibition of neurosphere formation

Using the conditions described in previous examples, HSC were plated onto laminin for neuronal differentiation in NBM medium (3 days), with or without LPA (10 μ M), with or antibody to LPA at 1 mg/ml (B3, B7, or the humanized antibody LT3015, tested singly with or without LPA).

As before, the number of neuron-forming spheres was significantly decreased in the presence of 10 μ M LPA, to approximately 26% of control. None of the antibodies when tested alone had any effect on number of neuron-forming spheres (all were equivalent to control, which was 100%). However, all of the anti-LPA antibodies were able to block the inhibition of neuronal differentiation by LPA. Cells treated with B3 and LPA or with LT3015 and LPA had neuron-forming neurospheres equal to 75% of control. Cells treated with B7 and LPA had neuron-forming neurospheres equal to 50% of control. Pool of data results are similar: LPA 25.00 $\pm$ 6.45%; B3+LPA: 86.66 $\pm$ 5.65; B7+LPA 37.00 $\pm$ 5.31%; Humanized B7 (LT3015): 87.5 $\pm$ 12.5 (however although differentiation occurs there are fewer neurons observed than with B3) n=2 for hB7 and n>3 for B3 and B7. Thus, all three LPA antibodies, including LT3015, a humanized antibody to LPA, inhibit LPA's effect on neuronal differentiation, as measured by neurosphere formation. It was noted that neurospheres from cells treated with B3 and LPA had the greatest number of neurons (indicating further differentiation), followed by neurospheres from LT3015-treated cells, with a lesser number of neurons in neurospheres from cells treated with B7 antibody.

EXAMPLE 6: Immunohistochemical staining of LPA using monoclonal antibody to LPA

Immunohistochemical methods can be used to determine the presence and location of LPA in cells. Spinal cords (adult (3 months old) male C57BL/6 mice) from animals with and without spinal cord injury were immunostained 4 days after injury. Adult C57BL/6 mice (20-30g) were anaesthetized with a mixture of ketamine and xylazine (100mg/kg and 16mg/kg, respectively) in phosphate buffered saline (PBS) injected intraperitoneally. The spinal cord was exposed at the low thoracic to high lumbar area, at level T12, corresponding to the level of the lumbar enlargement. Fine forceps were used to remove the spinous process and lamina of the vertebrae and a left hemisection was made at T12. A fine scalpel was used to cut the spinal cord, which was cut a second time to ensure that the lesion was complete, on the left side of the spinal cord, and the overlying muscle and skin were then sutured. This resulted in paralysis of the left hindlimb. After 2 or 4 days the animals were re-anaesthetized as above and then perfused with PBS through the left ventricle of the heart, followed by 4% paraformaldehyde (PFA). After perfusion, the spinal cords were gently removed using fine forceps and post-fixed for 1 hour in cold 4% PFA followed by paraffin embedding or cryo-preserving in 20% sucrose in PBS overnight at 4°C for frozen sections. Tissues for taken from n=3 uninjured mice and n=3 injured mice at 2 and 4 days post-injury. As described in Goldshmit Y, Galea MP, Wise G, Bartlett PF, Turnley AM: Axonal regeneration and lack of astrocytic gliosis in EphA4-deficient mice. *J Neurosci* 2004, **24**(45):10064-10073.

IHC frozen spinal cord sagittal sections (10 μ m) were examined using standard immunohistochemical procedures to determine expression and localization of the different LPA receptors. Frozen sections were postfixated for 10min with 4% PFA and washed 3 times with PBS before blocking for 1 hour at room temperature (RT) in blocking solution containing 5% goat serum (Millipore) and 0.1% Triton-X in PBS in order to block non-specific antisera interactions. Primary antibodies used were B3 (0.1 mg/ml) rabbit anti-LPA₁ (1:100, Cayman

Chemical, USA), rabbit anti-LPA₂ (1:100, Abcam, UK) and mouse anti-GFAP (1:500, Dako, Denmark). Primary antibodies were added in blocking solution and sections incubated over night at 4°C. They were then washed and incubated in secondary antibody for 1hr at RT, followed by Dapi counterstain. Sections were coverslipped in Fluoromount (Dako) and examined using an Olympus BX60 microscope with a Zeiss Axiocam HRc digital camera and Zeiss Axiovision 3.1 software capture digital images. Some double labeled sections were also examined using a Biorad MRC1024 confocal scanning laser system installed on a Zeiss Axioplan 2 microscope. All images were collated and multi-colored panels produced using Adobe Photoshop 6.0.

After injury, non-neuronal glial cells in the CNS called astrocytes respond to many damage and disease states resulting in a "glial response". Glial Fibrillary Acidic Protein (GFAP) antibodies are widely used to see the reactive astrocytes which form part of this response, since reactive astrocytes stain much more strongly with GFAP antibodies than normal astrocytes. LPA was revealed by immunohistochemistry using antibody B3 (0.1 mg/ml overnight). Fluorescence microscopy showed that reactive astrocytes are present in spinal cords 4 days after injury, and these cells stain positively for LPA. In contrast, uninjured (control) spinal cords have little to no staining for astrocytes or LPA. Thus LPA is present in reactive astrocytes of the spinal cord. In both injured and control animals, the central canal (hypothesized to be a stem cell niche) does not stain for LPA.

EXAMPLE 7: Immunohistochemical confirmation that anti-LPA antibodies block LPA inhibition of neuronal differentiation

Neurospheres grown and treated as in above examples were immunostained for CD133 (1/1000, Abcam, Inc., Cambridge MA), β -tubulin (1/500, Millipore, Billerica MA) or LPA (0.1 mg/ml) as described in the previous example. β -tubulin staining is indicative of differentiation of neurons. In contrast, CD133 staining is lost upon differentiation. With LPA treatment, CD133-positive cells are observed as the cells migrating out of the neurosphere. In control cells, the migrating cells are either weakly CD133 positive or are negative for CD133 staining. Expression of CD133 was seen to be reduced by the LPA antibodies (not quantitated).

Example 8: Anti-LPA antibody in murine cortical impact model of traumatic brain injury (TBI)

The mouse is an ideal model organism for TBI studies because there is an accepted model of human TBI, the type I IFN system in the mouse is similar to that in human, and the ability to generate gene-targeted mice helps to clarify cause and effect rather than mere correlations. Adult mice were anaesthetised with a single ip injection of Ketamine/Xylazine and the scalp above the parietal bones shaved with clippers. Each scalp was disinfected with chlorhexidine solution and an incision made to expose the right parietal bone. A dentist's drill with a fine burr tip was then used to make a 3mm diameter circular trench of thinned bone centred on the centre of the right parietal bone. Fine forceps were then used to twist and remove the 3mm plate of parietal bone to expose the parietal cortex underneath. The plate of bone removed was placed into sterile saline and retained. The mouse was mounted in a stereotaxic head frame and the tip of the impactor (2mm diameter) positioned in the centre of the burr hole at right angles to the surface of the cortex and lowered until it just touches the dura mater membrane covering the cortex. A single impact injury (1.5mm depth) was applied using the computer

controller. The mouse was removed from the head frame and the plate of bone replaced. Bone wax was applied around the edges of the plate to seal and hold the plate in position. The skin incision was then closed with fine silk sutures and the area sprayed with chlorhexidine solution. The mouse was then returned to a holding box underneath a heat lamp and allowed to regain consciousness (total time anaesthetised = 30-40 minutes).

Treatments: Treatments or isotype controls were injected at various time points, either before or after TBI. Anti-LPA antibody (B3 or other) was injected by tail-IV (0.5 mg). Following 24-48 hours, the animals were sacrificed and their brains analysed.

Analysis: Neuronal death/survival (TUNEL analysis), reactive astrogliosis (revealed by Ki67 positive cells co-labelled with GFAP) and NS/PC responses (proliferation by CD133/Ki67, migration to the injury site by CD133 and differentiation) are analysed. The immune response is assessed by CD11b immunostaining. Quantification is performed by density measurement using ImageJ (NIH).

Preliminary results: Preliminary data in this model show that anti-LPA antibody treatment (B3) reduces the degree of hemorrhage normally seen in the mouse brain following TBI in this cortical impact model (Figure 1).

Example 9: LPA inhibits the neuronal differentiation of adult mouse NSC

In mouse adult neurospheres generated from mouse subventricular zone NSC, expression analysis of the LPA receptors indicated the presence of the mRNA transcripts for LPA receptors LPA₁, LPA₃ and LPA₄ and absence or low level expression of the mRNA transcripts for LPA receptors LPA₂ and LPA₅, indicating that adult mNS/PC are also potential targets for LPA. Contrary to what was observed in human NSC, LPA did not modify neurosphere formation or growth of mouse NSC. However, and similarly to data obtained in human NSC, LPA inhibited the neuronal differentiation of adult mouse NSC by maintaining them as NSC when plated in conditions normally inducing neuronal differentiation. After three days, LPA (10 μ M) -treated mouse NSC only showed low levels of expression of β III-tubulin, a marker for differentiated neurons (26.25 ± 2.08 % of total cells), and remained mainly positive for nestin, a marker for undifferentiated NSCs (87.55 ± 3.20 % of total cells). In contrast, untreated cells showed greater levels of differentiated neurons (β III-tubulin expressed by 57.12 ± 18.42 % of cells) and lower levels of undifferentiated NSCs (nestin was expressed by 58.01 ± 6.20 of total cells). These effects were independent of apoptosis or proliferation.

* * *

All of the compositions and methods described and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit and scope of the invention as defined by the appended claims.

All patents, patent applications, and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents, patent applications, and publications, including those to which priority or another benefit is claimed, are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms “comprising”, “consisting essentially of”, and “consisting of” may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

We claim:

1. A method for increasing neuronal differentiation of neural stem cells, comprising delivering an antibody, or a fragment, variant, or derivative thereof, that binds lysophosphatidic acid, to an environment that also comprises neural stem cells so that levels of lysophosphatidic acid in the environment and/or in said cells are decreased and neuronal differentiation is increased.
2. A method according to claim 1 wherein an increase in neuronal differentiation of said neural stem cells is determined by an increase in neuron formation.
3. A method according to claim 1 wherein an increase in neuronal differentiation of said neural stem cells is determined by a decrease in gliogenesis.
4. A method according to claim 1 wherein the increase in neuronal differentiation or the decrease in gliogenesis occurs in vivo.
5. A method according to claim 1 wherein the increase in neuronal differentiation or the decrease in gliogenesis occurs in vitro.
6. A method according to claim 1 wherein the increase in neuronal differentiation or the decrease in gliogenesis occurs in neurospheres.
7. A method according to claim 1 wherein the antibody that binds lysophosphatidic acid is a monoclonal antibody, or a fragment, variant, or derivative thereof, that binds lysophosphatidic acid.
8. A method according to claim 7 wherein the monoclonal antibody is a humanized monoclonal antibody, or a fragment, variant, or derivative thereof.
9. A method for treating a disease, condition, or injury of the nervous system in an animal, preferably a human, wherein said condition is associated with undesirably high levels of lysophosphatidic acid, said method comprising treating said animal with an antibody that binds lysophosphatidic acid, so that levels of said lysophosphatidic acid in the nervous system of the animal are reduced.
10. A method according to claim 9 wherein said disease or condition is selected from the group consisting of traumatic brain injury, stroke, brain or spinal cord hemorrhage, spinal cord injury, cancer of the central nervous system, and a neurodegenerative disease.

11. A method according to claim 10 wherein the neurodegenerative disease is selected from the group consisting of Parkinson's disease, Alzheimer's disease, and Huntington's disease.
12. A method for treating a disease, condition, or injury of the nervous system in an animal, preferably a human, wherein said condition is associated with insufficient neuronal differentiation, said method comprising treating said animal with an antibody that binds lysophosphatidic acid, so that levels of said lysophosphatidic acid in the nervous system of the animal are reduced and neuronal differentiation is increased.
13. A method according to claim 12 wherein said disease or condition is selected from the group consisting of traumatic brain injury, stroke, brain or spinal cord hemorrhage, spinal cord injury, cancer of the central nervous system, and a neurodegenerative disease.
14. A method according to claim 13 wherein the neurodegenerative disease is selected from the group consisting of Parkinson's disease, Alzheimer's disease, and Huntington's disease.

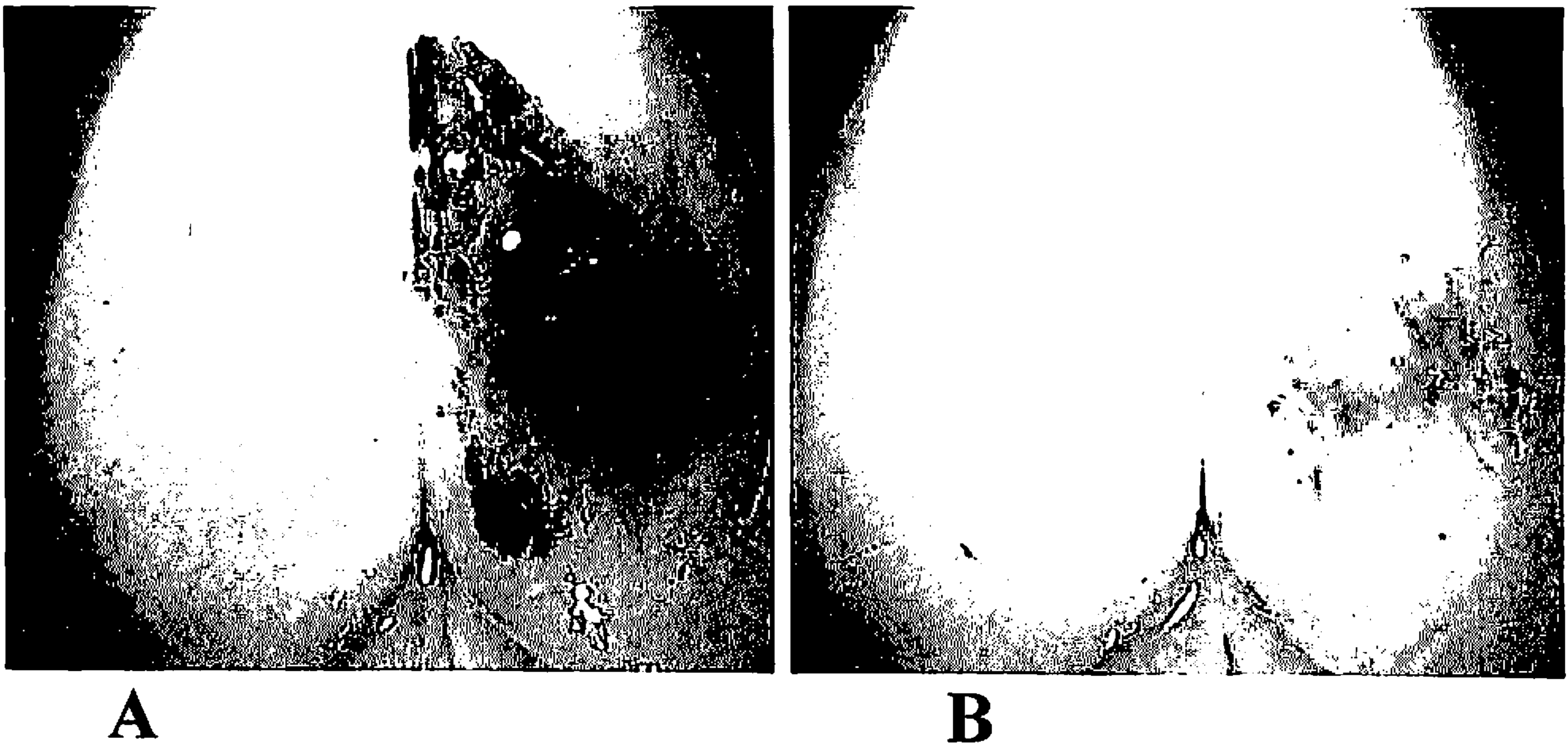


Figure 1