Title: COMPOSITIONS AND METHODS OF TREATING ONCOLOGICAL, INFLAMMATORY AND AUTOIMMUNE DISEASES MEDIATED BY SEMA4A

Abstract: Disclosed are compositions and methods of treating diseases by modulating the interaction of Sema4A with Plexin-B2, and methods to identify compounds that modulate the interaction of Sema4A with Plexin-B2.
Compositions and Methods of Treating Oncological, Inflammatory and Autoimmune Diseases Mediated by Sema4a

BACKGROUND OF THE INVENTION

1. TECHNICAL FIELD
This invention relates to the involvement of Sema4A in activation and effector functions of CD8+ T-cells and NK-cells.

2. BACKGROUND INFORMATION
We have previously shown that a class IV Semaphorin, Sema4A is involved in activation and differentiation of CD4+ T-cells. Kumanogoh, A. et al. *Immunity* Vol. 22, 305-316, March 2005. The present invention demonstrates the role of Sema4A in regulation of CD8+ T-cell and NK-cell functions which up until the present invention has been unclear.

BRIEF SUMMARY OF THE INVENTION

Enhancing Sema4A/Plexin-B2 interaction or signaling may be useful in the treatment of diseases where increased T lymphocyte and/or NK cell activation and/or lytic activity is beneficial, for example cancer and viral infections, or for enhancing the immune response to vaccines. Inhibition of Sema4A/Plexin-B2 interaction or signaling may be useful in the treatment of diseases where decreased T lymphocyte and/or NK cell activation and/or lytic activity is beneficial, for example inflammatory and autoimmune diseases, including sepsis, rheumatoid arthritis, multiple sclerosis, psoriasis, Lupus, and inflammatory bowel diseases.

It is therefore an object of the invention to provide a method of treating an oncological disease, by administering to a patient a composition which enhances or promotes Sema4A/Plexin-B2 interaction.
It is another object of the invention to provide a method of treating an inflammatory or autoimmune disease, by administering to a patient a composition which inhibits Sema4A/Plexin-B2 interaction.

It is yet still another object of the invention to provide a method to identify a compound that regulates interaction of Sema4A/Plexin-B2 activity in a cell, comprising: (1) contacting a cell with a putative regulatory compound, wherein the cell includes a Plexin-B2 protein and a Sema4A protein; and (2) assessing the ability of the putative regulatory compound to inhibit or enhance the interaction of Sema4A/Plexin-B2.

It is yet still another object of the invention to provide a composition that controls interaction of Sema4A/Plexin-B2 activity in a cell wherein the composition is therapeutically useful in treating an oncological, inflammatory or autoimmune disease or enhances the immunological response to a vaccine.

Another object of the invention is to provide a use of a composition which inhibits Sema4A - Plexin-B2 interaction or signaling for the treatment of an inflammatory or autoimmune disease. Another object of the invention is to provide a use of a composition which inhibits Sema4A - Plexin-B2 interaction or signaling for the manufacture of a medicament for the treatment of an inflammatory or autoimmune disease.

Another object of the invention is to provide a use of a composition which enhances Sema4A -Plexin-B2 interaction or signaling for the treatment of an oncological disease. Another object of the invention is to provide a use of a composition which enhances Sema4A -Plexin-B2 interaction or signaling for the manufacture of a medicament for the treatment of an oncological disease.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Binding of Sema4A-Fc to activated T cells are shown.
**Figure 2:** Sema4A promotes T-cell activation. (A). Sema4A-Fc dose-dependently enhances *in vitro* T-cell activation of purified CD4+ and CD8+ T-cells that were stimulated with anti-CD3. The effects of various concentrations of Sema4A-Fc are shown. (B) Sema4A-Fc dose-dependently induces IFN-gamma by anti-CD3-stimulated CD8+ T-cells.

**Figure 3:** NK cells express a receptor for Sema4A other than Tim-2. An analysis of the binding of Sema4A-Fc to NK cells, and expression of Sema4A and TIM molecules on NK cells is shown.

**Figure 4:** Sema4A treatment induces IFN-gamma from NK cells. Purified NK cells were cultured in presence of IL-2 or IL-12 or in combination with and without addition of Sema4A fusion proteins.

**Figure 5:** Sema4A enhances CTL activity of NK cells against YAC-I cells. Purified NK cells were treated with 50 U/ml of recombinant IL-2 for 24 h either with 20 ug/ml of Sema4A-Fc fusion protein or human IgGl and mixed with target YAC-I cells at various effector to target ratios as indicated. Cytotoxicity was measured using an LDH-calorimetric assay.

**Figure 6:** Generation of P815 mastocytoma cells stably expressing Sema4A. P815 were transfected with full length Sema4A expression vector and selected for 10 days in 0.5 mg/ml neomycin. Sema4A expressing cells were separated by FACS sorting. Stable P815 cells expression was tested using anti-Sema4A antibody. Anti-CD100 and mouse isotype were used as controls.

**Figure 7:** Expression of Sema4A in P815 cells results in increased primary CTL generation. DBA/2 syngeneic splenocytes were cultured for 9 days with 20,000 rad irradiated P815 cells that were non-transfected or stably expressing B7.1 or Sema4A. Recombinant IL-2, 10 U/ml or IL-4, 20 U/ml was added separately or in combination to the cultures every alternate day. A CTL assay was performed using non-transfected P815 cells at a 1:10 ratio of Effector to Target cells.
Figure 8: Effect of Sema4A on tumorigenicity of P815 tumor. (A), $10^6$ non-transfected or Sema4A expressing cells were injected subcutaneously and tumor size was measured after 4 weeks. (B) Mice with regressed tumors were challenged with a subcutaneous injection of $5 \times 10^5$ non-transfected P815 cells (circles) 8 weeks after primary tumor inoculation. Naïve DBA/2 mice were inoculated with same number of non-transfected P815 cells as control (squares).

Figure 9: Naïve and activated CD8$^+$ T cells and NK cells do not express Tim-2. FACS analysis was performed with purified CD8$^+$ T-cells before and after activation with anti-CD3 and anti-CD28 antibodies (5 mg/ml each) for 24h and with purified NK cells before and after activation with 200 U/ml of recombinant IL-2 for 24h. Cells were stained with biotinylated anti-TIM-2 or biotinylated Sema4A-Fc and streptavidin-APC. Biotinylated human IgG1 and rat IgG2a were used respectively as isotype controls.

Figure 10: Purified NK cells were treated with IL-2, IL-12 or both for 16 hours. RT-PCR was performed from cDNA isolated from the treated NK cells for analysis of the expression of various plexin-B family members. An expression library made from NK cells was used as another source of NK cell cDNA.

Figure 11: cDNA isolated from NK cells was analyzed for the expression of various TIM family members.

Figure 12: Purified NK cells were infected with either control lentivirus or Plexin-B2 silencing shRNA lentivirus particles. NK cells were selected for 6 days using 100 U/ml recombinant IL-2 and 2.5 mg/ml Puromycin followed by 48 h activation with Sema4A or control IgG. IFN-gamma production was measured in culture supernatants by ELISA.

Figure 13: OT-I transgenic purified CD8$^+$ T-cells were infected with either control lentivirus or Plexin-B2 silencing lentivirus particles. CD8$^+$ T-cells were selected for 6 days using 100 U/ml recombinant IL-2 and 2.5 mg/ml Puromycin followed by 48 h activation with Sema4A or control IgG. IFN-gamma production was measured in culture supernatants by ELISA.
Figure 14: Sigma shRNA lentiviral Vector used for Plexin-B2 silencing.

DETAILED DESCRIPTION OF THE INVENTION

This invention demonstrates that Plexin-B2 is a ligand for Semaphorin 4A on CD8+ T cells and NK cells. Treatment with Sema4A-Fc augments production of IFN-gamma by NK cells and CD8+ T lymphocytes. Sema4A-Fc treatment also enhances NK cell lytic activity. Expression of Sema4A on tumor cells leads to increased generation of cytotoxic T lymphocytes and resultant tumor regression. Knockdown of Plexin-B2 expression on CD8+ T cells or NK cells abrogates the ability of Sema4A-Fc to enhance IFN-gamma production from these cells. Collectively these findings reveal the important role of Sema4A/Plexin-B2 interaction and signaling in the activation of CD8+ T-cells and NK-cells and in the generation of lytic activity of CD8+ T-cells and NK-cells. Enhancing Sema4A/Plexin-B2 interaction or signaling may be useful in the treatment of diseases where increased T lymphocyte and/or NK cell activation and/or lytic activity is (are) beneficial, for example cancer and viral infections, or for enhancing the immune response to vaccines. Inhibition of Sema4A/Plexin-B2 interaction or signaling may be useful in the treatment of diseases where decreased T lymphocyte and/or NK cell activation and/or lytic activity is (are) beneficial, for example inflammatory and autoimmune diseases, including sepsis, rheumatoid arthritis, multiple sclerosis, psoriasis, systemic lupus erythematosus, and inflammatory bowel diseases.

As demonstrated in the examples below and shown in the figures, recombinant Sema4A-Ig fusion protein bound to activated CD8+ T-cells and NK-cells, induced IFN-γ production and enhanced their CTL activities. Adoptive transfer of OT-I CD8+ T-cells into Sema4A-/- mice and subsequent challenge with peptide antigen in CFA revealed that the expansion of CD8+ T-cells was dependent on Sema4A. In addition, the stable expression of Sema4A in P815 tumor cells enhanced in vivo tumor immunogenecity in inoculated mice. When syngeneic DBA/2 mice were inoculated with parental P815 cells, mice developed progressively large tumors and died. In contrast, when inoculated with P815 cells stably expressing Sema4A (Sema4A-P815 cells), mice did not develop large tumors due to Sema4A-enhanced CTL activity. Furthermore, the mice that
previously experienced regression of Sema4A-P815 tumors were resistant to tumor development when freshly inoculated with parental P815 cells. Boehringer Ingelheim application WO03080673 demonstrates that Sema4A binds to Tim-2 expressed on activated CD4+ T-cells. See also Kumanogoh, A. et al. *Nature* Vol. 419 October 10, 2002. However, CD8+ T-cells and NK-cells do not express Tim-2. Instead, we found that Sema4A bound to Plexin-B2 receptor expressed on CD8+ T-cells and NK-cells. Consistent with this observation, knockdown of Plexin-B2 expression by RNAi inhibited the effects of Sema4A on both CD8+ T-cells and NK-cells, suggesting the involvement of Plexin-B2 in the activities of Sema4A. Collectively these findings reveal the important role of Sema4A in activation and CTL functions of CD8+ T cells and NK cells.

In a first generic embodiment, there is provided a method of treating an oncological disease, by administering to a patient a composition which enhances Sema4A/Plexin-B2 interaction.

One embodiment of the present invention relates to a method to identify a compound that controls interaction of Sema4A with Plexin-B2 activity in a cell, comprising: (1) contacting a cell with a putative regulatory compound, wherein the cell includes a Plexin-B2 protein or a Sema4A protein; and (2) assessing the ability of the putative regulatory compound to inhibit or enhance the interaction of Sema4A with Plexin-B2. The assessment step preferably involves either i) determining the cytokine production as described herein-below, or ii) *in vitro* assays performed as described previously and methods known in the art.

The term "regulate" refers to controlling the activity of a molecule and/or biological function, such as enhancing or diminishing such activity or function.

The term "patient" includes both human and non-human mammals.

The terms "treating" or "treatment" mean the treatment of a disease-state in a patient, and include:
(i) preventing the disease-state from occurring in a patient, in particular, when such patient is genetically or otherwise predisposed to the disease-state but has not yet been diagnosed as having it;

(ii) inhibiting or ameliorating the disease-state in a patient, i.e., arresting or slowing its development; or

(iii) relieving the disease-state in a patient, i.e., causing regression or cure of the disease-state.

Yet another embodiment of the present invention relates to an antibody or antibody binding site which binds Sema4A, Plexin-B2 or fragments thereof. Embodiments of the present invention further include polyclonal and monoclonal antibodies. Preferred embodiments of the present invention include a monoclonal antibody such an anti-Plexin-B2 monoclonal antibody. The above antibody or antibody binding site which binds Plexin-B2 or Sema4a inhibits binding of Sema4A with Plexin-B2.

Yet another embodiment of the present invention relates to a biotherapeutic comprising Sema4A or Plexin-B2 or fragments thereof, wherein the biotherapeutic is useful for treating inflammatory, autoimmune or oncological diseases.

Another embodiment of the present invention provides a method of enhancing an immunological response to a vaccine comprising administering to a patient a biotherapeutic composition as described immediately above. The composition which enhances immunological response to a vaccine can be administered together or separately with the vaccine. Examples of vaccines include any vaccine whose effectiveness is enhanced by stimulation of the cell-mediated lytic immune response including but not limited to HIV vaccine.

The term "composition" as referred to herein include a putative compound, or a substantially pure protein selected from Sema4A, Plexin-B2 or fragments thereof, an antibody or antibody binding site which binds Sema4A, Plexin-B2 or fragments thereof, to an expression vector encoding Sema4A, Plexin-B2 or fragments thereof, a fusion protein comprising Sema4A, Plexin-B2 or fragments thereof. In the antibody binding site embodiments, the antibody binding site may be: specifically immunoreactive with a mature protein selected from the group consisting of the Sema4A and Plexin-B2; raised
against a purified or recombinantly produced human or mouse Sema4A or Plexin-B2; in a monoclonal antibody, Fab, or F(ab)2; immunoreactive with denatured antigen; or in a labeled antibody. In certain embodiments; the antibody binding site is detected in a biological sample by a method of: contacting a binding agent having an affinity for Sema4A or Plexin-B2 with the biological sample; incubating the binding agent with the biological sample to form a binding agent: Sema4A, Plexin-B2 protein complex; and detecting the complex. In a preferred embodiment, the biological sample is human, and the binding agent is an antibody.

Putative compounds as referred to herein include, for example, compounds that are products of rational drug design, natural products and compounds having partially defined signal transduction regulatory properties. A putative compound can be a protein-based compound, a carbohydrate-based compound, a lipid-based compound, a nucleic acid-based compound, a natural organic compound, a synthetically derived organic compound, an anti-idiotypic antibody and/or catalytic antibody, or fragments thereof. A putative regulatory compound can be obtained, for example, from libraries of natural or synthetic compounds, in particular from chemical or combinatorial libraries (i.e., libraries of compounds that differ in sequence or size but that have the same building blocks; see for example, U.S. Pat. Nos. 5,010,175 and 5,266,684 of Rutter and Santi, which are incorporated herein by reference in their entirety) or by rational drug design.

In a rational drug design procedure, the three-dimensional structure of a compound, such as a signal transduction molecule can be analyzed by, for example, nuclear magnetic resonance (NMR) or x-ray crystallography. This three-dimensional structure can then be used to predict structures of potential compounds, such as putative regulatory compounds by, for example, computer modelling. The predicted compound structure can then be produced by, for example, chemical synthesis, recombinant DNA technology, or by isolating a mimetope from a natural source (e.g., plants, animals, bacteria and fungi). Potential regulatory compounds can also be identified using SELEX technology as described in, for example, PCT Publication Nos. WO 91/19813; WO 92/02536 and WO 93/03172 (which are incorporated herein by reference in their entirety).
In particular, a naturally-occurring intracellular signal transduction molecule can be modified based on an analysis of its structure and function to form a suitable regulatory compound. For example, a compound capable of regulating Sema4A-Plexin-B2 binding or signaling can comprise a compound having similar structure to the amino acid residues in the intracellular domain of Sema4A or Plexin-B2. Such a compound can comprise a peptide, a polypeptide or a small organic molecule. In addition, a compound capable of regulating Sema4A-Plexin-B2 binding or signaling can comprise a compound that binds to the intracellular domain or Sema4A or Plexin-B2. Such a compound can comprise a peptide, a polypeptide or a small organic molecule.

Putative regulatory compounds can also include molecules designed to interfere with Sema4A and Plexin-B2. For example, mutants of Plexin-B2 can be created that interfere with the coupling of the protein with Sema4A. Putative regulatory compounds can include agonists and antagonists of Sema4A and Plexin-B2 binding. Such agonists and antagonists can be selected based on the structure of a naturally-occurring ligand to these proteins.

The technology for producing monoclonal antibodies is well known. In general, an immortal cell line (typically myeloma cells) is fused to lymphocytes (typically splenocytes) from a mammal immunized with whole cells expressing a given antigen, e.g., Plexin-B2, and the culture supernatants of the resulting hybridoma cells are screened for antibodies against the antigen. See, generally, Kohler et al., 1975, Nature 265: 295-497, "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity".

Immunization may be accomplished using standard procedures. The unit dose and immunization regimen depend on the species of mammal immunized, its immune status, the body weight of the mammal, etc. Typically, the immunized mammals are bled and the serum from each blood sample is assayed for particular antibodies using appropriate screening assays. For example, anti-integrin antibodies may be identified by immunoprecipitation of 1251-labeled cell lysates from integrin-expressing cells. Antibodies, including for example, anti-Plexin-B2 antibodies, may also be identified by flow cytometry, e.g., by measuring fluorescent staining of antibody-expressing cells incubated with an antibody believed to recognize Plexin-B2 molecules. The
lymphocytes used in the production of hybridoma cells typically are isolated from immunized mammals whose sera have already tested positive for the presence of anti-Plexin-B2 antibodies using such screening assays.

Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same mammalian species as the lymphocytes. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using 1500 molecular weight polyethylene glycol ("PEG 1500"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridomas producing a desired antibody are detected by screening the hybridoma culture supernatants. For example, hybridomas prepared to produce anti-Plexin-B2 antibodies may be screened by testing the hybridoma culture supernatant for secreted antibodies having the ability to bind to a recombinant Plexin-B2-expressing cell line.

To produce antibody homologs which are within the scope of the invention, including for example, anti-Plexin-B2 antibody homologs, that are intact immunoglobulins, hybridoma cells that tested positive in such screening assays were cultured in a nutrient medium under conditions and for a time sufficient to allow the hybridoma cells to secrete the monoclonal antibodies into the culture medium. Tissue culture techniques and culture media suitable for hybridoma cells are well known. The conditioned hybridoma culture supernatant may be collected and the anti-Plexin-B2 antibodies optionally further purified by well-known methods.

Alternatively, the desired antibody may be produced by injecting the hybridoma cells into the peritoneal cavity of an unimmunized mouse. The hybridoma cells proliferate in the peritoneal cavity, secreting the antibody which accumulates as ascites fluid. The antibody may be harvested by withdrawing the ascites fluid from the peritoneal cavity with a syringe.

Fully human monoclonal antibody homologs against, for example Plexin-B2, are another preferred binding agent which may block antigens in the method of the
invention. In their intact form these may be prepared using in vitro-primed human splenocytes, as described by Boerner et al, 1991, J. Immunol. 147:86-95, "Production of Antigen-specific Human Monoclonal Antibodies from In Vitro-Primed Human Splenocytes".


In yet another method for producing fully human antibodies, U.S. Pat. No. 5,789,650 (Aug. 4, 1998, "Transgenic non-human animals for producing heterologous antibodies") describes transgenic non-human animals capable of producing heterologous antibodies and transgenic non-human animals having inactivated endogenous immunoglobulin genes. Endogenous immunoglobulin genes are suppressed by antisense polynucleotides and/or by antiserum directed against endogenous immunoglobulins. Heterologous antibodies are encoded by immunoglobulin genes not normally found in the genome of that species of non-human animal. One or more transgenes containing sequences of unrearranged heterologous human immunoglobulin heavy chains are introduced into a non-human animal thereby forming a transgenic animal capable of functionally rearranging transgenic immunoglobulin sequences and producing a repertoire of antibodies of various isotypes encoded by human immunoglobulin genes. Such heterologous human antibodies are produced in B-cells which are thereafter immortalized, e.g., by fusing with an immortalizing cell line such as a myeloma or by manipulating such B-cells by other techniques to perpetuate a cell line capable of producing a monoclonal heterologous, fully human antibody homolog.
The conditions under which the cell of the present invention is contacted with a putative regulatory compound, such as by mixing, are conditions in which the cell can exhibit Plexin-B2, Sema4A activity if essentially no other regulatory compounds are present that would interfere with such activity. Achieving such conditions is within the skill in the art, and includes an effective medium in which the cell can be cultured such that the cell can exhibit Sema4A with Plexin-B2 activity. For example, for a mammalian cell, effective media are typically aqueous media comprising RPMI 1640 medium containing 10% fetal calf serum.

Cells of the present invention can be cultured in a variety of containers including, but not limited to, tissue culture flasks, test tubes, microtiter dishes, and petri plates. Culturing is carried out at a temperature, pH and carbon dioxide content appropriate for the cell. Such culturing conditions are also within the skill in the art. For example, for Ramos cells, culturing can be carried out at 37°C, in a 5% CO₂ environment.

Acceptable protocols to contact a cell with a putative regulatory compound in an effective manner include the number of cells per container contacted, the concentration of putative regulatory compound(s) administered to a cell, the incubation time of the putative regulatory compound with the cell, the concentration of ligand and/or intracellular initiator molecules administered to a cell, and the incubation time of the ligand and/or intracellular initiator molecule with the cell. Determination of such protocols can be accomplished by those skilled in the art based on variables such as the size of the container, the volume of liquid in the container, the type of cell being tested and the chemical composition of the putative regulatory compound (i.e., size, charge etc.) being tested.

In one embodiment of the method of the present invention, a suitable number of cells are added to a 96-well tissue culture dish in culture medium. A preferred number of cells includes a number of cells that enables one to detect a change in Sema4A with Plexin-B2 binding activity using a detection method of the present invention (described in detail below). A more preferred number of cells includes between about 1 and 1 x 10⁶ cells per well of a 96-well tissue culture dish. Following addition of the cells to the
tissue culture dish, the cells can be preincubated at 37° C, 5% CO₂ for between about 0
to about 24 hours.

A suitable amount of putative regulatory compound(s) suspended in culture medium is
added to the cells that is sufficient to regulate the activity of a Sema4A, Plexin-B2
protein in a cell such that the regulation is detectable using a detection method of the
present invention. A preferred amount of putative regulatory compound(s) comprises
between about 1 nM to about 10 mM of putative regulatory compound(s) per well of a
96-well plate. The cells are allowed to incubate for a suitable length of time to allow the
putative regulatory compound to enter a cell and interact with Sema4A or Plexin-B2
protein. A preferred incubation time is between about 1 minute to about 48 hours.

In another embodiment of the method of the present invention, cells suitable for use in
the present invention are stimulated with a stimulatory molecules capable of binding to
Sema4A or Plexin-B2 protein of the present invention to initiate a signal transduction
pathway and create a cellular response. Preferably, cells are stimulated with a
stimulatory molecule following contact of a putative regulatory compound with a cell.
Suitable stimulatory molecules can include, for example, antibodies that bind
specifically to Sema4A or Plexin-B2 protein. A suitable amount of stimulatory molecule
to add to a cell depends upon factors such as the type of ligand used (e.g., monomeric or
multimeric; permeability, etc.) and the abundance of Sema4A or Plexin-B2 protein.
Preferably, between about 1.0 nM and about 1 mM of ligand is added to a cell.

The method of the present invention include determining if a composition is capable of
regulating Sema4A and Plexin-B2 protein activation. Such methods include assays
described in detail in the Examples section. The method of the present invention can
further include the step of performing a toxicity test to determine the toxicity of the
composition.

Another aspect of the present invention includes a kit to identify compositions capable
of regulating Sema4A and Plexin-B2 protein activity in a cell. Such a kit includes: (1) a
cell comprising Sema4A and Plexin-B2 protein; and (2) a means for detecting
regulation of either the Sema4A and/or Plexin-B2 protein. Such a means for detecting
the regulation of Sema4A or Plexin-B2 protein include methods and reagents known to
those of skill in the art, for example, Plexin-B2 protein activity can be detected using, for example, activation assays. Means for detecting the regulation of Sema4A with Plexin-B2 protein also include methods and reagents known to those of skill in the art. Suitable cells for use with a kit of the present invention include cells described in detail herein. A preferred cell for use with a kit includes a human cell.

METHODS OF THERAPEUTIC USE

It has been found for the first time by the present inventors that that Plexin-B2 is a ligand for Semaphorin 4A on CD8+ T cells and NK cells. Collectively the findings by the present inventors reveal the important role of Sema4A/Plexin-B2 interaction and signaling in the activation of CD8+ T-cells and NK-cells and in the generation of lytic activity of CD8+ T-cells and NK-cells.

Enhancing Sema4A/Plexin-B2 interaction or signaling may be useful in the treatment of diseases where increased T lymphocyte and/or NK cell activation and/or lytic activity is (are) beneficial, for example cancer and viral infections, or for enhancing the immune response to vaccines.

A composition according to the invention will also be useful for treating oncological diseases. These diseases include but are not limited to solid tumors, such as cancers of the breast, respiratory tract, brain, reproductive organs, digestive tract, urinary tract, eye, liver, skin, head and neck, thyroid, parathyroid and their distant metastases. Those disorders also include lymphomas, sarcomas, and leukemias.

Examples of breast cancer include, but are not limited to invasive ductal carcinoma, invasive lobular carcinoma, ductal carcinoma in situ, and lobular carcinoma in situ.

Examples of cancers of the respiratory tract include, but are not limited to small-cell and non-small-cell lung carcinoma, as well as bronchial adenoma and pleuropulmonary blastoma and mesothelioma.
Examples of brain cancers include, but are not limited to brain stem, optic and hypothalamic glioma, cerebella and cerebral astrocytoma, medulloblastoma, ependymoma, as well as pituitary, neuroectodermal and pineal tumor.

Examples of peripheral nervous system tumors include, but are not limited to neuroblastoma, ganglioneuroblastoma, and peripheral nerve sheath tumors.

Examples of tumors of the endocrine and exocrine system include, but are not limited to thyroid carcinoma, adrenocortical carcinoma, pheochromocytoma, and carcinoid tumors.

Tumors of the male reproductive organs include, but are not limited to prostate and testicular cancer.

Tumors of the female reproductive organs include, but are not limited to endometrial, cervical, ovarian, vaginal, and vulvar cancer, as well as sarcoma of the uterus.

Tumors of the digestive tract include, but are not limited to anal, colon, colorectal, esophageal, gallblader, gastric, pancreatic, rectal, small-intestine, and salivary gland cancers.

Tumors of the urinary tract include, but are not limited to bladder, penile, kidney, renal pelvis, ureter, and urethral cancers.

Eye cancers include, but are not limited to intraocular melanoma and retinoblastoma.

Examples of liver cancers include, but are not limited to hepatocellular carcinoma (liver cell carcinomas with or without fibrolamellar variant), hepatoblastoma, cholangiocarcinoma (intrahepatic bile duct carcinoma), and mixed hepatocellular cholangiocarcinoma.

Skin cancers include, but are not limited to squamous cell carcinoma, Kaposi's sarcoma, malignant melanoma, Merkel cell skin cancer, and non-melanoma skin cancer.

Head-and-neck cancers include, but are not limited to laryngeal/hypopharyngeal/nasopharyngeal/oropharyngeal cancer, and lip and oral cavity cancer.
Lymphomas include, but are not limited to AIDS-related lymphoma, non-Hodgkin's lymphoma, Hodgkin's lymphoma, cutaneous T-cell lymphoma, and lymphoma of the central nervous system.

Sarcomas include, but are not limited to sarcoma of the soft tissue, osteosarcoma, Ewing's sarcoma, malignant fibrous histiocytoma, lymphosarcoma, angiosarcoma, and rhabdomyosarcoma. Leukemias include, but are not limited to acute myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, chronic myelogenous leukemia, and hairy cell leukemia.

Plasma cell dyscrasias include, but are not limited to multiple myeloma, and Waldenstrom's macroglobulinemia.

A composition that would block the interaction of Plexin-B2 with Sema4a would block inflammatory cytokine production from cells. The inhibition of cytokine production is an attractive means for preventing and treating a variety of cytokine mediated diseases or conditions associated with excess cytokine production, e.g., diseases and pathological conditions involving inflammation, autoimmune responses or bone resorption. Thus, the compositions are useful for the treatment of diseases and conditions including the following:

- osteoarthritis, atherosclerosis, contact dermatitis, bone resorption diseases including osteoporosis, reperfusion injury, asthma, multiple sclerosis, Guillain-Barre syndrome, Crohn's disease, ulcerative colitis, psoriasis, graft versus host disease, systemic lupus erythematosus and insulin-dependent diabetes mellitus, rheumatoid arthritis, toxic shock syndrome, Alzheimer's disease, diabetes, inflammatory bowel diseases, acute and chronic pain as well as symptoms of inflammation and cardiovascular disease, stroke, myocardial infarction, alone or following thrombolytic therapy, thermal injury, adult respiratory distress syndrome (ARDS), multiple organ injury secondary to trauma, acute glomerulonephritis, dermatoses with acute inflammatory components, acute purulent meningitis or other central nervous system disorders, syndromes associated with hemodialysis, leukopheresis, granulocyte transfusion associated syndromes, and necrotizing enterocolitis, complications including restenosis following percutaneous transluminal coronary angioplasty, traumatic arthritis, sepsis, chronic obstructive pulmonary disease and congestive heart failure. Said composition may also be useful for...
anticoagulant or fibrinolytic therapy (and the diseases or conditions related to such therapy).


These disorders have been well characterized in man, but also exist with a similar etiology in other mammals, and can be treated by pharmaceutical compositions of the present invention.

For therapeutic use, the compositions may be administered in any conventional dosage form in any conventional manner. Routes of administration include, but are not limited to, intravenously, intramuscularly, subcutaneously, intrasynovially, by infusion, sublingually, transdermally, orally, topically or by inhalation. The preferred modes of administration are oral and intravenous.

The compositions may be administered alone or in combination with adjuvants that enhance stability of the inhibitors, facilitate administration of pharmaceutical compositions containing them in certain embodiments, provide increased dissolution or dispersion, increase inhibitory activity, provide adjunct therapy, and the like, including other active ingredients. Advantageously, such combination therapies utilize lower dosages of the conventional therapeutics, thus avoiding possible toxicity and adverse side effects incurred when those agents are used as monotherapies. The above described compositions may be physically combined with the conventional therapeutics or other adjuvants into a single pharmaceutical composition. Advantageously, the compositions may then be administered together in a single dosage form. In some embodiments, the pharmaceutical compositions comprising such combinations of compositions contain at least about 5%, but more preferably at least about 20%, of a composition (w/w) or a combination thereof. The optimum percentage (w/w) of a composition of the invention may vary and is within the purview of those skilled in the art. Alternatively, the compositions may be administered separately (either serially or in parallel). Separate dosing allows for greater flexibility in the dosing regime.
As mentioned above, dosage forms of the compositions described herein include pharmaceutically acceptable carriers and adjuvants known to those of ordinary skill in the art. These carriers and adjuvants include, for example, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, buffer substances, water, salts or electrolytes and cellulose-based substances. Preferred dosage forms include, tablet, capsule, caplet, liquid, solution, suspension, emulsion, lozenges, syrup, reconstitutable powder, granule, suppository and transdermal patch. Methods for preparing such dosage forms are known (see, for example, H.C. Ansel and N.G. Popovish, *Pharmaceutical Dosage Forms and Drug Delivery Systems*, 5th ed., Lea and Febiger (1990)). Dosage levels and requirements are well-recognized in the art and may be selected by those of ordinary skill in the art from available methods and techniques suitable for a particular patient. In some embodiments, dosage levels range from about 1-1000 mg/dose for a 70 kg patient. Although one dose per day may be sufficient, up to 5 doses per day may be given. For oral doses, up to 2000 mg/day may be required. As the skilled artisan will appreciate, lower or higher doses may be required depending on particular factors. For instance, specific dosage and treatment regimens will depend on factors such as the patient's general health profile, the severity and course of the patient's disorder or disposition thereto, and the judgment of the treating physician.

**EXPERIMENTAL METHODS**

Splenic CD4+ T cells and CD8+ T cells were stained with biotinylated Sema4A-Fc (blue) or biotinylated human IgGl (red) and streptavidin-APC before and after 24 h of 10 mg/ml of anti-CD3 and ConA activation as shown in Figure 1: Binding of Sema4A-Fc to activated T cells. **Figure 2** shows that Sema4A promotes T-cell activation. In (A), Sema4A-Fc enhances *in vitro* T-cell activation of purified CD4+ and CD8+ T cells that were stimulated with anti-CD3, with or without various concentrations of Sema4A-Fc. (B) Dose dependent induction of IFN-gamma by Sema4A-Fc treated CD8+ T-cells, culture supernatants were estimated by ELISA.

Analysis of binding of Sema4A-Fc, expression of Sema4A and TIM molecules on NK cells is shown in **Figure 3**. Purified NK cells were stained with biotinylated Sema4A-Fc (red) or human IgGl biotinylated (blue), biotinylated anti-Sema4A, anti-TIM-1, anti-
TIM-2 and streptavidin-APC. **Figure 4** shows Sema4A treatment induces IFN-gamma from NK cells. Purified NK cells were cultured in presence of IL-2 and IL-12 or in combination with and without addition of Sema4A fusion proteins. 48h later culture supernatants were estimated by ELISA. In **Figure 5**, purified NK cells were treated with 50 U/ml of recombinant IL-2 for 24 h either with 20 mg/ml of Sema4A-Fc fusion protein or human IgGl and mixed with target YAC-I cells at various effector to target ratio as indicated and cytotoxicity was measured using LDH-calorimetric assay. In **Figure 6**, P815 were transfected with full length Sema4A expression vector and selected for 10 days in 0.5 mg/ml neomycin. Sema4A expressing cells were separated by FACS sorting. Stable P815 cells expression was tested using anti-Sema4A antibody. Anti-CD100 and mouse isotype were used as controls. DBA/2 syngeneic spleenocytes were cultured for 9 days with 20,000 rad irradiated parental or P815 stably expressing B7.1 or Sema4A. Recombinant IL-2, 10 U/ml or IL-4, 20 U/ml was added separately or in combination to the cultures every alternate day. CTL assay was performed using parental P815 cells using 1:10 ratio of Effector to Target cells as shown in **Figure 7**. The effect of Sema4A on tumorigenicity of P815 tumor demonstrated in **Figure 8**. (A), 10^6 non-transfected or Sema4A expressing cells were injected subcutaneously and tumor size was measured after 4 weeks. (B) Mice with regressed tumors were challenged with s.c injection of 5x10^5 non-transfected P815 cells (circles) 8 weeks after primary tumor inoculation. Naïve DBA/2 mice were inoculated with same number of non-transfected P815 cells as control (squares). FACS analysis was performed with purified CD8^+ T-cells and NK cells before and after activation with anti-CD3 and anti-CD28 antibodies (5 mg/ml) and 200 U/ml of recombinant IL-2 respectively for 24h and stained with biotinylated anti-TIM-2 or biotinylated Sema4A-Fc and straptavidin-APC, see **Figure 9**. Biotinylated human IgGl and rat IgG2a as isotype controls were used. **Figure 10** shows purified NK cells were treated with either IL-2 and IL-12 or in combination for 16 hours. RT-PCR was performed from cDNA isolated from treated NK cells for analysis of various plexin-B family members. Expression library made from NK cells was used as another source of NK cell cDNA. In **Figure 11**, cDNA was isolated from NK cells was analyzed for expression of various TIM family members.

Purified NK cells were infected with either controls lentivirus or Plexin-B2 silencing ShRNA lentivirus particles. NK cells were selected for 6 days using 100 U/ml
recombinant IL-2 and 2.5 mg/ml Puromycin followed by 48 h activation with Sema4A or con-Ig. IFN-g was estimated from culture supernatants by ELISA, see Figure 12. OT-I transgenic purified CD8+ T-cells were infected with either controls lentivirus or Plexin-B2 silencing lentivirus particles shown in Figure 13. CD8+ T-cells were selected for 6 days using 100 U/ml recombinant IL-2 and 2.5 mg/ml Puromycin followed by 48 h activation with Sema4A or con-Ig. IFN-g was estimated from culture supernatants by ELISA.

All publications cited in this application are hereby incorporated by reference in their entirety.
Claims

1. A method to identify a compound that inhibits or enhances interaction of Sema4A with Plexin-B2 activity in a cell, comprising: (1) contacting a cell with a putative regulatory compound, wherein the cell includes a Sema4A protein and Plexin-B2 protein; and (2) assessing the ability of the putative regulatory compound to inhibit or enhance the interaction of Sema4A with Plexin-B2.

2. An antibody or antibody binding site which effectively binds Sema4A or Plexin-B2 or fragments thereof, wherein the above antibody or antibody binding site which binds Sema4A or Plexin-B2 and inhibits Sema4A - Plexin-B2 binding or signaling.

3. A biotherapeutic composition for treating an inflammatory, autoimmune or oncological disease comprising a therapeutically effective amount of Plexin-B2 protein, Sema4A protein or fragments thereof and one or more pharmaceutically acceptable carriers and/or adjuvants.

4. A method of treating an inflammatory or autoimmune disease, the method comprising administering to a patient a therapeutically effective amount of a composition which inhibits Sema4A - Plexin-B2 interaction or signaling.

5. A method of treating an oncological disease, the method comprising administering to a patient a therapeutically effective amount of a composition which enhances Sema4A - Plexin-B2 interaction or signaling.

6. A method of enhancing an immunological response to a vaccine comprising administering to a patient a biotherapeutic composition according to claim 6 together or separately with the vaccine.

7. Use of a composition which inhibits Sema4A - Plexin-B2 interaction or signaling for the treatment of an inflammatory or autoimmune disease.
8. Use of a composition which inhibits Sema4A - Plexin-B2 interaction or signaling for the manufacture of a medicament for the treatment of an inflammatory or autoimmune disease.


10. Use of a composition which enhances Sema4A - Plexin-B2 interaction or signaling for the manufacture of a medicament for the treatment of an oncological disease.
Fig. 1

No treatment  Anti-CD3  Con-A

CD4+ T cells

CD8+ T cells

---

Sema 4A

Sema4A binds to activated CD4 and CD8+ T cells
Fig. 3

Dx-5 + NK cells express Sema4A receptor other than Tim-2.
Sema4A treatment enhances IFN-γ production by NK cells.
Fig. 5

LDH-calorimetric assay

- ▲ Sema-4A-Fc+ IL-2
- ■ HulgG-Fc+IL-2
- ○ IL-2 alone

% cytotoxicity

Sema4A enhances CTL activity of NK cells against YAC-1 cells
Fig. 6

Stained with anti-CD100.

Stained with anti-Sema4A.

Generation of P815 mastocytoma cells stably expressing Sema4A.
Expression of Sema4A in P815 cells results in increased primary CTL generation.
P815-Sema4A enhances tumor regression by CTL and NK cell activation.
P815-Sema4A enhances tumor regression by CTL and NK cell activation.

**Fig. 8b**

Tumor size (mm)

Days after tumor inoculation

- Sema4A P815
- Parental P815
Fig. 9

CD8 T cells

NK cells

Naïve and activated CD8 T cells and NK cells do not express Tim-2
Plexin-B2 is dominantly expressed by NK cells.
Naïve or activated NK cells does not express Tim-2.
Fig. 12

Plexin-B2 silencing effect on Sema4A activity on NK cells.
Fig. 13

Plexin-B2 silencing effect:
Sema4A activity on CD8+ T-cells.
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

| INV. | C07K16/18 | C07K16/28 | C07K14/47 | A61P37/06 | A61P35/00 |

**B. CLASSIFICATION OF SUBJECT MATTER**

According to International Patent Classification (IPC) and both national classification and IPC.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>WO 03/080673 A (BOEHRINGER INGELHEIM INT [DE]); KIKUTANI HITOSHI [JP]; KUMANOGOH ATSUSHI) 2 October 2003 (2003-10-02) page 25; claims 1-20</td>
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**Date of the actual completion of the international search**

4 January 2008

**Name and mailing address of the ISA**

European Patent Office, P B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx 31 651 epo nl, Fax (+31-70) 340-3016

**Authorized officer**

Bernhardt, Wiebke

**Date of mailing of the international search report**

11/02/2008
### DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>A</td>
<td>KIKUTANI H ET AL: &quot;SEMAPHORINS IN INTERACTIONS BETWEEN T CELLS AND ANTIGEN-PRESENTING CELLS&quot; NATURE REVIEWS. IMMUNOLOGY, XX, XX, vol. 3, no. 2, February 2003 (2003-02), pages 159-167, XP008055307 ISSN: 1474-1733 the whole document</td>
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Continuation of Box II.1

Although claims 4-7 and 9 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.1

Claims Nos.: -

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
   see FURTHER INFORMATION sheet PCT/ISA/210

2. ☐ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.: 

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos:

Remark on Protest
☐ The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.
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