LIGANDS OF HVEM FOR TREATING HEMATOLOGIC MALIGNANCIES AND AUTOIMMUNE DISEASES

The present invention relates to ligands of HVEM for the treatment of hematologic malignancies, in particular Chronic lymphocytic leukaemia, and for the treatment of autoimmune diseases.
Ligands of HVEM for Treating Hematologic Malignancies and Autoimmune Diseases.

FIELD OF THE INVENTION
5 The present invention relates to ligands of HVEM for the treatment of hematologic malignancies, in particular Chronic lymphocytic leukemia (CLL), and for the treatment of autoimmune diseases.

BACKGROUND OF THE INVENTION
10 Cells of the Immune System
The cells of the immune system originate in the bone marrow, where many of them also mature. They then migrate to patrol the tissues, circulating in blood and in a specialized system of vessels called the lymphatic system.

All the cellular elements of blood including the red blood cells, platelets, and white blood cells of the immune system derive ultimately from the same progenitor or precursor cells, the hematopoietic stem cells in the bone marrow. As these stem cells can give rise to all of the different types of blood cells, they are often referred to as pluripotent hematopoietic stem cells. Initially, they give rise to stem cells of more limited potential which include: i) erythroblasts which give rise erythrocytes or red blood cells, ii) megakaryocytes which are the precursors of platelets, iii) myeloid progenitors which are precursors of the granulocytes, macrophages and mast cells of the innate immune system, and iv) the common lymphoid progenitors which give rise to lymphocytes which are the main components of the adaptive immune system.

There are two main types of lymphocytes: B lymphocytes or B cells, which when activated differentiate into plasma cells, and T lymphocytes or T cells, of which there are two main classes. Within the T cells, one class differentiate into cytotoxic T cells which kill virally infected cells, whereas the second class differentiate into helper T cells. Helper T cells provide help to the adaptive immune system by activating other lymphocytes such as B cells, and interact with the innate immune system by activating some of the myeloid lineage cells. Although all lymphocytes originate within the bone marrow, which is a primary or central lymphoid organ in humans,
only B lymphocytes mature there. T lymphocytes migrate to the thymus, the other central lymphoid organ in humans, to undergo maturation. Both B and T cells express on their surface, specialized receptors that allow them to recognize antigens. Although collectively these receptors are highly diverse in their antigen specificity, each fully differentiated lymphocyte, and all its progeny, is designed to express receptors that recognize only one antigen. Collectively, the receptors on all the lymphocytes are capable of recognizing a very large repertoire of antigens. The B-cell antigen receptor (BCR) is a membrane-bound form of the antibody that will be secreted when the cells are activated. The T cell antigen receptor (TCR), although related to BCR because of their common structural immunoglobulin roots, is quite distinct from BCR in its antigen binding region, and in the way it interacts with the antigen. A third class of lymphoid cells, called natural killer cells or NK cells, lack antigen-specific receptors and so form part of the innate immune system.

Mature antigen-responsive B lymphocytes develop in the bone marrow prior to their encounter with antigen. The maturation process goes through an orderly series of differentiation stages from the common lymphoid progenitors through the pro-B, pre-B, transitional or immature B, to mature B lymphocytes. Following their encounter with antigen, B cells undergo antigen-induced proliferation and differentiation whose hallmark is the re-arrangement of the immunoglobulin gene locus and expansion of B cell clones. This process ultimately results in the progeny of the B cells secreting antibodies of different heavy and light chain isotypes, or becoming memory cells.

**Hematologic Malignancies**

With respect to pathological conditions which involve the immune system, the diversity in the lineages and differentiation stages of hematopoietic cells results in a large number of distinct and heterogeneous tumors generally referred to as hematologic malignancies. Thus, hematologic malignancies or hematologic neoplasia affect cells and tissues of the immune and hematopoietic system, including blood, bone marrow and lymph nodes. Hematologic malignancies include both leukemias and lymphomas.
The term leukemia has generally been used to define hematologic malignancies of the blood or bone marrow characterized by abnormal proliferation of leukocytes. The principal subtypes of leukemia are identified on the basis of malignancy involving lymphoid (e.g. T or B lymphocytic lineage) or myeloid (e.g. granulocytic, erythroid or megakaryocyte lineage) cells, and whether the disease is acute or chronic in onset [Freireich, E.J. et al., 1991].

The term lymphoma covers a heterogeneous group of neoplasms of lymphoid tissue. Lymphomas are broadly categorized under Hodgkin lymphoma, and T-cell (T-NHL) and B-cell (B-NHL) non-Hodgkin lymphomas. A World Health Organization (WHO) classification has recently been published (discussed later in this application), and diagnostic guidelines have been established based on this classification [Jaffe, E.S. et al., 2004 (see Table 3 and 4 hereinafter)]. Chronic Lymphocytic Leukemia (CLL) is a form of lymphocytic leukemia characterized by slow but progressive accumulation of lymphocytes in the bone marrow and blood. Depending on the stage of the disease, lymph node and spleen enlargement occur commonly. Although CLL may be of T cell or B cell origin, over 85% of the cases are of B-cell origin. Current understanding suggests that CLL is a heterogeneous disease originating from B lymphocytes that differ in their activation and maturation states and cellular subgroup (see [Kuppers, R., 2005]). The disease may result both from decreased apoptosis as well as increased proliferation of the leukemic B cells. CLL cells are usually clonal in origin, and express the following cell surface markers: CD19, CD20, CD21, and CD24. In addition, they express CD5 which is more typically found on T cells (see [Chiorazzi, N, and al., 2005]).

CLL is considered a subgroup of "non-Hodgkin's lymphoma" (NHL) and together with the closely related disease "small lymphocytic lymphoma" (SLL) which presents primarily in the lymph nodes, corresponds to around 20% of all NHL cases. CLL is the most common leukemia in adults in the US and most of Western Europe. The National Cancer Institute (NCI) estimate for the incidence of CLL is about 10,000 new cases in the US per year. Clinical manifestations of CLL occur predominantly after the age of 55. The incidence rate for men is higher than for women, with men almost twice as likely to acquire the disease as women.

CLL represents an unmet medical need as there are limited options for treatment
The most common treatments for NHL are chemotherapy, in particular a combination regimen called CHOP (for Cytoxan, Hydroyrubincin [Adriamycin], Oncovin [Vincristine], Prednisone), and radiation therapy. In some cases, surgery and bone marrow transplantation have also been used. More recently, there has been an increase in the use of biopharmaceutical agents, especially monoclonal antibodies, such as rituximab and alemtuzumab. Other combination approaches include the use of biopharmaceuticals such as rituximab with chemotherapy. Although these treatments have significantly improved the management of B-lymphoid malignancies, among their deficiencies include non-responsiveness of many patients to these regimens (some patients become refractory to some or all these approaches), and the side effects and complications which result from the use of these treatments. Among the most common side effects of chemotherapy are nausea and vomiting (which is generally managed with the use of antiemetics), alopecia (which is generally reversed over time after completion of treatment), and leukopenia, especially neutropenia. Neutropenia generally develops in the second week. During this period, many clinicians recommend prophylactic use of ciprofloxacin. If a fever develops in the neutropenic period, urgent medical assessment is required for neutropenic sepsis, as infections in patients with low neutrophil counts may progress rapidly. With respect to rituximab, first infusion reaction, lymphopenia, infectious complications such as viral reactivation including Hepatitis B and Progressive Multifocal Leukoencephalopathy (PML), mucocutaneous reactions, and renal complications have been reported. In the case of alemtuzumab, serious hematologic toxicities can occur, including pancytopenia, bone marrow hypoplasia, autoimmune idiopathic thrombocytopenia, and autoimmune hemolytic anemia. In some cases, these toxicities can accelerate morbidity and mortality rates.

**Autoimmune Diseases**

The immune system has control mechanisms which prevent it from attacking self tissue. When these mechanisms do not function properly or when they break down, they can result in the development of autoimmunity or autoimmune diseases. Autoimmunity represents a broad spectrum of diseases from the organ specific to the non-organ specific. At one end of the spectrum, Hashimoto's thyroditis typifies the
highly organ specific diseases where the destructive lesion is directed at one organ only. At the other end of the spectrum, lupus erythematosus (SLE) represents the non-organ specific diseases where the tissues involved are widespread throughout the body. With improvements in our understanding of immunobiology, and advances in molecular and diagnostic tools, it is becoming progressively evident that most organ or tissue systems can be subject to the autodestructive potential of autoimmune diseases as is shown in the following list. Thus among the autoimmune diseases are included: Addison's disease, ankylosing spondylitis, aplastic anemia, autoimmune hemolytic anemia, autoimmune hepatitis, coeliac disease, Crohn's disease, dermatomyositis, Goodpasture's syndrome, Graves' disease, Guillain-Barre syndrome, Hashimoto's disease, idiopathic leucopenia, idiopathic thrombocytopenic purpura, insulin dependent diabetes mellitus (Type 1 diabetes), male infertility, mixed connective tissue disease, multiple sclerosis (MS), myasthenia gravis, pemphigoid, pemphigus vulgaris, pernicious anemia, phacogenic uveitis, primary biliary cirrhosis, primary myxoedema, Reiter's syndrome, rheumatoid arthritis (RA), scleroderma, Sjogren's syndrome, stiff man syndrome, systemic lupus erythematosus (SLE), thyrotoxicosis, ulcerative colitis, and Wegener's granulomatosis.

The etiology of autoimmune diseases is not completely understood. In some instances, mechanisms of molecular mimicry have been proposed whereby a productive anti-bacterial or anti-viral response may inadvertently result in the development of immunological responses to self tissue. In addition, inherited or genetic predispositions are known to contribute to the development of many of these diseases.

Both lymphoid and myeloid lineage cells have been implicated in the development of autoimmune diseases. Autoreactive T and B lymphocytes determine the principal clinico-pathologic features of each disease and the tissue involved. T lymphocytes may attack self tissue directly whereas B cells secrete autoreactive antibodies. In SLE, copious of self reactive antibodies including antibodies to double-stranded DNA are produced which are believed to cause or exacerbate kidney damage.

Myeloid lineage cells such are macrophages help to maintain, amplify and extend the immune attack against self tissue by providing cytokine and chemokine responses such as TNF-α and IL-8, as well as by serving as effector cells for the
autodestructive processes. A role for TNF-α has been clearly established for RA and Crohn's disease which are now known to respond to anti-TNF-α therapies. In the case of RA, myeloid lineage cells are believed to differentiate to osteoclasts thus causing bone damage and destruction of synovial linings with the inflamed joints. RA patients have also been shown to respond to treatments directed against B cells, such as anti-CD20 antibody therapy.

**SUMMARY OF THE INVENTION**

The invention relates to a ligand of HVEM for therapeutic use, wherein said ligand of HVEM is selected from the group consisting of LIGHT, a fragment of LIGHT which induces apoptosis in chronic lymphocytic leukemia B cells, an anti-HVEM antibody and a fragment thereof which binds to HVEM. In particular the invention relates to a ligand of HVEM for the treatment of hematologic malignancies or autoimmune diseases. The invention also relates to a method of treatment of hematologic malignancies or autoimmune diseases which comprises administering to a subject in need thereof a therapeutically effective amount of a ligand of HVEM.

**DEFINITIONS**

As used herein, references to specific proteins (e.g., antibodies or LIGHT) can include a polypeptide having a native amino acid sequence, as well as variants and modified forms regardless of their origin or mode of preparation. A protein that has a native amino acid sequence is a protein having the same amino acid sequence as obtained from nature (e.g., a naturally occurring LIGHT). Such native sequence proteins can be isolated from nature or can be prepared using standard recombinant and/or synthetic methods. Native sequence proteins specifically encompass naturally occurring truncated or soluble forms, naturally occurring variant forms (e.g., alternatively spliced forms), naturally occurring allelic variants and forms including post-translational modifications. A native sequence protein includes proteins following post-translational modifications such as glycosylation, or phosphorylation, or other modifications of some amino acid residues.
The term "HVEM", as used herein, is intended to encompass all synonyms including, but not limited to, "Herpes Virus Entry Mediator", "HVEA", "Herpes Virus Entry Mediator A", "TNFRSF 14", "Tumor Necrosis Factor Receptor Superfamily Member 14", "TNRI 4", "LIGHT", "LIGHT receptor", "TR2", "TNF Receptor-like", "ATAR", "Another TRAF-Associated Receptor". TNFRSF 14 is the HUGO (Human Genome Organization) Gene Nomenclature Committee (HGNC) approved symbol. The UniProtKB/Swiss-Prot "Primary Accession Number" for HVEM is Q92956. The "Secondary Accession Numbers" are Q8WXR1, Q96J31 and Q9UM65.

By "ligand" is meant a natural or synthetic compound which binds to a receptor molecule to form a receptor-ligand complex. So far, four ligands have been identified which bind to HVEM. Two of these ligands, LIGHT and LTα, are member of the TNF family of molecules (Morel, Y. et al., 2000; Mauri, D.N. et al., 1998 and Harrop, J.A. et al., 1998). Structurally, members of the TNF family are generally expressed as single-pass type 2 transmembrane, homotrimer or heterotrimer, glycoproteins. Following their expression as transmembrane proteins, they are cleaved by proteolytic action to produce a soluble form of the ligand. The third ligand for HVEM, BTLA, a type 1 transmembrane glycoprotein, is a member of the immunoglobulin (Ig) superfamily of molecules and is closely related to CD28 (Gonzalez, L.C. et al., 2005). The fourth ligand, glycoprotein D (gD), is a structural component of the herpes simplex virus (HSV) envelope, and is essential for HSV entry into host cells (Montgomery, R.I. et al., 1996; Hsu, H. et al., 1997; Kwon, B.S. et al., 1997; Tan, K.B. et al., 1997; Marsters, S.A. et al., 1997; Wallach, D. et al., 1999; Collette, Y. et al., 2003; Harrop, J.A. et al., 1998; Gonzalez, L.C. et al., 2005 and Whitbeck, J.C. et al., 1997)

Binding studies (Gonzalez, L.C. et al., 2005 and Sedy, J.R. et al., 2005) which were later supported by crystallography (Compaan, D.M. et al., 2005) indicate that BTLA interacts with the most membrane-distal CRD region of HVEM. The membrane-distal CRD1 region of HVEM has also been implicated in the interactions with HSV-gD, with additional contributions from CRD2 (Compaan, D.M. et al., 2005 and Carfì, A. et al., 2001). Despite the sequence and structural dissimilarities between BTLA and HSV-gD, the crystal structure studies also show that their binding sites on

The term "LIGHT", as used herein, is intended to encompass all synonyms including, but not limited to, "lymphotoxins, inducible, competes with herpes simplex virus (HSV) glycoprotein D for HVEM, expressed by T cells", "TNFSF14", "Tumor Necrosis Factor Ligand Superfamily Member 14", "TNF14_HUMAN", "HVEM-L", "HVEM-Ligand", "Herpes Virus Entry Mediator Ligand", "Herpesvirus entry mediator-ligand", "TL4", "TNF-like 4", "TN14", "LTγ" and "CD258". TNFSF14 is the HGNC approved symbol. CD258 is the cluster designation assignment of the HLDA (Human Leukocyte Differentiation Antigens) Workshop. The UniProtKB/Swiss-Prot "Primary Accession Number" for LIGHT is 043557. The "Secondary Accession Numbers" are 075476, Q8WVF8 and Q96LD2.

In natural antibodies, the two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. There are two types of light chains, lambda (λ) and kappa (κ). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. Each chain contains distinct sequence domains. The light chain includes two domains, a variable domain (VL) and a constant domain (CL). The heavy chain includes four domains, a variable domain (VH) and three constant domains (CH1, CH2 and CH3, collectively referred to as CH). The variable regions of both light (VL) and heavy (VH) chains determine binding recognition and specificity to the antigen. The constant region domains of the light (CL) and heavy (CH) chains confer important biological properties such as antibody chain association, secretion, trans-placental mobility, complement binding, and binding to Fc receptors (FcR). The Fv fragment is the N-terminal part of the Fab fragment of an immunoglobulin consisting of the variable portions of one light chain and one heavy chain. The specificity of the antibody resides in the structural complementarity between the antibody combining site and the antigenic determinant. Antibody combining sites are made up of residues that are primarily from the hypervariable or complementarity determining regions (CDRs). Occasionally, residues from nonhypervariable or framework regions (FR) influence the overall domain structure.
and hence the combining site. Complementarity Determining Regions or CDRs refer to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site. The light and heavy chains of an immunoglobulin each have three CDRs, designated L-CDR1, L-CDR2, L-CDR3 and H-CDR1, H-CDR2, H-CDR3, respectively. An antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. Framework Regions (FRs) refer to amino acid sequences interposed between CDRs. The term "antibody" is further intended to encompass antibodies, digestion fragments, specified portions and variants thereof, including antibody mimetics or portions of antibodies that mimic the structure and/or function of an antibody or specified fragment or portion thereof, including single chain antibodies and fragments thereof. Functional fragments include antigen-binding fragments that bind to a mammalian HVEM.

As used herein, the term "human antibody" refers to an antibody in which a substantial portion of the antibody molecule resembles, in amino acid sequence or structure, that of an antibody derived from human origin. The term "humanized antibody" refers to an antibody which has been modified by genetic engineering or by other means to be similar in structure or amino acid sequence to naturally occurring human antibodies. A "human antibody" or a "humanized antibody" may be considered more suitable in instances where it is desirable to reduce the immunogenicity of the antibody for administration to humans for therapeutic, prophylactic or diagnostic purposes.

A "monoclonal antibody" or "mAb" in its various names refers to a population of antibody molecules that contains only one species of antibody combining site capable of immunoreacting with a particular epitope. A monoclonal antibody thus typically displays a single binding affinity for any epitope with which it immunoreacts. Monoclonal antibody may also define an antibody molecule which has a plurality of antibody combining sites, each immunospecific for a different epitope. For example, a bispecific antibody would have two antigen binding sites, each recognizing a different interacting molecule, or a different epitope. As used herein, the terms "antibody fragment", "antibody portion", "antibody variant" and the like include any protein or polypeptide containing molecule that comprises at
least a portion of an immunoglobulin molecule such as to permit specific interaction between said molecule and an antigen (e.g. HVEM). The portion of an immunoglobulin molecule may include, but is not limited to, at least one complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework region, or any portion thereof, or at least one portion of a ligand or counter-receptor (e.g. LIGHT, BTLA or HSV-gD) which can be incorporated into an antibody of the present invention to permit interaction with the antigen (e.g. HVEM).

The term "hybridoma" denotes a cell, which is obtained by subjecting a B cell, prepared by immunizing a non-human mammal with an antigen, to cell fusion with a myeloma cell derived from a mouse or the like which produces a desired monoclonal antibody having an antigen specificity.

As used herein, the term "subject" denotes a mammal, such as a rodent, a feline, a canine, and a primate. Preferably a subject according to the invention is a human.

DETAILED DESCRIPTION OF THE INVENTION

Therapeutic methods and uses

A first object of the invention relates to a ligand of HVEM for therapeutic use, wherein said ligand of HVEM is selected from the group consisting of LIGHT, a fragment of LIGHT which induces apoptosis in chronic lymphocytic leukemia B cells, an anti-HVEM antibody and a fragment thereof which binds to HVEM.

Typically said ligand of HVEM may be used in combination with radiotherapy and hormone therapy.

Typically said ligand of HVEM may also be used in combination with a one or more agents selected from the group consisting of an anticancer agent, an antiemetic agent, an hematopoietic colony stimulating factor, an analgesic agent and an anxiolytic agent.
In a preferred embodiment, the invention relates to a ligand of HVEM for the treatment of hematologic malignancies or autoimmune diseases.

The invention also relates to the use of a ligand of HVEM for the manufacture of a medicament for the treatment of hematologic malignancies or autoimmune diseases, wherein said ligand of HVEM is selected from the group consisting of LIGHT, a fragment thereof, which induces apoptosis in chronic lymphocytic leukemia B cells, an anti-HVEM antibody and a fragment thereof which binds to HVEM.

In one embodiment, hematologic malignancies include but are not limited to lymphoid cell neoplasms such as chronic lymphocytic leukaemia (CLL), non-Hodgkin lymphoma (NHL), small lymphocytic lymphoma (SLL), and mantle cell lymphoma (MCL). More specifically, non-Hodgkin lymphoma (NHL) include B and T non-Hodgkin lymphoma. Furthermore, cell lymphoid neoplasms include B, NK and T cell lymphoid neoplasms.

In one embodiment, autoimmune diseases include but are not limited to Addison's disease, ankylosing spondylitis, aplastic anemia, autoimmune hemolytic anemia, autoimmune hepatitis, coeliac disease, Crohn's disease, dermatomyositis, Goodpasture's syndrome, Graves' disease, Guillain-Barre syndrome, Hashimoto's disease, idiopathic leucopenia, idiopathic thrombocytopenic purpura, insulin dependent diabetes mellitus (Type 1 diabetes), male infertility, mixed connective tissue disease, multiple sclerosis (MS), myasthenia gravis, pemphigoid, pemphigus vulgaris, pernicious anemia, phacogenic uveitis, primary biliary cirrhosis, primary myxoedema, Reiter's syndrome, rheumatoid arthritis (RA), scleroderma, Sjogren's syndrome, stiff man syndrome, systemic lupus erythematosus (SLE), thyrotoxicosis, ulcerative colitis, and Wegener's granulomatosis.

In a preferred embodiment, said ligand of HVEM is LIGHT or a fragment thereof, which induces apoptosis in chronic lymphocytic leukemia B cells.

Specifically, said ligand may consist of a polypeptide, comprising a sequence with at least 90% identity with the sequence whose accession number is Q92956 and which
induces apoptosis in chronic lymphocytic leukemia B cells. In another preferred embodiment, the ligand of the invention is LIGHT that may be used in a soluble form.

Polypeptides of the invention may be produced by any technique known per se in the art, such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination(s).

Knowing the amino acid sequence of the desired sequence, one skilled in the art can readily produce said polypeptides, by standard techniques for production of polypeptides. For instance, they can be synthesized using well-known solid phase method, preferably using a commercially available peptide synthesis apparatus (such as that made by Applied Biosystems, Foster City, California) and following the manufacturer's instructions.

Alternatively, the polypeptides of the invention can be synthesized by recombinant DNA techniques as is now well-known in the art. For example, these fragments can be obtained as DNA expression products after incorporation of DNA sequences encoding the desired (poly)peptide into expression vectors and introduction of such vectors into suitable eukaryotic or prokaryotic hosts that will express the desired polypeptide, from which they can be later isolated using well-known techniques.

Polypeptides of the invention can be used in an isolated (e.g., purified) form or contained in a vector, such as a membrane or lipid vesicle (e.g. a liposome).

In another preferred embodiment, said ligand of HVEM is an anti-HVEM antibody or a fragment thereof which binds to HVEM.

Said ligand may induce death and/or elimination of malignant lymphocytes expressing HVEM by mechanisms such as induction of apoptosis, antibody-dependent cellular cytotoxicity, complement-mediated cytotoxicity, or recruitment and/or activation of immune effector cells through the production of cytokines or chemokines.

In a preferred embodiment, said ligand induces apoptosis in malignant lymphocytes expressing HVEM, in chronic lymphocytic leukemia B cells in particular.
Malignant lymphocytes expressing HVEM may be obtained from patients suffering from acute leukemia, chronic lymphocytic leukemia, plasma cell leukemia, multiple myeloma, B cell lymphoma or T cell lymphoma.

In a preferred embodiment said anti-HVEM antibody or said fragment is an antibody or a fragment thereof which recognizes an epitope selected from the group consisting of groups I, II, III, IV, or V. In another preferred embodiment said anti-HVEM antibody or said fragment is an antibody or a fragment thereof which recognizes an epitope selected from the group consisting of groups II, IV, or V.

The epitopes recognized by the HVEM mAbs are characterized by the following features:

i) The ability of the mAbs to inhibit the binding of LIGHT, HSV-gD and/or BTLA to HVEM.

ii) Mutagenesis experiments whereby the mAbs were tested for their ability to bind to mutants of HVEM.

The HVEM mutants used include:

i) Two deletion mutants
   a. CRD1 domain deletion and
   b. Deletion of amino acids 129-133 within the CRD3 domain (dell29-133)

ii) An alanine substitution mutant with substitution of residues 131-133 (mutl31-133)

The above set of experiments defines 5 distinct groups of mAbs and thus 5 epitopes:

1. Group I mAbs correspond to those which do not bind to the CRD1 and del 129-133 deletion mutants and do not block binding of HVEM to its three ligands.

2. Group II mAbs correspond to those which bind to the CRD1 deletion but not to the dell29-133 deletion, or the mutl31-133 mutant, and are able to block HVEM binding to LIGHT.

3. Group III mAbs correspond to those which do not bind to the CRD1 deletion mutant but are not affected by the dell29-133 deletion, and do not inhibit the binding of the three HVEM ligands.
4. Group IV mAbs correspond to those which are not affected by the CRD1 and dell29-133 deletion mutants, but do not inhibit the binding of the three HVEM ligands.

5. Group V mAbs correspond to those which bind to the CRD I deletion but not to the dell29-133 deletion, are not affected by the mutI31-133 mutant, and are able to block HVEM binding to LIGHT.

In a preferred embodiment said anti-HVEM antibody is a monoclonal antibody obtainable from a hybridoma deposited at the COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM) selected from the group consisting of CNCM 1-3752, CNCM 1-3753 and CNCM 1-3754.

In a further embodiment, the invention relates to a hybridoma cell line suitable for obtaining anti-HVEM monoclonal antibodies, which induce death and/or elimination of malignant lymphocytes by mechanisms such as induction of apoptosis, antibody-dependent cellular cytotoxicity, complement-mediated cytotoxicity, or recruitment and/or activation of immune effector cells through the production of cytokines or chemokines.

In a preferred embodiment, the invention relates to a hybridoma cell line suitable for obtaining anti-HVEM monoclonal antibodies, which induce apoptosis in malignant lymphocytes expressing HVEM, in chronic lymphocytic leukemia B cells in particular.

In a preferred embodiment, the invention relates to a hybridoma cell line suitable for obtaining anti-HVEM monoclonal antibodies, which recognize an epitope selected from the group consisting of groups I, II, III, IV, or V.

In a preferred embodiment said hybridoma cell line is selected from the group consisting of CNCM 1-3752, CNCM 1-3753 and CNCM 1-3754.

Whereas polyclonal antibodies may be used, monoclonal antibodies are preferred.

Antibodies capable of specific binding to HVEM may be derived from a number of species including, but not limited to, rodent (mouse, rat, rabbit, guinea pig, hamster, and the like), porcine, bovine, equine or primate and the like. Antibodies from
primate (monkey, baboon, chimpanzee, etc.) origin have the highest degree of similarity to human sequences and are therefore expected to be less immunogenic. Antibodies derived from various species can be "humanized" by modifying the amino acid sequences of the antibodies while retaining their ability to bind the desired antigen. Antibodies may also be derived from transgenic animals, including mice, which have been genetically modified with the human immunoglobulin locus to express human antibodies. Procedures for raising "polyclonal antibodies" are well known in the art. For example, polyclonal antibodies can be obtained from serum of an animal immunized against HVEM, which may be produced by genetic engineering for example according to standard methods well-known by one skilled in the art. Typically, such antibodies can be raised by administering HVEM protein subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be injected at a total volume of 100 µl per site at six different sites. Each injected material may contain adjuvants with or without pulverized acrylamide gel containing the protein or polypeptide after SDS-polyacrylamide gel electrophoresis. The rabbits are then bled two weeks after the first injection and periodically boosted with the same antigen three times at six weeks' interval. A sample of serum is then collected 10 days after each boost. Polyclonal antibodies are then recovered from the serum by affinity chromatography using the corresponding antigen to capture the antibody. This and other procedures for raising polyclonal antibodies are disclosed by (Harlow et al., 1988), which is hereby incorporated in the references.

Although historically monoclonal antibodies were produced by immortalization of a clonally pure immunoglobulin secreting cell line, a monoclonally pure population of antibody molecules can also be prepared by the methods of the present invention.

Laboratory methods for preparing monoclonal antibodies are well known in the art (see, for example, Harlow et al., 1988). Monoclonal antibodies (mAbs) may be prepared by immunizing a mammal such as mouse, rat, primate and the like, with purified HVEM protein. The antibody-producing cells from the immunized mammal are isolated and fused with myeloma or heteromyeloma cells to produce hybrid cells (hybridoma). The hybridoma cells producing the monoclonal antibodies are utilized as a source of the desired monoclonal antibody. This standard method of hybridoma
culture is described in (Kohler and Milstein, 1975). Alternatively, the immunoglobulin genes may be isolated and used to prepare a library for screening for reactive specifically reactive antibodies. Many such techniques including recombinant phage and other expression libraries are known to one skilled in the art.

While mAbs can be produced by hybridoma culture the invention is not to be so limited. Also contemplated is the use of mAbs produced by cloning and transferring the nucleic acid cloned from a hybridoma of this invention. That is, the nucleic acid expressing the molecules secreted by a hybridoma of this invention can be transferred into another cell line to produce a transformant. The transformant is genotypically distinct from the original hybridoma but is also capable of producing antibody molecules of this invention, including immunologically active fragments of whole antibody molecules, corresponding to those secreted by the hybridoma. See, for example, U.S. Pat. No. 4,642,334 to Reading; PCT Publication No.; European Patent Publications No. 0239400 to Winter et al. and No. 0125023 to Cabilly et al.

In a particular embodiment, mAbs recognizing HVEM may be generated by immunization of Balb-c mice with the respective recombinant human Fc-IgGl fusion proteins. Spleen cells were fused with X-63 myeloma cells and cloned according to already described procedures (Olive D, 1986). Hybridoma supernatants were then screened by staining of transfected cells and for lack of reactivity with untransfected cells.

Antibody generation techniques not involving immunisation are also contemplated such as for example using phage display technology to examine naive libraries (from non-immunised animals); see (Barbas et al., 1992, and Waterhouse et al. (1993). Antibodies of the invention are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, affinity, ion exchange and/or size exclusion chromatography, and the like.

In a particular embodiment, the antibody of the invention may be a human chimeric antibody. Said human chimeric antibody of the present invention can be produced by obtaining nucleic sequences encoding VL and VH domains, constructing a human chimeric antibody expression vector by inserting them into an expression vector for animal cell having genes encoding human antibody CH and human antibody CL, and expressing the expression vector by introducing it into an animal cell. The CH
domain of a human chimeric antibody may be any region which belongs to human immunoglobulin, but those of IgG class are suitable and any one of subclasses belonging to IgG class, such as IgG1, IgG2, IgG3 and IgG4, can also be used. Also, the CL of a human chimeric antibody may be any region which belongs to Ig, and those of kappa class or lambda class can be used. Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques are well known in the art (See Morrison SL. et al. (1984) and patent documents US5,202,238; and US5,204,244).

In another particular embodiment, said antibody may be a humanized antibody. Said humanized antibody may be produced by obtaining nucleic acid sequences encoding for CDRs domain by inserting them into an expression vector for animal cell having genes encoding a heavy chain constant region identical to that of a human antibody; and a light chain constant region identical to that of a human antibody, and expressing the expression vector by introducing it into an animal cell.

The humanized antibody expression vector may be either of a type in which a gene encoding an antibody heavy chain and a gene encoding an antibody light chain exist on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of a humanized antibody expression vector, easiness of introduction into animal cells, and balance between the expression levels of antibody H and L chains in animal cells, a tandem type of the humanized antibody expression vector is more preferable (Shitara K et al. 1994). Examples of the tandem type humanized antibody expression vector include pKANTEX93 (WO 97/10354), pEE18 and the like. Methods for producing humanized antibodies based on conventional recombinant DNA and gene transfection techniques are well known in the art (See, e.g. Riechmann L. et al. 1988; Neuberger MS. et al. 1985). Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan EA (1991); Studnica GM et al. (1994); Roguska MA. et al. (1994)), and chain shuffling (U.S. Pat. No.5,565,332). The general recombinant DNA technology for preparation of such antibodies is also known (see European Patent Application EP 125023 and International Patent Application WO 96/02576).
For example, antibody fragments capable of binding to HVEM or portions thereof, including, but not limited to Fab (e.g., by papain digestion), Fab' (e.g., by pepsin digestion and partial reduction) and F(ab')2 (e.g., by pepsin digestion), facb (e.g., by plasmin digestion), pFc' (e.g., by pepsin or plasmin digestion), Fd (e.g., by pepsin digestion, partial reduction and reaggregation), Fv or scFv (e.g., by molecular biology techniques) fragments, are encompassed by the invention (see, e.g., Colligan, Immunology, supra).

Such fragments may be produced by enzymatic cleavage, synthetic or recombinant techniques, as known in the art and/or as described herein. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. The various portions of antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques.

Said Fab fragment of the present invention can be obtained by treating an antibody which specifically reacts with human HVEM with a protease, papain. Also, the Fab may be produced by inserting DNA encoding Fab of the antibody into a vector for prokaryotic expression system or for eukaryotic expression system, and introducing the vector into a procaryote or eucaryote to express the Fab.

Said F(ab')2 of the present invention may be obtained by treating an antibody which specifically reacts with HVEM with a protease, pepsin. Also, the F(ab')2 can be produced by binding Fab' described below via a thioether bond or a disulfide bond.

Said Fab' may be obtained by treating F(ab')2 which specifically reacts with HVEM with a reducing agent, dithiothreitol. Also, the Fab' can be produced by inserting DNA encoding Fab' fragment of the antibody into an expression vector for prokaryote or an expression vector for eukaryote, and introducing the vector into a prokaryote or eukaryote to effect its expression.

Said scFv fragment may be produced by obtaining cDNA encoding the \( V_H \) and \( V_L \) domains as previously described, constructing DNA encoding scFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the scFv. To generate a humanized scFv fragment, a well known technology called CDR grafting may be used, which involves selecting the complementary determining
regions (CDRs) from a donor scFv fragment, and grafting them onto a human scFv fragment framework of known three dimensional structure (see, e. g., WO98/45322; WO 87/02671; US5,859,205; US5,585,089; US4,816,567; EP0173494).

In a particular embodiment, monoclonal antibodies of the invention are monovalent, bivalent, multivalent, monospecific, bispecific, or multispecific. In another preferred embodiment, the antibody to HVEM is a binding fragment or a conjugate. For examples antibodies of the invention may be conjugated to a growth inhibitory agent, cytotoxic agent, or a prodrug-activating enzyme.

It may be also desirable to modify the antibody of the invention with respect to effector functions, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing inter-chain disulfide bond formation in this region.

The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and/or antibody-dependent cellular cytotoxicity (ADCC) (Caron PC. et al. 1992; and Shopes B. 1992) Another type of amino acid modification of the antibody of the invention may be useful for altering the original glycosylation pattern of the antibody.

By "altering" is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically N-linked. "N-linked" refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites).
Another type of covalent modification involves chemically or enzymatically coupling glycosides to the antibody. These procedures are advantageous in that they do not require production of the antibody in a host cell that has glycosylation capabilities for N-or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. For example, such methods are described in WO87/05330.

Removal of any carbohydrate moieties present on the antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the antibody intact. Chemical deglycosylation is described by Sojahr H. et al. (1987) and by Edge, AS. et al. (1981). Enzymatic cleavage of carbohydrate moieties on antibodies can be achieved by the use of a variety of endo-and exo-glycosidases as described by Thotakura, NR. et al. (1987).

Another type of covalent modification of the antibody comprises linking the antibody to one of a variety of non-proteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in US Patent Nos. 4,640, 835; 4,496, 689; 4,301, 144; 4,670, 417; 4,791, 192 or 4,179,337.

A further object of the invention relates to a method of treating hematologic malignancies and autoimmune diseases comprising administering in a subject in need thereof a therapeutically effective amount of ligand of HVEH as defined above. In the context of the invention, the term "treating" or "treatment", as used herein, means reversing, alleviating, inhibiting the progress of, or preventing the disorder or condition to which such term applies, or one or more symptoms of such a disorder or condition.
According to the invention, the term "patient" or "patient in need thereof" is intended for a human or non-human mammal affected or likely to be affected by a hematologic malignancie or by an autoimmune disease.

By a "therapeutically effective amount" of the ligand of HVEM according to the invention is meant a sufficient amount of the ligand of HVEM to treat said hematologic malignancie or autoimmune disease, at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood, however, that the total daily usage of the ligand of HVEM and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder, activity of the specific ligand of HVEM employed; the specific composition employed, the age, body weight, general health, sex and diet of the patient, the time of administration, route of administration, and rate of excretion of the specific antibody employed, the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed, and like factors well known in the medical arts. For example, it is well known within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

Ligands of HVEM according to the invention may be used in combination with any other therapeutic strategy for treating the disorders or conditions as above described (e.g. external radiotherapy, chemotherapy or cytokine therapy).

**Pharmaceutical compositions:**

A further object of the invention relates to a pharmaceutical composition comprising an effective dose of a ligand of HVEM.

Any therapeutic agent of the invention as above described may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.
"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, intranasal, intraocular, intravenous, intramuscular or subcutaneous administration and the like. Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment.

To prepare pharmaceutical compositions, an effective amount of a ligand of HVEM may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.
Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

A ligand of HVEM of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the
preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The preparation of more, or highly concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution may be suitably buffered and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; time release capsules; and any other form currently used.

Compositions of the present invention may comprise a further therapeutic active agent. The present invention also relates to a kit comprising a ligand of HVEM as defined above and a further therapeutic active agent.
In one embodiment said therapeutic active agent is an anticancer agent. For example, said anticancer agents include but are not limited to fludarabine, gemcitabine, capecitabine, methotrexate, taxol, taxotere, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, platinum complexes such as cisplatin, carboplatin and oxaliplatin, mitomycin, dacarbazine, procarbazine, etoposide, teniposide, camptotecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, mitomycin, mitoxantrone, L-asparaginase, doxorubicin, epimibcim, 5-fluorouracil, taxanes such as docetaxel and paclitaxel, leucovorin, levamisole, irinotecan, estramustine, etoposide, nitrogen mustards, BCNU, nitrosoureas such as carmustine and lomustine, vinca alkaloids such as vinblastine, vincristine and vinorelbine, imatimb mesylate, hexamethylmelamine, topotecan, kinase inhibitors, phosphatase inhibitors, ATPase inhibitors, tyrphostins, protease inhibitors, inhibitors herbimycm A, genistein, erbstatin, and lavendustin A.

In one embodiment, additional anticancer agents may be selected from, but are not limited to, one or a combination of the following class of agents: alkylating agents, plant alkaloids, DNA topoisomerase inhibitors, anti-folates, pyrimidine analogs, purine analogs, DNA antimetabolites, taxanes, podophyllotoxin, hormonal therapies, retinoids, photosensitizers or photodynamic therapies, angiogenesis inhibitors, antimitotic agents, isoprenylation inhibitors, cell cycle inhibitors, actinomycins, bleomycins, anthracyclines, MDR inhibitors and Ca2+ ATPase inhibitors.

Additional anticancer agents may be selected from, but are not limited to, cytokines, chemokines, growth factors, growth inhibitory factors, hormones, soluble receptors, decoy receptors, monoclonal or polyclonal antibodies, mono-specific, bi-specific or muti-specific antibodies, monobodies, polybodies.

Additional anticancer agent may be selected from, but are not limited to, growth or hematopoietic factors such as erythropoietin and thrombopoietin, and growth factor mimetics thereof.

In the present methods for treating cancer the further therapeutic active agent can be an antiemetic agent. Suitable antiemetic agents include, but are not limited to, metoclopromide, domperidone, prochlorperazine, promethazine, chlorpromazine, trimethobenzamide, ondansetron, granisetron, hydroxyzine, acetylleucine monoemanolamine, alizapride, azasetron, benzquinamide, bietanautine, bromopride,
buclizine, clebopride, cyclizine, dufenhydrinate, diphenidol, dolasetron, meclizine, methallatal, metopimazine, nabilone, oxypemdy, pipamazine, scopolamine, sulpiride, tetrahydrocannabinols, thieflhyperazine, thiopropazine and tropisetron.

In a preferred embodiment, the antiemetic agent is granisetron or ondansetron.

In another embodiment, the further therapeutic active agent can be an hematopoietic colony stimulating factor. Suitable hematopoietic colony stimulating factors include, but are not limited to, filgrastim, sargramostim, molgramostim and epoietin alpha.

In still another embodiment, the other therapeutic active agent can be a hematopoietic colony stimulating factor. Suitable hematopoietic colony stimulating factors include, but are not limited to, filgrastim, sargramostim, molgramostim and epoietin alpha.

In yet another embodiment, the further therapeutic active agent can be an opioid or non-opioid analgesic agent. Suitable opioid analgesic agents include, but are not limited to, morphine, heroin, hydromorphone, hydrocodone, oxymorphone, oxycodone, metopon, apomorphine, nomioiphine, etoipbine, buprenorphine, mepeddine, lopermide, aniledidine, ethoheptazine, piminidine, betaprodine, diphenoxylate, fentani, sufentani, alfentani, remifentani, levorphanol, dextromethorphan, phenazodne, pemazocine, cyclazocine, methodone, isomethadone and propoxyphene. Suitable non-opioid analgesic agents include, but are not limited to, aspirin, celecoxib, rofecoxib, diclofinac, diflusinal, etodolac, fenoprofen, flurbiprofen, ibuprofen, ketoprofen, indomethacin, ketorolac, meclofenamate, mefanamic acid, nabumetone, naproxen, piroxicam and sulindac.

In yet another embodiment, the further therapeutic active agent can be an anxiolytic agent. Suitable anxiolytic agents include, but are not limited to, buspirone, and benzodiazepines such as diazepam, lorazepam, oxazapam, chlorazepate, clonazepam, chlordiazepoxide and alprazolam.

**Screening methods**

Fragments of LIGHT which induce apoptosis in chronic lymphocytic leukemia B cells, anti-HVEM antibodies or fragments thereof which bind to, and induce apoptosis in malignant lymphocytes expressing HVEM, in chronic lymphocytic leukemia B cells in particular may be selected by any screening methods well known in the art.

For example, a method for the in vitro screening of ligands of HVEM which induce apoptosis in malignant lymphocytes expressing HVEM, in chronic lymphocytic leukemia B cells in particular, may comprise the following steps:
(a) adding fragments of LIGHT, anti-HVEM antibodies, or fragments thereof to malignant lymphocytes expressing HVEM e.g. chronic lymphocytic leukemia B cells; 
(b) selecting the fragments or the antibodies which induce the apoptosis of the cells.

The invention will be further illustrated through the following examples, figures and tables.

**FIGURES**

*Figure 1*: LIGHT Induces Apoptosis of CLL Cells.
B-CLL cells were incubated with LIGHT transfected L cells or control CD32 transfected cells. After 24 hours of incubation, cells were analyzed by flow cytometry with AnnexinV/PI double staining. The figure shows one experiment representative of 15 experiments. Apoptotic cells include Annexin \( \text{V}^4 \text{TPF} \) (early apoptosis) and Annexin \( \text{V}^4 \text{TPI}^+ \) (late apoptotic /necrotic) cells. The error bars indicate the standard error of the mean (SEM).

*Figure 2*: HVEM and LT\( \beta \)R Expression on CLL-B Cells.
B-CLL cells were stained with CD19, HVEM and LT\( \beta \)R mAbs. The results are expressed as mean of the percentage of positive cells \( \pm \) SEM performed on 9 samples

*Figure 3*: LIGHT AND HVEM mAb Induce Death of CLL Cells.
B-CLL were incubated with LIGHT transfected L cells or CD32 transfected L cell as control or anti-HVEM mAb and analyzed by Annexin V/PI staining. The bars depict the mean percentage of apoptotic cells corresponding to 25 independent experiments.

*Figure 4*: Comparison of HVEM mAb & Rituximab on Apoptosis of CLL Cells.
B-CLL cells were either left untreated or stimulated with anti-HVEM or therapeutic anti CD20 mAb (rituximab). After 24 hours, cells were collected, and apoptotic cells were analyzed by Annexin V/PI staining. Values represent the percentage of
maximum apoptosis: (sample value - control value)/(maximum value - control value). The errors bars correspond to the SEM.

**Figure 5**: HVEM mAb Induced Activation of Caspase 3 in CLL Cells.

B-CLL cells from two different patients were either left untreated or stimulated with anti-HVEM Mab. After 24 hours of incubation cells were collected, and protein lysates prepared. Immunoblot membranes prepared from such samples were probed for cleaved caspase 3 or β-actin mAbs as indicated. Jurkat cells unstimulated or treated with anti-Fas mAb CHl 1 were used as positive control.

**Figure 6**: Activation of Caspase 3 in CLL Cells Induced by HVEM mAb.

B-CLL cells were pretreated or not with the pan-caspase inhibitor Z-VAD-FMK for 30 min at 37°C and then after either left unstimulated or stimulated with anti-HVEM mAb. After 24 hours, cells were collected, permeabilized and stained for active caspase-3. Cells were analyzed by flow cytometry. Data represent the mean percentage of cells positive for active caspase-3 observed for 10 independent experiments.

**Figure 7**: Activation of Caspase 8 and 9 in CLL Cells Induced by HVEM mAb.

B-CLL cells were either left unstimulated or stimulated with anti-HVEM mAb for 24 hours cells were then incubated with specific caspase-8 or-9 fluorescent inhibitor at the indicated time points for 1 hour at 37°C, and analyzed by flow cytometry. Data represent the mean percentage of cells positive for active caspase-8 or - 9 (after subtraction of the background corresponding to the untreated condition) ± SEM in 6 different patients.

**Figure 8**: Mitochondrial Membrane Depolarization Caused by HVEM mAb

B-CLL cells were pretreated or not with the pan-caspase inhibitor Z-VAD-FMK for 30 min at 37°C and then after either left unstimulated or stimulated with anti-HVEM mAb. After 24 hours, cells were collected, incubated for 50 nM of DiOC2(3) for 30 min at 37°C, and analyzed for the loss of mitochondrial membrane potential by flow
cytometry. Data represent the mean percentage of cells positive for DiOC2(3) green fluorescence ± SEM observed for 6 patients.

**Figure 9:** Effect of HVEM mAb on Bax and Bcl-2 Expression.

B-CLL cells were either left untreated or stimulated with anti-HVEM mAb. After 24 hours of incubation cells were collected, permeabilized and stained with anti-Bax or anti-Bcl-2 mAb, and analyzed by flow cytometry. Cells were analyzed by flow cytometry. Data represent the mean percentage of cells positive after subtraction of the background corresponding to the isotype matched control) ± SEM in 6 different patients.

**Figure 10:** HVEM mAb Induced Activation of FADD in CLL Cells.

B-CLL cells from two different patients were either left untreated or stimulated with anti-HVEM Mab. After 24 hours of incubation cells were collected, and protein lysates prepared. Immunoblot membranes prepared from such samples were probed for FADD or β-actin mAbs as indicated. TFl erythroleukemic cell line was used as positive control.

**Figure 11:** HVEM mAb Induced IL-8 Production by CLL Cells.

B-CLL cells from 6 patients were either left unstimulated or stimulated with anti-HVEM mAb for 24 hours cells. Supernatants were then collected and production of IL-8 was assessed by the CBA immunoassay. Results are expressed as fold induction obtained by dividing the IL-8 production values for anti-HVEM mAb treated cells by values determined for unstimulated cells.
TABLES

Table 1: Chemokine, Cytokine & Receptor Genes Upregulated in CLL by Stimulation with HVEM mAb.

B-CLL cells were either left unstimulated or stimulated with anti-HVEM mAb before RNA extraction. RNA content was evaluated by qRT-PCR as described in material and methods section. RNA expression was considered as positive when superior to 1.5. The experiments were performed on 4 different B-CLL samples.

Chemokine, Cytokines & Receptors Genes Upregulated in CLL by Stimulation with HVEM mAb

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>Chemokine Receptors</th>
<th>Cytokines</th>
<th>Cytokine Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>CCR4</td>
<td>IL-1α</td>
<td>TGF-βR1</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>CXCR1</td>
<td>IL-1β</td>
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<tr>
<td>IP10</td>
<td>XCR1</td>
<td>IL-10</td>
<td></td>
</tr>
<tr>
<td>Eotaxin 2</td>
<td></td>
<td>IL-24</td>
<td></td>
</tr>
<tr>
<td>GRO1</td>
<td></td>
<td>IL-25</td>
<td></td>
</tr>
<tr>
<td>MCP1</td>
<td></td>
<td>CSF2</td>
<td></td>
</tr>
<tr>
<td>MCP2</td>
<td></td>
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</table>

A total number of 180 cytokine, chemokine, receptor and adhesion genes were analyzed, β-actin was used to normalize cDNA concentrations.

Table 2: Characterization Summary of HVEM mAb Clones.

Fourteen mAb clones were characterized for a number of methods. This list represents a summary of a subset of data generated on 3 mAb clones. The data in this table includes the Ig isotype of the mouse heavy chain, the type of the light chain, the EC50 value which represents the 50% saturation binding to HVEM by flow cytometry, the epitope cluster, and the ability of the mAb to induce apoptosis of CLL cells. The epitopes for this panel of mAbs represent 3 of 5 which were determined by binding studies with HVEM mutants, and competition studies with LIGHT, BTLA and HSV-gD.
<table>
<thead>
<tr>
<th>mAb Clone</th>
<th>Isotype</th>
<th>EC50 (µM)</th>
<th>H V E M Binding</th>
<th>Epitope Cluster</th>
<th>CLL Apoptosis</th>
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</thead>
<tbody>
<tr>
<td>HVEM4-4</td>
<td>IgG2b κ</td>
<td>0.12</td>
<td>+</td>
<td>II</td>
<td>+</td>
</tr>
<tr>
<td>HVEM11-8</td>
<td>IgG1 κ</td>
<td>0.10</td>
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<td>+</td>
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<tr>
<td>HVEM20-4</td>
<td>IgG1 κ</td>
<td>0.24</td>
<td>+</td>
<td>IV</td>
<td>+</td>
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</tbody>
</table>
Table 3: **WHO classification of B-cell lymphoid neoplasms** (Jaffe, E.S. et al., 2004).

<table>
<thead>
<tr>
<th>5</th>
<th>Precursor B-cell neoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Precursor B-lymphoblastic leukemia/lymphoma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>10</th>
<th>Mature B-cell neoplasms</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Chronic lymphocytic leukemia/small lymphocytic lymphoma</td>
</tr>
<tr>
<td>10</td>
<td><em>Variant:</em> with plasmacytoid differentiation or monoclonal gammopathy</td>
</tr>
<tr>
<td>10</td>
<td>B-cell prolymphocytic leukemia</td>
</tr>
<tr>
<td>10</td>
<td>Lymphoplasmacytic lymphoma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>15</th>
<th>Splenic marginal zone B-cell lymphoma (± villous lymphocytes)</th>
</tr>
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<tbody>
<tr>
<td>15</td>
<td>Hairy cell leukemia</td>
</tr>
<tr>
<td>15</td>
<td><em>Variant:</em> hairy cell variant</td>
</tr>
<tr>
<td>15</td>
<td>Plasma cell myeloma/plasmacytoma</td>
</tr>
<tr>
<td>15</td>
<td>Extranodal marginal zone B-cell lymphoma of MALT type</td>
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Table 4: WHO classification of T-cell and NK-cell lymphoid neoplasms (Jaffe, E.S. et al, 2004).

**Precursor T-cell neoplasm**
- Precursor T-lymphoblastic lymphoma/leukemia

5

**Mature (peripheral) T-cell and NK-cell neoplasms**
- T-cell prolymphocytic leukemia
- **Morphologic variants:** small cell, cerebriform cell
- T-cell granular lymphocytic leukemia
- Aggressive NK-cell leukemia

10

- Blastic 'NK-cell' lymphoma
- Adult T-cell leukemia/lymphoma (HTLV-1+)

**Clinical variants**
- Acute
- Lymphomatous
- Chronic
- Smoldering
- Hodgkin-like

15

- Extranodal NK/T-cell lymphoma, nasal type
- Enteropathy-type T-cell lymphoma

20

- Hepatosplenic T-cell lymphoma
- Subcutaneous panniculitis-like T-cell lymphoma
- Mycosis fungoides/Sezary syndrome

**Variants**
- Pagetoid reticulosis
- MF-associated follicular mucinosis
- Granulomatous slack skin disease

25

- Primary cutaneous CD30+T-cell lymphoproliferative disorder

**Variants**
- Lymphomatoid papulosis (type A and B)

30

- Primary cutaneous anaplastic large-cell lymphoma
- Borderline lesions

**Peripheral T-cell lymphoma, not otherwise characterized**
- **Morphologic variants:** lymphoepithelioid (Lennert's), T-zone

35

- Angioimmunoblastic T-cell lymphoma
- Anaplastic large cell lymphoma, (ALK+/ ALK-)

**Morphologic variants:** lymphohistiocytic, small cell
EXAMPLES

Materials and Methods

Cells and culture conditions:
This study was approved by the review board of the Institut Paoli-Calmettes, Marseille, France. Upon informed consent, and following institutional procedures and the Helsinki Declaration, peripheral-blood mononuclear cells (PBMCs) were isolated from heparinized blood obtained from untreated patients diagnosed with CLL on the basis of clinical and immunophenotypic criteria. PBMCs were isolated by density gradient centrifugation (Lymphoprep) and subjected to a preliminary phenotypic characterization. When residual non-B cells exceeded 10%, B cells were enriched by negative selection with antibody-coated magnetic beads (CD2-beads; Dynal, Oslo, Norway) to obtain a purified population of CD19+/CD5+ B cells.

mAbs:
BALB/c mice were immunised by IP injection of human HVEM-Ig fusion protein, after the last injection the spleen cells were fused with X63Ag8 myeloma cells according to standard procedures. The hybridoma supernatants were screened by cell surface staining of human HVEM cells lines.

Co-culture of CLL B cells with CD40L-transfected cells or mAbs
Murine L cells stably transfected by the human LIGHT cDNA were cultured in RPMI 1640 supplemented with penicillin/streptomycin, L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS) in flasks until confluency. CLL B cells were co-cultured with CD40L-transfected L cells or monoclonal antibodies (mAbs) in 24-well plates for 24 to 48 hours. Then, cells were collected, checked for viability, and re-suspended in fresh medium before use.

Immunofluorescence analysis of cell-surface antigens
Flow cytometry analysis of total CLL PBMCs and of B-cell-enriched fractions were performed by 3- to 4-color immunofluorescence, by staining 105 cells/sample at 4°C for 30 minutes, with the following mAbs: FITC- or PE-labeled anti-CD19, anti-CD4,
anti-CD8, anti-CD3, anti-CD19, anti-CD38, anti-HVEM, anti-LTβR (Beckman Coulter, BD Biosciences, R&D). FITC- or PE-labeled isotype-matched Ig's were used as negative controls. After 2 washings in phosphate-buffered saline (PBS) plus 2% FBS, cells were analyzed by flow cytometry (FACSCANTO; BD Biosciences). The percentage of activated Caspase-3+ cells was assessed by immunofluorescence on CLL cells first stained by anti-CD3-PE (Beckman Coulter), fixed and permeabilized by "Fix and Perm" reagents (BD Biosciences), and then stained with activated Caspase-3-FITC conjugated mAb.

**Western blotting** analysis

B cells (10^7 cells/sample) from CLL patients were incubated with or without LIGHT-transfected L cells or HVEM mAbs for 24 to 48 hours. Then, cells were collected, washed in PBS, and lysed in 50 µL lysis buffer for 30 minutes on ice. Equal amounts (50 µg) of protein extracts were analyzed under reducing conditions on 10% or 12% polyacrylamide gels and transferred onto Hybond-C membranes (Amersham Biosciences, Little Chalfont, United Kingdom). After overnight saturation, blots were stained. The blots were then washed with Tris-buffered saline (TBS) supplemented with 0.05% Tween 20 and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit Ig antisera (Dako, Glostrup, Denmark) for 2 hours. Bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

**Statistical analysis**

All statistical calculations were performed using the statistical package SPSS for Windows, release 11.5, 2002 software (SPSS UK, Working, Surrey, United Kingdom). When ZAP-70, CD38, mutational status, and IL-21R were considered as binary variables, statistical comparisons were performed using 2-way tables for the Fisher exact test and multiway tables for the Pearson chi-square test. The nonparametric Mann-Whitney U test was used to test the difference between 2 groups (CD38+ [> 30%] and CD38- [< 30%], and VH-mutated [> 2%] and VH-unmutated [< 2%] cases). Paired 2-tailed Student t test was used for analysis of IL-21R expression on resting or activated CLL B cells. A p value of less than 0.05 was
considered significant. Paired 2-tailed Student t test was used for analysis of IL-21R expression on resting or activated CLL B cells. A p value of 0.05 or less was considered significant.

**Determination of apoptosis**

CLL B cells were stimulated using LIGHT-expressing L cells or HVEM mAbs. Following stimulation, apoptosis was determined at different time points (1-2 days) by annexin V-FITC and propidium iodide (PI) double-staining (BD Biosciences) and flow cytometry analysis.

In experiments with chemotherapeutic agents such as fludarabine, cells were incubated for 24 hours with the drug, with or without stimulation via HVEM (using HVEM mAb or LIGHT). Cell death was evaluated by flow cytometry as described above.

**Determination of mitochondrial depolarization**

Changes in the inner mitochondrial membrane potential were evaluated by staining CLL B cells at 37°C for 15 minutes with 40 nM of the green fluorescent dye DiOC2(3) (Invitrogen). After 2 washes, cells were analyzed by flow cytometry. Viable cells display bright DiOC2(3) fluorescence, while apoptotic cells display dull DiOC2(3) fluorescence. The positive control was CCCP (Invitrogen).

**Determination of caspase, Bcl-2 and Bax activation** by flow cytometry

Caspase activation was evaluated using the following cell-permeable fluoresceinated caspase inhibitors (Caspglow, Invitrogen): FAM-LETD-FMK (caspase-8 inhibitor) and FAM-LEHD-FMK (caspase-9 inhibitor). Cells were stained for 1 hour at 37°C in the dark. After staining, the cells were washed with washing buffer, resuspended in 0.5 mL buffer, and immediately analyzed by flow cytometry. Activated intracellular caspase-3, Bcl-2 and Bax were detected by staining permeabilized CLL B cells with the Alexa-Fluor-labeled anti-human active caspase-3 rabbit antiserum (Ozyme, Cedex, France), or with FITC-conjugated anti-Bcl-2 or anti-Bax mAbs (BD Biosciences) and subsequent analysis by flow cytometry.
QRT-PCR
Before RNA isolation, all cells were selected for their ability to be killed by LIGHT stimulation. The method used has been previously described [1]. Briefly, total RNA was isolated from CCL cells using the standard TRIzol reagent protocol (Invitrogen Life Technologies) 1 to 2 days poststimulation. The isolated RNA was reverse transcribed using random hexamers.

QRT-PCR analysis was performed with the ABI Prism 7900HT thermocycler (Applied Biosystems) using SYBR Green detection. Each reaction was performed in a 10 µL reaction containing 3 mM MgC12, 50 nM dNTP, 20 nM primers, 40 ng of cDNA, Ix Rox reference dye (Invitrogen Life Technologies), Ix SYBR green reagent, and 0.125 U/µL Jumpstart Taq polymerase (Sigma-Aldrich). The following protocol was used: denaturation program (95°C for 3 min), followed by the amplification and quantification program (95°C for 10 s, 60°C for 15 s, 72°C for 20 s) repeated for 40 cycles, with one cycle of a finishing program (72°C for 1 min). Amplification was followed by melting curve analysis (95°C for 15 s, 60°C for 15 s, and 95°C for 15 s) to ensure the presence of a single PCR product. The expression of b-actin was used to normalize starting cDNA concentrations. The primers used for all QRT-PCR were selected using Primer Express software (Applied Biosystems).

Cytokine and Chmeokine production
CLL-B cells were incubated with LIGHT transfected L cells or HVEM mAbs in RPMI 1640 (Bioproducts, MA, U.S.A) with 10% FBS. Supernatants were harvested after a 1 day incubation. The IL-8 was quantified using an immuno-enzymatic assay with a sensitivity of 5 pg/ml (Beckman Coulter).

Results

LIGHT triggering induces death of CLL cells
LIGHT has been shown to inhibit growth of serum-starved, IFN-g-primed cell lines of solid tumor origin. In the current study, we investigated the effect of LIGHT on freshly isolated primary CLL cells. Using flow cytometry with Annexin V and
propidium iodide staining, we show that LIGHT induces apoptotic death of CLL cells (Figure 1). These results were reproduced in 15 CLL samples.

HVEM expression on B cells and T cells and on CLL-B cells in particular
LIGHT is expressed on stromal and epithelial cells, but is absent on lymphoid cells. However, its two known receptors, HVEM and LTβR, are expressed on cells of the hematopoietic lineage. To better understand the role of these two receptors in LIGHT-induced CLL cell-death, we evaluated their expression on CLL-B cells from 9 patients. Data summarized in Figure 2 shows that HVEM is expressed, at uniformly high levels, on CLL-B cells. For comparison, Figure 2 also shows the high level of expression of the B cell marker, CD19. In sharp contrast, LTβR expression was either infrequent or low (Figure 2). LTβR was also not expressed on normal B cells (data not shown).

We also investigated, and have determined that HVEM is expressed in a number of hematological malignancies. Among the lymphoid malignancies we studied, we observed expression in several B cell malignancies including on acute leukemias, B cell lymphomas, chronic lymphocytic leukemia, plasma cell leukemias, and multiple myeloma. HVEM expression has also been observed in T cell lymphomas.

LIGHT-mediated CLL cell death depends on HVEM
To further explore the receptors involved in LIGHT-induced CLL cell death, CLL cells were treated by with LIGHT or mAb to HVEM. Figure 3 shows both LIGHT and the HVEM mAb were able to induce CLL cell death. LIGHT or HVEM mAb-induced CLL cell death does not require priming of the cells with IFN-g. A role for HVEM was further inferred by the inability of mAbs to LTalb2 to induce CLL cell death. As further corroborating evidence for the involvement of HVEM, CLL cells which did not express LTβR were nevertheless killed by HVEM mAb. In addition, another B cell lymphadenopathy, Mantle cell lymphoma, which is also negative for the expression of LTβR, was killed by treatment with LIGHT (data not shown).

HVEM mAb-induced CLL cell death compares favorably to that of rituximab
HVEM mAb and a CD20 mAb were compared for their effectiveness in inducing CLL cell death. The HVEM mAb-induced CLL cell death compared favorably with that of the pan-B cell therapeutic mAb rituximab (Figure 4).

**HVEM-mediated killing of CLL cells can be enhanced by chemotherapeutic drugs**

Many anti-cancer drugs are used in combination with each other in order to enhance their effectiveness. However, combining two or more drugs requires some level of understanding of the mechanism of action of each agent or experimental determination of how effectively such agents can be combined. With that in mind, experiments were conducted to determine whether, HVEM mAb-induced killing of CLL cells can be enhanced by combining it with anti-cancer drugs such as fludarabine. These experiments show that fludarabine enhances HVEM-mediated killing of CLL cells.

**HVEM-mediated CLL cell death involves apoptotic mechanisms**

Apoptosis induced by the death-domain containing TNFR family members such as Fas depend on the activation of effector caspases such as caspases-8 and caspases-9. The activation of the effector caspases has in turn been described to depend on the presence of the intracytoplasmic death-domain, a prominent feature of Fas, TNFRI and the TRAIL receptors DR4 and DR5. The death-domain is however, absent from HVEM.

Downstream of the effector caspases, cleavage of caspase-3 depends on the activation of caspase-8 and caspase-9. Western blot analysis (Figure 5) shows that stimulation of CLL cells by HVEM mAb results in the cleavage of caspase-3. This observation was confirmed by another assay using flow cytometry and a fluorescent substrates (Figure 6). Figure 6 also shows that HVEM mAb induced caspase-3 activation is completely abrogated by the pan-caspase inhibitor z-VAD.

**Pathways involved in the induction of caspase-3 activation**

We analyzed by flow cytometry (Figure 7) and Western blotting (not shown) the activation of caspases 8 and 9. Both caspase-8 and caspase-9 were activated in
response to treatment of CLL cells by HVEM mAb. Interestingly, caspase-8 and caspase-9 were activated with similar kinetics.

HVEM-triggering of CLL cells causes mitochondrial membrane depolarization

Mitochondrial membrane depolarization is part of the "intrinsic" pathway of apoptotic mechanisms. Figure 8 shows that treatment of CLL cells with HVEM mAb results in mitochondrial membrane depolarization. Interestingly, the HVEM-mediated mitochondrial depolarization was not inhibited by the pan-caspase inhibitor z-VAD (Figure 8), suggesting that this step is independent of caspase activation.

HVEM-induced cell death of CLL is associated with increase in Bax

Bax is one of the key bcl-2 members involved in cell apoptosis. Its action is mainly through interaction with mitochondrial membrane and activation of the permeability transition pore. Its action is counter-acted by Bcl-2. This latter molecule is increased in CLL. HVEM stimulation did not decrease Bcl-2 but in contrast induced a major decrease in Bax cytosolic levels (Figure 9). This increase may be associated with the mitochondrial membrane depolarization described above.

Putative other molecules involved HVEM-mediated CLL cell death

Since HVEM is devoid of a death domain, the pathways involved in the HVEM-induced cell death are unclear. Based on the known mechanisms of apoptosis, we analyzed the expression of FADD, the major adaptor molecule involved in Fas and TNFR mediated cell death. Interestingly, treatment of CLL cells with HVEM mAb caused a major increase in FADD expression (Figure 10).

HVEM-stimulation induces production of chemokines and cytokines on CLL-B cells

We investigated whether HVEM stimulation of CLL-B cells was associated with cytokine and chemokine production, as soluble factors are critical for the recruitment of immune effectors. Previously, LTβR has been associated with chemokine production in a rhabdomyosarcoma cell line.
HVEM stimulation induces chemokine and cytokine production from CLL cells. Figure 1 shows upregulation of IL-8 both at the transcriptional and post-transcriptional levels.

Although the more detailed analysis was conducted primarily on the master chemokine IL-8, 180 chemokine, cytokine, receptor and adhesion genes were evaluated in a gene array format. An overall summary is presented in Table 1 which shows upregulation of a number of chemokine and cytokine genes in CLL-B cells in response to stimulation with HVEM mAb.

**LIGHT-induced gene regulation in lymphoma cells**

In addition to CLL-B cells, we also investigated the effect of LIGHT on genes in freshly isolated Mantle Cell Lymphoma cells. LIGHT-stimulation of lymphomas induced the increased transcription of several genes including chemokines (IL-8, GRO-CC, GRO-CC, MDC and ENA-78), TNF- (LIGHT) and TNFR-family (CD30 and DR6) members, and adhesion molecules (α5β1, β4 and β8 integrins). In contrasts to the upregulated genes, several other genes were also down regulated including the protease MMP9, the adhesion molecule PECAM, the tyrosine kinase receptor EphB6, angiogenic factors like VEGF and the cytokine receptor M-CSF.

**Characterization of anti-HVEM mAb Clones**

Fourteen mAb clones were characterized for a number of methods including: i) typing of the mouse immunoglobulin heavy and light chain types, ii) flow cytometry analysis for binding cell surface HVEM and determination of mean fluorescent intensity (MFI) values, maximum saturation binding, and 50% saturation binding (EC50 value), iii) competitive blocking studies with LIGHT, BTLA and HSV-gD, iv) mutagenesis analysis through binding of mAbs to a panel of HVEM mutants, and v) the ability of the mAb to induce apoptosis of CLL cells. Based on criteria from the competitive blocking and mutagenesis experiments, five epitope clusters were identified as epitopes I, II, III, IV and V. The data represented in Table 2 is a summary subset for 3 mAbs.
References:

Barbas CF, Bain JD, Hoekstra DM, Lerner RA. (1992), Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. PNAS USA, 89, 4457-4461.


Claims

1. A ligand of HVEM for therapeutic use, wherein said ligand of HVEM is selected from the group consisting of LIGHT or a fragment of LIGHT which induces apoptosis in chronic lymphocytic leukemia B cells, an anti-HVEM antibody, and a fragment thereof which binds to HVEM.

2. A ligand of HVEM according to claim 1 for the treatment of hematologic malignancies.

3. A ligand of HVEM according to claim 1 for the treatment of autoimmune diseases.

4. Use of a ligand of HVEM for the manufacture of a medicament for the treatment of hematologic malignancies or autoimmune diseases, wherein said ligand of HVEM is selected from the group consisting of LIGHT, a fragment thereof which induces apoptosis in chronic lymphocytic leukemia B cells, an anti-HVEM antibody and a fragment thereof which binds to HVEM.

5. A ligand of HVEM according to claim 2 or use according to claim 4, wherein the hematologic malignancy is selected from the group consisting of B-cell lymphoid neoplasm, T-cell lymphoid neoplasm, non-Hodgkin lymphoma (NHL), B-NHL, T-NHL, chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), mantle cell lymphoma (MCL), NK-cell lymphoid neoplasm and myeloid cell lineage neoplasm.

6. A ligand of HVEM according to any one of claims 1, 2, 3 and 5 or use according to claim 4 or 5, wherein said ligand of HVEM is LIGHT or a fragment thereof which induces apoptosis in chronic lymphocytic leukemia B cells.

7. A ligand of HVEM or use according to claim 6, wherein said ligand of HVEM is a soluble form of LIGHT.
8. A ligand of HVEM according to any one of claims 1, 2, 3 and 5 or use according to claim 4 or 5, wherein said ligand of HVEM is an anti-HVEM antibody or a fragment thereof which binds to HVEM.

9. A ligand of HVEM or use according to claim 8, wherein said anti-HVEM antibody or said fragment thereof induces death and/or elimination of a malignant lymphocyte expressing HVEM by induction of apoptosis, antibody-dependent cellular cytotoxicity, complement-mediated cytotoxicity and/or activation of immune effector cells through the production of cytokines and/or chemokines.

10. A ligand of HVEM or use according to claim 8, wherein said anti-HVEM antibody or said fragment thereof induces apoptosis in a malignant lymphocyte.

11. A ligand of HVEM or use according to claim 10, wherein said malignant lymphocyte is a chronic lymphocytic leukemia B cell.

12. A ligand of HVEM or use according to any one of claims 8 to 11, wherein said ligand of HVEM is an antibody which recognizes an epitope selected from the group consisting of groups I, II, III, IV, or V.

13. A ligand of HVEM or use according to any one of claims 8 to 12, wherein said ligand of HVEM is a monoclonal antibody.

14. A ligand of HVEM or use according to claim 13, wherein said monoclonal antibody is obtainable from a hybridoma deposited at the COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM) selected from the group consisting of CNCM 1-3752, CNCM 1-3753 and CNCM 1-3754.

15. A hybridoma cell line suitable for obtaining anti-HVEM monoclonal antibodies which induce death and/or elimination of a malignant lymphocyte expressing HVEM by induction of apoptosis, antibody-dependent cellular cytotoxicity, complement-
mediated cytotoxicity and/or activation of immune effector cells through the production of cytokines and/or chemokines.

16. A hybridoma cell line suitable for obtaining anti-HVEM monoclonal antibodies which induce apoptosis of a malignant lymphocyte expressing HVEM.

17. A hybridoma cell line suitable for obtaining anti-HVEM monoclonal antibodies which induce apoptosis in chronic lymphocytic leukemia B cells.

18. A hybridoma cell line according to any one of claims 15 to 17, wherein said anti-HVEM monoclonal antibodies recognize an epitope selected from the group consisting of groups I, II, III, IV, or V.

19. A hybridoma cell line selected from the group consisting of CNCM 1-3752, CNCM 1-3753 and CNCM 1-3754.

20. An anti-HVEM antibody or a fragment thereof which binds to HVEM, wherein said antibody or said fragment thereof induces death and/or elimination of a malignant lymphocyte expressing HVEM by induction of apoptosis, antibody-dependent cellular cytotoxicity, complement-mediated cytotoxicity and/or activation of immune effector cells through the production of cytokines and/or chemokines.

21. An anti-HVEM antibody or a fragment thereof which binds to HVEM, wherein said antibody or said fragment thereof induces apoptosis in malignant lymphocytes.

22. An anti-HVEM antibody or a fragment thereof which binds to HVEM, wherein said antibody or said fragment thereof induces apoptosis in chronic lymphocytic leukemia B cells.

23. An anti-HVEM antibody or a fragment thereof according to any one of claims 20 to 23, wherein said antibody recognizes an epitope selected from the group consisting of groups I, II, III, IV, or V.
24. An anti-HVEM antibody according to any one of claims 20 to 23, wherein said antibody is a monoclonal antibody.

25. A monoclonal antibody obtainable from a hybridoma deposited at the COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM) selected from the group consisting of CNCM 1-3752, CNCM 1-3753 and CNCM 1-3754.
Figure 10

- 27 kDa
- 43 kDa

FADD

Actin

CLL2 Control
CLL1 Control
Positive Control
CLL2 + HVEL Map
CLL1 + HVEL Map
**INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL**  
(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 49, line 24-27.

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<td>Institut Pasteur, 25, rue du Docteur Roux, F-75724 Paris Cedex 15, FRANCE</td>
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C. ADDITIONAL INDICATIONS (leave blank if not applicable)  
This information is continued on an additional sheet

of deposit receipt, viability statement enclosed

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

The applicant requests that the furnishing of a sample shall only be effected to an expert in the art.

This request should apply in all countries where such a request is possible according to their respective legal provisions (in particular in EP, AU, CA...)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use only  
Authorized officer

For International Bureau use only  
Authorized officer

Form PCT/RO/134 (July 1998; reprint January 2004)
TRAITE DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES
Aux FINS DE LA PROCEDURE EN MATIERE DE BREVETS

FORMULB INTERNATIONALE

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I. IDENTIFICATION DU MICRO-ORGANISME

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II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSÉE

Le micro-organisme identifié sous chiffre I était accompagné :

- [X] d'une description scientifique
- [X] d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I qu'elle a reçu le 26 avril 2007 (date du dépôt initial)

IV. RECEPTION D'UNE REQUETE EN CONVERSION

La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial) et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)

V. AUTORITE DE DEPOT INTERNATIONALE

Nom : COLLECTION NATIONALE DE CULTURES DE MICROORGANISMES (CNCM)
Adresse : Institut Pasteur 25, rue du Docteur Roux F-75724 Paris Cedex 15 (France)

[Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité nationale ou de l'(des) National(s(s) autorisé(s) : Georges Wagener]

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)
### FORMULE INTERNATIONALE

**DESTINATAIRE :**

MME DENISE HIRSCH

INSENM

**Département Valorisation et transfert de technology**

101, RUE DE TOLBIAC

75654 PARIS CEDEX 13

---

**1. DEPOSANT**

INSTITUT NATIONAL de la SANTE et de la RECHERCHE MEDICALE

Adresse :

101, rue de Tolbiac

75654 PARIS CEDEX 13

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**11. IDENTIFICATION DU MICRO-ORGANISME**

Kuméro d'ordre attribué par l'AUTORITÉ DE DEPOT INTERNATIONAL :

CNCM 1-3752

Date du dépôt ou du transfert :

26 avril 2007

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**III. DECLARATION SUR LA VIABILITE**

La viabilité du micro-organisme identifié sous chiffre II a été évaluée par l'AUTORITÉ DE DEPOT INTERNATIONAL aux dates suivantes :

- pour le dépôt initial ou un transfert aux dates suivantes :
  - le 30 avril 2007 :
    - X itait viable
    - n'itait plus viable

---

1. Interroger la date du dépôt initial ou un transfert aux dates pertinentes.

2. Pour les cas visés à la règle 10.2.a)ii) et iii), mentionner le contrôle de viabilité.

3. Cocher la case qui convient.
IV. CONDITIONS DANS LESQUELLES LE CONTROLE DE VIABILITE A ETTE EFFECTUE

V. AUTORITE DE DEPOT INTERNATIONALE

Nom :

COLLECTION NATIONALE
DE CULTURES DE MICROORGANISMES (CNCM)

Adresse :

Institut Pasteur
25, rue du Docteur Roux
F-75724 Paris Cedex 15 (France)

Signature(s) de la (des) personne(s)
concernee(s), pour represente r l'autorite
de depot internationale ou de l'(des)
estatut(s) autorise(s) :

Georges Wagner

Date : Paris, le 15 juin 2000

A remplir si cette information a ete demandee et si les resultats du controle etaient
negatifs.

Formule BP/9 ideuxieme et derniere page)
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K39/00  C07K14/705  C07K16/00

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K  C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
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<tbody>
<tr>
<td></td>
<td>page 10, line 38 - line 47</td>
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<td>page 47 - page 48; claims 1,2</td>
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Further documents are listed in the continuation of Box C

See patent family annex

* Special categories of cited documents

1A1 document defining the general state of the art which is not considered to be of particular relevance

1E earlier document but published on or after the international filing date

1L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

1O* document referring to an oral disclosure, use, exhibition or other means

1P* document published prior to the international filing date but later than the priority date claimed

1P1 later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

1X document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

1Y document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

1Z document member of the same patent family

Date of the actual completion of the international search

15 February 2008

Date of mailing of the International search report

27/02/2008

Name and mailing address of the ISA/

European Patent Office, P B 5818 Paterlsaan 2

NL - 2280 HV Rijswijk

Tel (+31-70) 340-2040, Tx 31 651 epo nl

Fax (+31-70) 340-3016

Authorized officer

Camil Ileri, A lain
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<td>Patent document cited in search report</td>
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<td>Patent family member(s)</td>
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<tr>
<td>EP 1336619 A</td>
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Form PCT/ISA/210 (patent family annex) (April 2005)