(54) Title: VARIANT PEPTIDE LIGANDS THAT SELECTIVELY INDUCE APOPTOSIS

(57) Abstract

The present invention provides a method for treating or preventing a T cell associated disorder in a subject comprising administering to the subject a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell–derived, non-death inducing cytokines by T cells which recognize the peptide ligand. Also provided is a method for enhancing immune tolerance in a transplant recipient comprising administering to the recipient a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell–derived, non-death inducing cytokines by T cells which recognize the peptide ligand. Further provided is a method for inducing apoptosis of T cells without release of T cell–derived, non-death inducing cytokines, comprising contacting the T cells with a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell–derived, non-death inducing cytokines and a method for improving transplantation of bone marrow cells, comprising inducing apoptosis of T cells without release of T cell–derived, non-death inducing cytokines, comprising contacting the T cells with a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell–derived, non-death inducing cytokines.
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VARIANT PEPTIDE LIGANDS THAT SELECTIVELY INDUCE APOPTOSIS

This application claims priority to U.S. provisional application Serial No. 60/072,952, filed January 29, 1998, and the 60/072,952 application is herein incorporated by this reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to selective modulation of specific T cell responses in a subject. Specifically, the present invention is directed to the identification and characterization of variant peptide ligands for the T cell receptor which act as partial agonists by inducing apoptosis in cycling T cells without concomitant production and release of non-death inducing cytokines. Such variant ligands can be used to treat or prevent T cell associated disorders such as autoimmune disease, allergic disorders, graft rejection and graft versus host disease by selectively eliminating specific T cell populations.

Background Art

T cell receptor (TCR) engagement can lead to several different responses in mature peripheral T lymphocytes including activation and proliferation, anergy, cytokine elaboration and programmed cell death (apoptosis). These events are controlled by signals resulting from interaction of the TCR and CD4 or CD8 coreceptors with peptide/ major histocompatibility complex (MHC) ligands and contribute to the quality and extent of the T cell response (1-5).

The activation state of a T cell also dictates the outcome of interactions between the TCR and the peptide/MHC ligand. Agonist ligands stimulate cytokine production and proliferation in resting T cells whereas in cycling cells, they cause cytokine production followed by death via apoptosis (7, 8). The uncoupling of apoptosis and non-death inducing cytokine production in response to a ligand has not been demonstrated.
The present invention provides variant peptide TCR ligands with selective T cell death-inducing activity without concomitant release of non-death inducing cytokines and methods for the use of these variant peptide ligands in treating or preventing T cell associated disorders such as autoimmune disease, allergic disorders, graft rejection and graft versus host disease in transplantation recipients.

**SUMMARY OF THE INVENTION**

The present invention provides a method for treating or preventing a T cell associated disorder in a subject comprising administering to the subject a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines by T cells which recognize the peptide ligand.

Also provided is a method for enhancing immune tolerance in a transplant recipient comprising administering to the recipient a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines by T cells which recognize the peptide ligand.

Further provided is a method for inducing apoptosis of T cells without release of T cell-derived, non-death inducing cytokines, comprising contacting the T cells with a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines.

Additionally provided is a method for improving transplantation of bone marrow cells, comprising inducing apoptosis of T cells without release of T cell-derived, non-death inducing cytokines, comprising contacting the T cells with a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines.

Furthermore, a method is provided for screening a variant peptide ligand for the T cell receptor for the ability to induce apoptosis of T cells without release of T cell-derived, non-death inducing cytokines by T-cells which recognize the peptide ligand, comprising: contacting cycling T-cells which recognize the variant peptide ligand with
the variant peptide ligand; detecting the presence or absence of apoptosis of the T cells;
and detecting the presence or absence of release of non-death inducing cytokines by the
T-cells, whereby the presence of apoptosis of the T cells and the absence of release of
non-death inducing cytokines by the T cells indicates a variant peptide ligand having
the ability to induce apoptosis of T cells without release of non-death inducing
cytokines by T cells which recognize the variant peptide ligand.

Various other objectives and advantages of the present invention will
become apparent from the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-D: (A) Variant ligands induce apoptosis of CD4+ Th1 cells
without the accompanying production of IL-2, IL-3 or IFN-\gamma. (B,C,D) Dose
response analyses of cycling A.E7 cells stimulated by P13.9 APC incubated
with PCC (88-104) WT peptide (B), variant peptide R99 (C) or variant peptide
Y99 (D). T cell apoptosis (solid square), IL-2 (diamond), IL-3 (circle) and IFN-\gamma
(triangle) production in cycling A-E7 cells were measured after stimulation for
24 hours with P13.9 APC in the presence of the indicated concentrations of WT
peptide or variant peptides. All values were expressed as a percentage of the
response obtained with 100 \(\mu\)M WT peptide.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "a" can include multiples.

The present invention is based on the surprising discovery of variant peptide
ligands which have the partial agonistic effect of inducing apoptosis of T cells without
production and release of non-death inducing cytokines by selective T cell populations.
Thus, the present invention provides a method for treating or preventing a T cell
associated disorder in a subject comprising administering to the subject a peptide ligand
for the T cell receptor which induces apoptosis of T cells without concomitant release
of T cell-derived, non-death inducing cytokines (i.e., cytokines which do not induce cell death) in T cells which recognize the peptide ligand. By “concomitant” is meant that the production and release of non-death inducing cytokines is associated with the binding of the peptide ligand under normal conditions. In this manner, a specific population of T cells capable of causing a T cell associated disorder can be eliminated while leaving the remainder of the subject’s T cells unaffected. In addition, adverse effects on the subject from the release of T cell derived, non-death inducing cytokines are avoided.

The peptide ligand of this invention can be a ligand that binds a very specific population of T cells for a very focused effect on the subject’s T cells or the peptide ligand can be a ligand that binds the T cell receptor of many T cell types for a broad effect on the subject’s T cells.

The T cells which recognize the peptide ligand can be cycling T cells in the subject or the T cells which recognize the peptide ligand can be induced to cycle by administering antigen to the subject which is specific for the population of T cells of interest. Whether the T cells of interest are already cycling in the subject can be determined by flow cytometric analysis, allowing for identification of cells expressing antigens characteristic of cycling cells, such as Ki-67 (75) or identification of cells having a cell surface phenotype manifesting DNA replication by the incorporation of labeled nucleotides. The antigen that can be administered to induce cycling of the specific T cell population of interest can be a peptide (e.g., the wild type peptide from which the variant peptide is produced), protein, polysaccharide, antibody, lectin, pharmacological mediator that can be administered to the subject, organic molecule or nucleic acid, as well as any other substance now known or identified in the future to function as an antigen or agonist for activating T cells.

As used herein, “activated” T cells are T cells which manifest a genetic response to an encounter with an antigen which comprises induction of various genes, including those encoding T cell growth factors such as interleukin-2 (IL-2) or other T cell growth cytokines (e.g., interleukin-4, interleukin-7, interleukin-10, interleukin-12
and interleukin-15) and components of the high affinity receptors which bind the
growth factors. Also as used herein, “cycling” T cells are T cells that are actively
progressing through the cell cycle as manifested by the synthesis of DNA, entry into
and completion of mitosis and an increase in cell numbers due to cell division. Cycling
occurs after and as a consequence of activation. When T cell growth factors are
secreted, they interact with their newly expressed high affinity receptors and prompt the

10 cell into cell cycle progression. Cell cycle progression is associated with distinct
molecular events including changes in cyclin and cyclin-dependent kinase molecules,
DNA synthesis, mitosis and cell division.

The peptide ligand for the T cell receptor can be a variant peptide having one or
more amino acid substitutions or modifications in the wild type peptide amino acid
sequence. The wild type peptide ligand can first be selected as described herein for its
ability to bind the TCR of a specific T cell subpopulation in association with a suitable

15 MHC molecule and to activate the T cells. The amino acid sequence of the wild type
peptide can be determined either by amino acid sequencing according to standard
protocols, by synthesizing a peptide having a specific amino acid sequence or by
isolating or synthesizing a nucleic acid encoding a specific amino acid sequence. Then
the wild type amino acid sequence can be substituted or modified as described herein to
generate a variant peptide ligand that can be screened for the partial agonist profile
described herein. The variant peptide can be produced according to protocols standard
in the art, such as peptide synthesis, cleavage of proteins into peptide sequences or
expression of nucleic acid encoding the peptide sequence.

Amino acid substitutions and/or modifications in the wild type amino acid
sequence can be produced by first determining the wild type amino acid sequence and
introducing substitutions or modifications into the amino acid sequence of the peptide
ligand at the level of synthesizing a peptide or at the level of synthesizing or isolating a
nucleic acid encoding the amino acid sequence containing the substitution and/or
modification. An example of a substitution in a peptide is replacement of one or more

25 amino acid of the wild type sequence with another amino acid. An example of a
modification in a peptide ligand is introduction of one or more amino acids not present
in the wild type sequence and/or deletion of one or more amino acids present in the wild type sequence. In addition, the variant peptide ligand of this invention can comprise non-natural amino acids. In particular, the substitutions and/or modifications to the wild type ligand amino acid sequence should not eliminate the binding of the peptide ligand to the MHC molecule or completely prevent binding to the TCR, as can be determined according to methods well known in the art.

The peptide ligands of the present invention can be administered to any subject having T cells which bind the peptide ligands. In a preferred embodiment, the subject of this invention is an animal and most preferably a human. Examples of non-human animals to which the peptide ligand of this invention can be administered include, but are not limited to, horses, sheep, rabbits and any other animal having T cells which bind peptide ligands and in which it is desirable to modulate a T cell response.

The therapeutic advantage of administering the peptide ligands of the present invention is that a specific T cell population or large numbers of T cells can be eliminated without concomitant production and release of T cell derived, non-death inducing cytokines, many of which have a role in the induction of an inflammatory response which can have an adverse effect on the subject. In particular, the production and release, by specific T cells which bind the peptide ligand of this invention, of non-death inducing cytokines such as interleukin-2 (IL-2), interleukin-3 (IL-3) and/or interferon gamma (IFN-γ), as well as other non-death inducing cytokines identified as having a role in the induction of an inflammatory response, can be inhibited according to the methods described herein. The inhibition of the production and release of T cell derived, non-death inducing cytokines by T cells by administering the ligand of this invention can be determined according to assays well known in the art, such as enzyme linked immunosorbent assays (ELISA) and ELISPOT (76) for detection and quantification of the production and/or release of specific cytokines, as described herein and in the literature.

The methods of the present invention can be employed to treat any T cell associated disorder. As used herein, a T cell associated disorder is any disease or
syndrome which is caused by or exacerbated by a T cell response. Thus, the present invention provides a method for modulating a T cell response in a subject. For example, the T cell disorder to be treated or prevented by the methods of this invention can be, but is not limited to, autoimmune disease, allergic disease, graft vs host disease, atopic disorder, transplantation rejection, viral infection, human immunodeficiency virus (HIV) associated disorder, T cell leukemia and T cell lymphoma.

In particular, the present invention provides a method for treating or preventing an autoimmune disease comprising administering to a subject a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines by T cells which bind the peptide ligand. As used herein, an autoimmune disease generally describes a disease state or syndrome whereby a subject’s body produces a dysfunctional immune response against the subject’s own body components, with adverse effects. This may include production of T cells bearing receptors recognizing self components and producing cytokines that cause inflammation. Examples of the autoimmune diseases which can be treated or prevented by the present invention include, but are not limited to, diabetes mellitus, ulcerative colitis, Crohn’s disease, multiple sclerosis, autoimmune uveitis, systemic vasculitis, polymyositis-dermatomyositis, systemic sclerosis (scleroderma), rheumatoid arthritis, rheumatic fever, hypersensitivity pneumonitis, allergic bronchopulmonary aspergillosis, inorganic dust pneumoconioses, sarcoidosis, autoimmune hemolytic anemia, immunological platelet disorders, cryopathies such as cryofibrinogenemia, autoimmune pneumoconioses, pernicious anemia, autoimmune gastritis, psoriasis, Bechet’s disease, idiopathic thrombocytopenic purpura, Wegener’s granulomatosis, autoimmune thyroiditis, autoimmune oophoritis, bullous pemphigoid, pemphigus, polyendocrinopathies, Still’s disease, Lambert-Eaton myasthenia syndrome, myasthenia gravis, Goodposture’s syndrome, autoimmune orchitis, autoimmune uveitis, systemic lupus erythematosus, Sjogren’s syndrome, ankylosing spondylitis and related spondyloarthropathies (23-29, 41, 43, 61), as well as any other autoimmune disease now known or identified in the future.
Also as used herein, an allergic disease or disorder describes a disease state or syndrome whereby the body produces a dysfunctional immune response to environmental antigens comprising immunoglobulin E (IgE) antibodies which evoke allergic symptoms. Examples of allergic diseases and disorders which can be treated or prevented by the methods of this invention include, but are not limited to, drug hypersensitivity, allergic rhinitis, bronchial asthma, ragweed pollen hayfever, anaphylactic syndrome, urticaria, angioedema, atopic dermatitis, erythema nodosum, erythema multiforme, Stevens-Johnson Syndrome, cutaneous necrotizing venulitis, bullous skin diseases, allergy to food substances and insect venom-induced allergic reactions (54,55,60), as well as any other allergic disease or disorder now known or identified in the future.

The methods of the present invention can additionally be employed to treat or prevent transplantation rejection in a transplant recipient, which is a disease state or syndrome whereby the transplant recipient’s body produces an immune response against the engrafted tissue, resulting in rejection. Transplantation rejection can occur, for example, with kidney, heart, lung or liver transplants as well as with any other transplanted tissue (58,62,63).

The present invention further provides a method for enhancing immune tolerance in a transplant recipient comprising administering to the recipient a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines by T cells which recognize the peptide ligand. By administration of particular peptide ligands to the transplant recipient, specific T cell populations which may adversely affect the transplant can be selectively destroyed without concomitant production of non-death inducing cytokines such as IL-2, IL-3 and IFN-γ, which can adversely affect the transplant recipient. Additionally, by administering a ligand to the transplant recipient which binds the TCR of many types of T cells, a large number of the recipient’s T cells can be destroyed, thereby enhancing immune tolerance in the recipient, without concomitant production of non-death inducing cytokines such as IL-2, IL-3 and IFN-γ, which can adversely affect the transplant recipient.
In vitro, ex vivo and in vivo applications are contemplated in the present invention. It will be recognized that the variant peptide ligand may be employed in any suitable form. For example, the variant peptide ligand may be used alone or as a peptide/MHC complex and may be used in association with other agents. The variant peptide ligand may be introduced by contacting with cells, expression in cells, presentation on the surface of antigen presenting cells, or introduction by any other appropriate means or combination of means.

It is also contemplated that the methods of the present invention can be employed to treat or prevent graft versus host (GvH) disease, which describes a disease state or syndrome whereby an immune response is initiated by engrafted cells and is directed against the recipient’s body with adverse effects. Examples of GvH disease include, but are not limited to, acute and chronic GvH disease following bone marrow and other organ transplants.

Thus, a method is provided for inducing apoptosis of T cells without release of T cell-derived, non-death inducing cytokines, comprising contacting the T cells with a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines. The T cells which recognize the peptide ligand can be cycling T cells or the T cells can be resting T cells which have been induced to cycle by contact with an antigen.

The present invention also provides a method for improving transplantation of bone marrow cells, comprising inducing apoptosis of T cells without release of T cell-derived, non-death inducing cytokines, comprising contacting the T cells with a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines. The T cells of this method can be a component of transplanted bone marrow cells in a transplantation recipient and/or can be the recipient’s own T cells. Alternatively, the T cells can be a component of bone marrow cells that are still in the bone marrow donor or that have been removed from the donor and are contacted with the peptide ligand in vitro or ex vivo prior to
transplantation into a recipient. The transplantation of bone marrow cells is improved by eliminating T cells included within the population of transplanted bone marrow cells that are capable of inducing GvH disease and/or by eliminating T cells in the recipient that are capable of causing rejection of the transplanted bone marrow cells.

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The source of the wild type peptide ligand for the T cell receptor of the present invention can be, for example, residues 84-102 and/or 143-168 of myelin basic protein (MBP) (for multiple sclerosis) (26,40-42), the human S antigen (for autoimmune uveitis) (29,43), type II collagen (for rheumatoid arthritis) (44), thyroglobulin (for thyroiditis) (45), Hepatitis B surface antigen (48-50), HIV gp120 (51), residues 109-145 of chorionic gonadotropin (52), malaria sporozoite antigen (53) and allergy antigens such as Amb aV, Amb tV, allergen M and antigen S (54,56,57,59), as well as any other source for a peptide ligand (64) which can be substituted or modified as described herein to have the effect of selectively inducing apoptosis of T cells without concomitant release of T cell-derived, non-death inducing cytokines, now known or identified in the future.

In addition, the identification of a wild type peptide ligand for the T cell receptor which causes the T cell diseases and disorders described herein and which can be modified and/or substituted according to the methods of the present invention can be carried out as follows: The T cells are taken from the peripheral blood of a subject diagnosed with a T cell associated disorder and maintained in culture and exposed to a variety of antigens (see, e.g., 23-43). After antigen exposure, T cells are assayed for activation or cycling according to assays standard in the art. An antigen that is identified as having an increased activating or cycling-inducing effect on the subject’s T cells, as compared to the activating or cycling-inducing effect of the antigen on the T cells of a subject which is not diagnosed with the T cell associated disorder of interest, can then be exposed to the cultured T cells in the form of a peptide to determine which peptide region of the antigen is the activating/cycling peptide ligand. For example, immunodominant peptides of the antigen can be identified according to methods standard in the art and such immunodominant peptides can be used to partially screen
the antigen for identifying the peptide region containing the activating/cycling peptide ligand.

Once the wild type peptide ligand is identified for a particular T cell associated disorder, the amino acid sequence can be determined according to methods standard in the art. Once the amino acid sequence is known, substitutions and/or modifications in the wild type amino acid sequence can be made, resulting in variant peptide ligands which can be screened according to the methods provided herein for the ability to induce apoptosis without release of T cell-derived, non-death inducing cytokines.

For example, the variant peptide ligands of the present invention can be screened for the ability to induce apoptosis without release of T cell-derived, non-death inducing cytokines by T-cells which recognize the variant peptide ligand as follows: Resting T cells can be isolated from a subject diagnosed with a particular T cell-associated disorder which is to be treated by the variant peptide ligand to be screened. T cell clones can then be prepared by 1) activating the T cells by repetitively stimulating the resting T cells with the wild type agonist peptide ligand from which the variant peptide ligand was produced; 2) inducing cell cycling by growing the activated T cells in the presence of IL-2 or other T cell growth cytokines (e.g., interleukin-4, interleukin-7, interleukin-10, interleukin-12 and interleukin-15); and 3) carrying out limited dilution cloning according to standard methods. The T cell clones can then be activated with wild type peptide ligand for two days, stimulated with IL-2 for 3-10 days to become cycling T cells and used to test the variant peptide ligands of this invention for the ability to induce death of the cycling, cloned T cells without stimulating the production of non-death inducing cytokines.

Thus the present invention provides a method for screening a variant peptide ligand for the ability to induce apoptosis of T cells without release of T cell-derived, non-death inducing cytokines by T-cells which recognize the peptide ligand, comprising: a) contacting cycling T cells which recognize the variant peptide ligand with the variant peptide ligand; b) detecting the presence or absence of apoptosis of the T cells; and c) detecting the presence or absence of release of non-death inducing
cytokines by the T cells, whereby the presence of apoptosis of the T cells and the absence of release of non-death inducing cytokines by the T cells indicates a variant peptide ligand having the ability to induce apoptosis of T cells without release of non-death inducing cytokines by T cells which recognize the variant peptide ligand.

The presence or absence of apoptosis of the T cells can be determined by a variety of methods, such as flow cytometry, exposure of phosphatidylserine, incorporation of propidium iodide and/or labeling of fragmented DNA according to protocols described herein and as are well known in the art for detecting molecular or cellular changes associated with apoptosis (8, 70-74). The presence or absence of apoptosis of T cells as a result of binding a variant peptide ligand of this invention is determined by comparison with the amount of apoptosis in a control population of T cells which have had no exposure to the variant peptide ligand. Thus, an increase in the amount of apoptosis in a T cell population exposed to a variant peptide ligand relative to the amount of apoptosis in a T cell population having no exposure to the variant peptide ligand indicates the presence of apoptosis as a result of binding a variant peptide ligand.

The presence or absence of the production and release of non-death inducing cytokines by the T cells can be determined by methods standard in the art for measuring cytokines (e.g., ELISA and ELISPOT). The presence or absence of the production and release of non-death inducing cytokines by the T cells as a result of binding a variant peptide ligand of this invention is determined by comparison with the amount of production and release of non-death inducing cytokines by a control population of T cells which have had no exposure to the variant peptide ligand. Thus, a decrease in the amount of production and release of non-death inducing cytokines in a T cell population exposed to a variant peptide ligand relative to the amount of production and release of non-death inducing cytokines in a T cell population having no exposure to the variant peptide ligand indicates the absence of production and release of non-death inducing cytokines by the T cells as a result of binding a variant peptide ligand.
The variant peptide ligands identified according to the screening methods described herein to have the partial agonistic effect of this invention can be administered to a subject in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject along with the selected peptide ligand without causing any substantial undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject.

The peptide ligand of this invention can be administered orally (46) or parenterally (48-50,52,53) to the subject (47). Suitable carriers for oral administration of the peptide ligand include one or more substances which may also act as flavoring agents, lubricants, suspending agents, or as protectants. Suitable solid carriers include calcium phosphate, calcium carbonate, magnesium stearate, sugars, starch, gelatin, cellulose, carboxypolymethylene, or cyclodextrans. Suitable liquid carriers may be water, pyrogen free saline, pharmaceutically accepted oils, or a mixture of any of these. The liquid can also contain other suitable pharmaceutical additions such as buffers, preservatives, flavoring agents, viscosity or osmo-regulators, stabilizers or suspending agents. Examples of suitable liquid carriers include water with or without various additives, including carboxypolymethylene as a pH-regulated gel. The peptide ligand may be contained in enteric coated capsules that release the peptide ligand into the intestine to avoid gastric breakdown.

For parenteral administration of the peptide ligand, a sterile solution or suspension is prepared in saline that may contain additives, such as ethyl
oleate or isopropyl myristate, and can be injected, for example, into subcutaneous or intramuscular tissues, as well as intravenously.

Alternatively, the peptide ligand may be microencapsulated with either a natural or a synthetic polymer into microparticles 4-8 µm in diameter, which target intestinal lymphoid tissues and produce a sustained release of ligand for up to four weeks (68,69).

The peptide ligand of this invention can be administered to the subject in amounts sufficient to selectively induce apoptosis of targeted T cell populations without concomitant production and release of non-death inducing cytokines by the targeted T cells. Optimal dosages used will vary according to the individual being treated and ligand being used. The amount of ligand will also vary among individuals on the basis of age, size, weight, condition, etc. One skilled in the art will realize that dosages are best optimized by the practicing physician and methods for determining dose amounts and regimens and preparing dosage forms are described, for example, in *Remington's Pharmaceutical Sciences* (21). For example, suitable doses and dosage regimens can be determined by comparison to agents presently used in the treatment or prevention of autoimmune disorders, allergic disorders, graft rejection and other T cell related disorders. The preferred or optimal dosage is the amount of peptide ligand which results in apoptosis of specific T cells without concomitant production of non-death inducing cytokines, in the absence of significant side effects.

Typically, the peptide ligand of this invention can be administered orally or parenterally in a dosage range of 0.1 to 100 mg/kg of body weight at about 3-10 day intervals, or more preferably, 3-5 day intervals, over a period of days, weeks or months, depending on the clinical response that is to be obtained. Administration of the peptide ligand can be stopped completely following a prolonged remission or
stabilization of disease signs and symptoms and readministered following a 
worsening of either the signs or symptoms of the disease, or following a 
significant change in immune status, as determined by routine follow-up 
immunological studies well known to a clinician in this field (e.g., a return to 
significant reactivity of T cells upon exposure to a particular antigen).

The efficacy of administration of a particular dose of peptide ligand in treating a 
T cell associated disorder as described herein can be determined by evaluating the 
particular aspects of the medical history, the signs, symptoms and objective laboratory 
tests that have a documented utility in evaluating pathophysiological activity of the 
particular T cell associated disorder being treated. These signs, symptoms and objective 
laboratory tests will vary depending on the particular disorder being treated, as will be 
well known to any clinician in this field. For example, if, based on a comparison with 
an appropriate control group and knowledge of the normal progression of the disorder 
in the general population or the particular individual, 1) a subject’s frequency or 
severity of recurrences is shown to be improved; 2) the progression of the disease or 
disorder is shown to be stabilized; or 3) the need for use of other immunosuppressive 
medications is lessened, then a particular treatment can be considered efficacious.

In a particular example, in using the ligands of the present invention to treat an 
autoimmune disease such as multiple sclerosis, clinical parameters and symptoms 
which can be monitored for efficacy can include the severity and number of attacks; or 
for continuously progressive disease, the worsening of symptoms and signs; the 
cumulative development of disability; the number or extent of brain lesions as 
determined by magnetic resonance imaging; and the use of immunosuppressive 
medications (65-67).

Once it is established that the T cell population of interest has been eliminated 
and/or that disease activity is significantly improved or stabilized by a particular 
peptide ligand treatment, specific signs, symptoms and laboratory tests can be 
evaluated in accordance with a reduced or discontinued treatment schedule. If a disease 
activity recurs, based on standard methods of evaluation of the particular signs,
symptoms and objective laboratory tests as described herein, peptide ligand treatment can be reinitiated.

Additionally, the efficacy of administration of a particular dose of a peptide ligand in preventing a T cell associated disorder in a subject not known to have a T cell associated disorder, but known to be at risk of developing a T cell associated disorder, can be determined by evaluating standard signs, symptoms and objective laboratory tests, known to one of skill in the art, over time. This time interval may be long (i.e., years/decades). The determination of who would be at risk for the development of a T cell associated disorder would be made based on current knowledge of the known risk factors for a particular disorder familiar to clinicians and researchers in this field, such as a particularly strong family history of a disorder or exposure to or acquisition of factors or conditions which are likely to lead to development of a T cell associated disorder.

The present invention is more particularly described in the following examples, which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

*T cell clones and antigen presenting cells.* CD4+ A.E7 Th1 cells (9,22) were maintained by bi-weekly stimulation with pigeon cytochrome c protein (5 μM) and irradiated splenocytes from B10.A (H-2b) mice. A.E7 cells stimulated in this manner two days previously were ficollied and transferred to fresh medium containing IL-2 (50-100 IU/ml recombinant human IL-2; Chiron, Emeryville, CA) or 10-15% T-Stim™ (Collaborative Biomedical Products, Bedford, MA) and incubated for an additional 2 to 5 days to generate cycling A.E7 cells that are predisposed to apoptosis following TCR engagement (8). P13.9 cells, which are L cell transfectants expressing I-Ek, ICAM-1, and B7.1 (CD80) molecules, were used as antigen-presenting cells (APC) (3).

*Peptides.* Wild type (WT) peptide PCC (88-104) has the amino acid sequence: KAERADLIAYLKQATAK (SEQ ID NO:1). Peptides Y99
(KAERADLIAYLQYKAK; SEQ ID NO:2), C99 (KAERADLIAAYLCQKAK; SEQ ID NO:3), R99 (KAERADLIAAYLQYKAK; SEQ ID NO:4) and A99 (KAERADLIAAYLQYKAK; SEQ ID NO:5) are synthetic peptides in which the primary TCR contact residue, lysine in position 99, is changed to tyrosine, cysteine, arginine, or alanine, respectively. All peptides were synthesized by the Peptide Synthesis Facility, NIAID, NIH, Bethesda, MD.

Cell death assays. 5 x 10^4 P13.9 cells were incubated with 5 x 10^4 cycling A.E7 cells in the presence of various concentrations of PCC peptides in quadruplicate in 96-well round bottom plates. To block apoptosis, Fas-Fc and TNFR-Fc (10 μg/ml; Immunex, Seattle, WA) were included in some assays. Cells were harvested after 24 hours incubation, two of each four wells were pooled and 10 μg/ml of propidium iodide were added. Ungated cells were acquired for 30 seconds at a constant flow rate using a FACScan equipped with CellQuest software (Becton-Dickinson, Mountain View, CA) and the number of viable A.E7 cells in each sample was calculated as previously described (10, 11). In some experiments, P13.9 cells were pre-loaded with 0.1 μM carboxyfluorescein diacetate-acetylene (CMFDA; Molecular Probes, Eugene, OR) to allow APC to be excluded from the analysis by gating on fluorescein-negative cells. In other experiments, A.E7 cells were stained with fluoresceinated-anti-CD4 before flow cytometry to differentiate them from APC.

Cytokine measurement. Supernatants from the apoptosis assays were harvested at 24 hours and assayed for cytokine production by ELISA. IL-2 and IL-3 were detected using antibodies purchased from Pharmingen, San Diego, CA, according to the manufacturer’s instructions. IFN-γ was measured by ELISA as previously described (6). Data are expressed as the mean of quadruplicate wells calculated as a percentage of the response to 100 μM WT peptide.

mRNA analysis by semi-quantitative RT-PCR. A.E7 cells were incubated with P13.9 cells pre-pulsed for 2 h with 100 μM of the indicated peptides and total RNA was isolated using RNAzol according to the manufacturer’s instructions (Tel-test, Inc, Friendswood, TX). The reverse transcriptase (RT) reaction was performed using
random hexamer primers and part of each sample was subjected to polymerase chain reaction (PCR) amplification using β-actin primers and 32P-labeled nucleotides (12). Serial dilutions of RT products were used to ensure comparisons were in the linear range. After normalizing the input cDNA to the β-actin signal, β-actin, Fas-L, TNFα, IL-2, IFN-γ and Bcl-X primers were then used to amplify their respective cDNA under the same conditions. The PCR protocol was 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds, for 30 cycles. Sequences of the primers are as follows: β-Actin, 5’ primer: 5’GAT GAC GAT ATC GCT GCG CTG3’ (SEQ ID NO:6); β-Actin, 3’ primer: 5’GTA CGA CCA GAG GCA TAC AGG3’ (SEQ ID NO:7); mIL-2, 5’ primer: 5’ATG TAC AGC ATG CAG CTC GCA TC3’ (SEQ ID NO:8); mIL-2, 3’ primer: 5’GGC TTG TTA AGA TGA TGC TTT GAG C3’ (SEQ ID NO:9); mFas-L, 5’ primer: 5’CTG GTG GCT CTT GTT GTT GGA AT3’ (SEQ ID NO:10); mFas-L, 3’ primer: 5’GTG TAG GGG CTT GGT GTT GC3’ (SEQ ID NO:11); mTNFα, 5’ primer: 5’ATG AGC ACA GAA AGC ATG ATG CGC3’ (SEQ ID NO:12); mTNFα, 3’ primer: 5’CCA AAG TAG ACC TGC CCG GAC TC3’ (SEQ ID NO:13); Bcl-X, 5’ primer: 5’CAA TGG TGG CTG AAG AGA3’ (SEQ ID NO:14); and Bcl-X, 3’ primer: 5’GGA GAG CGT TCA GTG ATC3’ (SEQ ID NO:15).

**Protein biochemistry.** 2-5 x 10^6 P13.9 APC were incubated for 2-4 hours in 200 μl complete medium alone (no peptide) or with the variant peptides added at a concentration of 100 μM. Pulsed APC were then centrifuged together with 1-1.25 x 10^7 cycling A.E7 cells in Eppendorf tubes and warmed to 37°C, as described (6). After stimulation, T cells + APC were lysed (6) and lysates were immunoprecipitated with anti-CD3e (500A2 mAb; Pharmingen), anti-ZAP-70 (rabbit antiserum; NICHD, NIH, Bethesda, MD), or anti-TCR-ζ (rabbit antiserum; DNAX, Palo Alto, CA,) (13). Immunoprecipitates were resolved by SDS-PAGE (12% under reducing conditions), transferred to nitrocellulose and blotted with anti-phosphotyrosine antibody (6). Blots were developed using SuperSignal™ chemiluminescent substrate (Pierce, Rockford, IL).

**Fluorescence microscopy.** 10^6 P13.9 APC were incubated for 2-4 hours in 200 μl complete medium alone (no peptide) or with the variant peptides added at a
concentration of 100 μM. Pulsed APC were then centrifuged together with 10^6 cycling
A.E7 cells in Eppendorf tubes and warmed to 37°C for 5 min. Cells were incubated on
ice for 5 min and for an additional 15 min in the presence of 10 μg/ml of anti-CD3 Ab
(Pharmingen), washed twice with cold PBS and fixed for 30 min at room temperature
in 1% PFA. For the detection of actin polymerization, A.E7 cells were similarly
stimulated with APC and with the different variant peptides. Cells were fixed for 30
min. at room temperature in 1% PFA, washed twice in PBS-1% FCS and incubated 30
min in PBS - 0.1% saponin -1% fetal calf serum (FCS)-Texas Red-X phalloidin (2
units/ml). Cells were washed twice with PBS and were mounted on slides using
Fluoromount-G™ (Electron Microscopy Sciences, Washington, PA). The images were
obtained on a Zeiss Axiophot with a CCD camera (Princeton Digital Instruments).

Variant ligands induce apoptosis without the production of activation cytokines
such as IL-2, IL-3, or IFN-γ. To determine whether variant ligands can selectively
elicit apoptosis responses, many peptides with single amino acid substitutions at the
major epitopic residue (lysine 99) of the agonist 88-104 peptide of pigeon cytochrome c
(PCC 88-104) have been previously screened for their effect on A.E7 cells that were
actively cycling under the influence of IL-2. Certain variants of PCC 88-104 with
substitutions at position 99 have been previously found to induce variant signaling in
unactivated, resting A.E7 cells (3). These substitutions do not affect binding to the I-E^k
molecule and therefore are assumed only to alter TCR recognition of peptide-MHC
ligand (3, 14).

T cell apoptosis, as well as IL-2, IL-3 and IFNγ production in cycling A.E7
cells, was measured after stimulation for 24 hours with P13.9 APC in the presence of
100 μM WT peptide or variant peptides in concentrations ranging from 0.0001 to 100
μM (Figure 1A). All values were expressed as a percentage of the response obtained
with 100 μM WT peptide. The actual 100% maximal values were as follows:
apoptosis: 55% dead cells at 24 hours; IL-2: 1.4 ng/ml; IL-3: ≥250 ng/ml; and IFN-γ:
120 ng/ml. The wild-type (WT) PCC 88-104 peptide induced both cell death and the
secretion of IL-2, IL-3 and IFN-γ. In contrast, at high concentrations, two of the
variant peptides tested, Y99 and C99, induced as much cell death as the WT peptide,
but failed to induce IL-2, IL-3, or IFN-γ production. T cell death under these circumstances has the morphological and nuclear fragmentation characteristics of apoptosis (8). Other variant peptides, such as A99 and R99, did not stimulate either apoptosis or cytokine secretion.

To address the possibility that the selective cell death induction by Y99 and C99 was due to weak stimulation of all effector functions, dose-response curves for apoptosis and cytokine responses were compared (Figure 1B-D). The apoptosis assay is slightly more sensitive than the cytokine ELISAs. However, 50% maximal apoptosis requires about 1 nM WT ligand or 1 µM Y99. Because half-maximal cytokine production in response to the WT peptide requires at most 10-fold more agonist than half-maximal apoptosis, cytokine production should be seen with 10 µM Y99 if the latter simply behaves as a weak agonist. Yet 100 µM of Y99 or C99 caused no production of IL-2, IL-3 or IFN-γ. Thus, Y99 and C99 are true partial agonists for A.E7 cells, because they induce apoptosis but not other typical effector functions. By contrast, R99 and A99 failed to induce these responses at any dose and therefore are non-agonists with respect to the responses examined.

_T cell death induced by partial agonists employs the Fas and TNF pathways._

Because T cell apoptosis can result from the action of Fas or tumor necrosis factor-alpha (TNF-α) (15-18), a role for these molecules in death induced by the Y99 and C99 partial agonists was assessed. Stimulation of cycling A.E7 cells for two hours with the apoptogenic WT, Y99, or C99 peptides (100 µl) induced the mRNAs for TNF-α and Fas-L but not for Bcl-X, a protein that prevents apoptosis (19). There was less induction of these mRNAs with the variant peptides compared to the WT peptide, in accordance with the latter’s greater lethality at low concentrations. Fas-L or TNF mRNA levels were not increased using the non-agonist peptides A99 and R99. Also, in contrast to the WT peptide, none of the variant peptides elicited IL-2 or IFN-γ mRNA, confirming the results obtained by ELISA.

In Fas-L and TNF blocking experiments, cycling A.E7 cells were stimulated with APC pre-pulsed with 2.5 nM WT peptide, 100 µM C99 peptide or 100 µM Y99
peptide, in medium alone or in the presence of either Fas-Fc (10 μg/ml), TNFR-Fc (10 μg/ml) or both Fas-Fc and TNFR-Fc. Data from these experiments indicated that both Fas-L and TNF were functionally important in the apoptosis caused by the WT, C99, and Y99 peptides because death was potently inhibited by these reagents that specifically block these cytokines. In control experiments, Fas-Fc and TNFR-Fc alone did not induce proliferation or apoptosis.

Tyrosine phosphorylation patterns induced by variant ligands. In resting T cells, partial agonist or antagonist ligands generate unique patterns of protein tyrosine phosphorylation (2, 3, 6). Hence, whether apoptosis-inducing variant peptides produce characteristic TCR-associated phosphorylation patterns in cycling T cells was determined. CD3ε, ZAP-70, or TCR-ζ and their associated proteins were immunoprecipitated from lysates prepared from cycling A.E7 cells that had been stimulated for 10 minutes with P13.9 APC pre-pulsed with WT or the variant peptide ligands. In cycling A.E7 cells, the WT peptide induced prominent tyrosine-phosphorylated species, including the three isoforms of phospho-ζ (p18, p21 and p23), phosphorylated CD3ε and phosphorylated ZAP-70. This is the same pattern as that obtained when resting A.E7 cells are stimulated with WT peptide (3). However, no correlation was found between the pattern of TCR-ζ tyrosine phosphorylation and programmed cell death. The apoptosis-inducing partial agonist, C99 and the non-agonist ligands, A99 and R99, all produced a modest increase in the p21 form of phospho-ζ. However, the partial agonist Y99 surprisingly failed to induce a detectable increase in p21 phospho-ζ, even upon examination by direct anti-ζ immunoprecipitation. None of the variant peptide ligands induced detectable levels of the other species of phospho-ζ (p18 and p23), phosphorylated CD3ε, or phosphorylated ZAP-70.

Ligands that deliver death-inducing signals induce TCR capping. Successful activation of resting T cells with an agonist peptide causes the redistribution of the TCR into polarized focal aggregates on the membrane, i.e. “capping” (20). The ability of the variant ligands to induce TCR aggregation and actin polymerization, after stimulating cycling A.E7 cells with P13.9 APC pulsed with medium alone (no peptide)
or with the variant peptides (100 μM) for 5 min at 37°C, was examined by fluorescence microscopy. Cells were stained with fluoresceinated anti-CD3 Ab and Texas Red-X phalloidin. TCR polarization was observed at points of contact with the APC after stimulation with the WT peptide and the apoptosis-inducing Y99 and C99 peptides but not by A99 peptide which, by itself, does not induce cell death. The aggregation of the TCR correlated closely with actin polymerization detected after intracellular staining of A.E7+APC cells with Texas Red-conjugated X phalloidin. However no distinct capping or TCR polarization was seen after stimulation with the variants R99 and A99 that fail to cause apoptosis, even though they induced readily detectable ζ-chain phosphorylation.

Treatment of multiple sclerosis with a variant peptide ligand of the present invention as an example of treatment of a T cell associated disorder with a variant peptide ligand of the present invention. A subject diagnosed with active relapsing/remitting or chronic progressive multiple sclerosis (MS), using established MS diagnostic criteria, can be treated with a variant peptide ligand of this invention as follows: T cells from the subject can be demonstrated to be reactive in increased numbers with myelin basic protein or other myelin antigens, preferably immunodominant peptides 84-102 or 143-168 according to standard in vitro assays, as described herein. The subject's T cells can then be cloned as described herein and the same wild type peptides can then be used to identify T cell clones which are reactive against these peptides by exposing the cloned T cells to these wild type peptides and assaying the cloned T cells for reactivity to the peptide as described herein.

Once reactive T cell clones from the subject have been identified, these clones can be used in screening assays as described herein to identify variant ligand peptides of this invention which have the ability to induce apoptosis in the cloned T cell populations without concomitant release of non-death inducing cytokines. Ideally, those variant peptides which induce apoptosis without concomitant release of non-death inducing cytokines in a majority of the T cell clones are selected for upscaled production for clinical use. The variant peptides identified to function as described
above can be produced in large quantities by standard chemical protocols and sterilized for administration to the subject.

The variant peptide ligands can be administered to the subject by oral or parenteral route in appropriate pharmaceutically acceptable delivery vehicles in a dosage range of 0.1 to 100 mg/kg body weight. The optimal dosage can be determined according to the protocols described herein and can depend on the particular pharmacological formula as well as the active concentration of the variant peptide ligand based on the average value that was needed to induce apoptosis in the majority of the T cell clones described above. The variant peptide ligand can be administered every 3-5 days, depending on the clinical setting and the subject’s response. Administration of the ligand can be continued for days, weeks or months, until a favorable clinical response is observed unless complications develop or an exacerbation of the disease occurs.

Clinical improvement can be determined according to standards recommended by the International Workshop on Outcomes Assessment sponsored by the National Multiple Sclerosis Society (65). Such standards can include an improved score on the Kurtzke expanded disability status scale (EDSS) and decreased numbers of lesions as detected by cranial magnetic resonance imaging (especially using quantitative imaging with gadolinium enhancement and T2 weighted images). Assessment of immunological responses in the subject’s T cells can also be carried out, using the subject’s post-treatment T cells and the original wild-type agonist peptides in ELISPOT assays. A decrease in the frequency of responding T cells in the ELISPOT assay would indicate the establishment in the subject of a type of immunological tolerance induced by the peptide ligand treatment.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.
Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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What is claimed is:

1. A method for treating or preventing a T cell associated disorder in a subject comprising administering to the subject a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines by T cells which recognize the peptide ligand.

2. The method of claim 1, wherein the T cells are cycling T cells.

3. The method of claim 1, further comprising the step of administering antigen to the subject to induce cycling of the T cells.

4. The method of claim 1, wherein the peptide ligand for the T cell receptor is a variant peptide ligand having one or more amino acid substitutions or modifications in the wild type peptide amino acid sequence.

5. The method of claim 1, wherein the T cell associated disorder is selected from the group consisting of autoimmune disease, allergic disease, graft vs host disease, atopic disorder, graft rejection, viral infection, human immunodeficiency virus associated disorder, T cell leukemia and T cell lymphoma.

6. The method of claim 1, wherein the T cell-derived, non-death inducing cytokines are selected from the group consisting of interleukin-2, interleukin-3 and interferon gamma.

7. A method for enhancing immune tolerance in a transplant recipient comprising administering to the recipient a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines by T cells which recognize the peptide ligand.

8. The method of claim 7, wherein the T cells are cycling T cells.
9. The method of claim 7, further comprising the step of administering antigen to the transplant recipient to induce cycling of the T cells.

10. The method of claim 7, wherein the peptide ligand for the T cell receptor is a variant peptide ligand having one or more amino acid substitutions or modifications in the wild type peptide amino acid sequence.

10. The method of claim 7, wherein the T cell-derived, non-death inducing cytokines are selected from the group consisting of interleukin-2, interleukin-3 and interferon gamma.

11. The method of claim 10, wherein the transplant recipient is human.

12. A method for inducing apoptosis of T cells without release of T cell-derived, non-death inducing cytokines, comprising contacting the T cells with a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines.

13. The method of claim 12, wherein the T cells are cycling T cells.

14. The method of claim 12, further comprising the step of contacting the T cells with antigen to induce cycling of the T cells.

15. The method of claim 12, wherein the T cell-derived, non-death inducing cytokines are selected from the group consisting of interleukin-2, interleukin-3 and interferon-gamma.

16. The method of claim 12, wherein the peptide ligand is a variant peptide ligand having one or more amino acid substitutions or modifications in the wild type peptide amino acid sequence.
17. A method for improving transplantation of bone marrow cells, comprising inducing apoptosis of T cells without release of T cell-derived, non-death inducing cytokines, comprising contacting the T cells with a peptide ligand for the T cell receptor which induces apoptosis of T cells without release of T cell-derived, non-death inducing cytokines.

18. The method of claim 17, wherein the T cells are a component of the bone marrow cells.

19. The method of claim 17, wherein the T cells are in a transplantation recipient.

20. The method of claim 18, wherein the bone marrow cells are in a bone marrow donor.

21. The method of claim 18, wherein the bone marrow cells are contacted with the peptide ligand in vitro.

22. The method of claim 17, wherein the peptide ligand is a variant peptide ligand having one or more amino acid substitutions or modifications in the wild type peptide amino acid sequence.

23. The method of claim 17, wherein the T cells are cycling T cells.

24. The method of claim 17, further comprising the step of contacting the T cells with antigen to induce cycling of the T cells.

25. The method of claim 17, wherein the T cell-derived, non-death inducing cytokines are selected from the group consisting of interleukin-2, interleukin-3 and interferon-gamma.
26. A method for screening a variant peptide ligand for the T cell receptor for
the ability to induce apoptosis of T cells without release of T cell-derived, non-death
inducing cytokines by T-cells which recognize the peptide ligand, comprising:

a) contacting cycling T-cells which recognize the variant peptide ligand with the
variant peptide ligand;

b) detecting the presence or absence of apoptosis of the T cells; and

c) detecting the presence or absence of cytokine release by the T-cells, whereby
the presence of apoptosis of the T cells and the absence of cytokine release by the T
cells indicates a variant peptide ligand having the ability to induce apoptosis of T cells
without release of cytokines by T cells which recognize the variant peptide ligand.

27. The method of claim 26, wherein the T cell-derived, non-death inducing
cytokine is selected from the group consisting of interleukin-2, interleukin-3 and
interferon-gamma.
SEQUENCE LISTING

110 The Government of the United States of America

120 VARIANT PEPTIDE LIGANDS THAT SELECTIVELY INDUCE APOPTOSIS

130 14014.0313/P

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**Description of artificial sequence:**

This sequence is a synthetic construct.
Lys Ala Glu Arg Ala Asp Leu Ile Ala Tyr Leu Ala Gln Ala Thr Ala
1 5 10 15
Lys

gatgacgata tcgctgcgct g
21

gtacgaccag aggcatacag g
21

atgtacgca tgcagctgc atc
23
<213> Artificial Sequence
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<400> 9
ggctgttga gatgatgcct tgaca 25

<210> 10
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## INTERNATIONAL SEARCH REPORT

### A. CLASSIFICATION OF SUBJECT MATTER

**IPC 6** A61K38/45   A61K38/17   G01N33/68

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

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols):

**IPC 6** A61K G01N 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).

### 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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<tr>
<td>A</td>
<td>WO 94 04171 A (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 3 March 1994 see page 128 - page 133</td>
<td>1-27</td>
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<tr>
<td>A</td>
<td>WO 96 36881 A (CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED) 21 November 1996 see the whole document</td>
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</table>

Further documents are listed in the continuation of box C. Patent family members are listed in annex.

Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance.
- "E" earlier document but published on or after the international filing date.
- "L" 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).
- "O" document referring to an oral disclosure, use, exhibition or other means.
- "P" document published prior to the international filing date but later than the priority date claimed.

- "X" 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.
- "P" 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.
- "Y" 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.
- "S" document member of the same patent family.

Date of the actual completion of the international search: **29 June 1999**

Date of mailing of the international search report: **12/07/1999**

Name and mailing address of the ISA:

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

Authorized officer: Moreau, J.
<table>
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<td>P,X</td>
<td>COMBADIÈRE B ET AL: &quot;Differential TCR signaling regulates apoptosis and immunopathology during antigen responses in vivo.&quot; IMMUNITY, (1998 SEP) 9 (3) 305-13, XP002107395 see the whole document</td>
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### Box I  Observations where certain claims were found unsearable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **X** Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
   **Remark:** Although claims 1-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. □ Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

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

### Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

1. □ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

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

3. □ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

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

**Remark on Protest**

- □ The additional search fees were accompanied by the applicant's protest.
- □ No protest accompanied the payment of additional search fees.
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<td>WO 9404171 A</td>
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