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(54) Title: IMMUNOTOLERIZING FUSION PROTEINS FOR TREATMENT OF MULTIPLE SCLEROSIS

(57) Abstract: The present invention relates to new immunotolerizing fusion proteins and pharmaceutical compositions comprising them, as well as uses thereof for the prevention or treatment of autoimmune demyelinating diseases, in particular multiple sclerosis. The immunotolerizing fusion proteins according to the present invention comprise a mutant ADP-ribosylating A1- subunit of the cholera toxin (CTA1), one or more autoantigens or epitopes thereof associated with said diseases, and a peptide capable of binding to a specific cellular receptor. Administration of a therapeutically or prophylactically effective amount of the immunotolerizing fusion protein to a subject elicits suppression of immune response against the antigen/s present in the fusion protein or against other antigen/s associated with the disease, thereby treating or preventing the disease.

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IMMUNOTOLERIZING FUSION PROTEINS FOR TREATMENT OF MULTIPLE SCLEROSIS

FIELD OF THE INVENTION

The present invention relates to new immunotolerizing fusion proteins comprising a mutant of the ADP-ribosylating A1-subunit of the cholera toxin (CTA1), one or more autoantigens associated with an autoimmune demyelinating disease, in particular multiple sclerosis, and a peptide capable of binding to a specific cellular receptor.

BACKGROUND

Autoimmune disease is any disease caused by immune cells that become misdirected at healthy cells and/or tissues of the body. Autoimmune disease affects 3% of the U.S. population, and likely a similar percentage of the industrialized world population (Jacobson et al., Clin Immunol Immunopathol 84: 223-43, 1997). Autoimmune diseases are characterized by T and B lymphocytes that aberrantly target self-proteins, -polypeptides, - peptides, and/or other self-molecules, causing injury and or malfunction of an organ, tissue, or cell-type within the body (for example, pancreas, brain, thyroid or gastrointestinal tract) to cause the clinical manifestations of the disease (Marrack et al., Nat Med 7: 899-905, 2001). Autoimmune diseases include diseases that affect specific tissues, as well as diseases that can affect multiple tissues. For some diseases, this may, in part, depend on whether the autoimmune responses are directed to an antigen confined to a particular tissue, or to an antigen that is widely distributed in the body. The characteristic feature of tissue-specific autoimmunity is the selective targeting of a single tissue or individual cell type. Nevertheless, certain autoimmune diseases that target ubiquitous self-proteins can also affect specific tissues. For example, in polymyositis, the autoimmune response targets the ubiquitous protein histidyl-tRNA synthetase, yet the clinical manifestations primarily involve autoimmune destruction of muscle.

The immune system employs a highly complex mechanism designed to generate responses to protect mammals against a variety of foreign pathogens, while at the same time preventing responses against self-antigens. In addition to deciding whether to respond (antigen specificity), the immune system must also choose appropriate effector functions to deal with each pathogen (effector specificity). A cell critical in mediating and regulating these effector functions is the $CD4^+$ T cell. Furthermore, it is the elaboration of specific cytokines from $CD4^+$ T cells that appears to be the major mechanism by which T cells mediate their functions. Thus, characterizing the types of cytokines made by $CD4^+$ T cells as well as how their secretion is controlled is extremely important in understanding how the immune response is regulated.

The characterization of cytokine production from long-term mouse $CD4^+$ T cell clones was first published more than 20 years ago (Mosmann et al., J Immunol 136: 2348- 2357, 1986). In these studies, it was shown that CD4+ T cells produced two distinct patterns of cytokine production, which were designated T helper 1 (Th1) and T helper 2 (Th2). Th1 cells were found to produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin (LT), while Th2 clones predominantly produced IL-4, IL-5, IL-6, and IL-13 (Cherwinski et al., J Exp Med 169:1229-1244, 1987). Somewhat later, additional cytokines, IL-9 and IL-10, were isolated from Th2 clones (Van Snick et al., J Exp Med 169:363-368, 1989) (Fiorentino et al., J Exp Med 170:2081-2095, 1989). Finally, additional cytokines, such as IL-3, granulocyte macrophage colony-stimulating factor (GM- CSF), and tumor necrosis factor-α (TNF-α) were found to be secreted by both Th1 and Th2 cells. Recently, it was reported that CD4+ T cells isolated from the inflamed joints of patients with Lyme disease contain a subset of IL-17–producing CD4+ T cells that are distinct from Th1 and Th2 (Infante-Duarte et al., J. Immunol 165:6107–6115, 2000). These IL-17–producing CD4+ T cells are designated Th17. IL-17, a proinflammatory cytokine predominantly produced by activated T cells, enhances T cell priming and stimulates fibroblasts, endothelial cells, macrophages, and epithelial cells to produce multiple proinflammatory mediators, including IL-1, IL-6, TNF-α, NOS-2, metalloproteases, and chemokines, resulting in the induction of inflammation. IL-17 expression is increased in patients with a variety of autoimmune diseases, such as multiple sclerosis, suggesting the contribution of IL-17 to the induction and/or development of such diseases.

There is ample evidence showing that suppressor T cells, now called regulatory T cells (Treg cells), suppress autoreactive T cells as an active mechanism for peripheral immune tolerance. Thus far, it is firmly established that Treg cells can be divided into two different subtypes, namely natural (or constitutive) and inducible (or adaptive) populations according to their origins (Mills, Nat Rev Immunol 4:841-855, 2004). In addition, a variety of Treg cell subsets have been identified according to their surface markers or cytokine products, such as CD4+ Treg cells (including natural CD4+CD25+ Treg cells, IL-10-producting Tr1 cells, and TGF-β- producing Th3 cells), CD8+ Treg cells, Veto CD8+ cells, γδ T cells, NKT (NK1.1+CD4-CD8-) cells, NK1.1- CD4-CD8- cells, etc. Accumulating evidence has shown that naturally occurring CD4+CD25+ Treg cells play an active role in down-regulating pathogenic autoimmune responses and in maintaining immune homeostasis (Akbari et al., Curr Opin Immunol 15:627-633, 2003).

Autoimmune disease encompasses a wide spectrum of diseases that can affect many different organs and tissues within the body (see, e.g., Paul, W.E. (1999), Fundamental Immunology, Fourth Edition, Lippincott-Raven, New York.)

Current therapies for human autoimmune disease include glucocorticoids, cytotoxic agents, and recently developed biological therapeutics. In general, the management of human systemic autoimmune disease is empirical and unsatisfactory. For the most part, broadly immunosuppressive drugs, such as corticosteroids, are used in a wide variety of severe autoimmune disorders. In addition to corticosteroids, other immunosuppressive agents are used in management of the systemic autoimmune diseases. Cyclophosphamide is an alkylating agent that causes profound depletion of both T- and B- lymphocytes and impairment of cell-mediated immunity.

Treatments for multiple sclerosis (MS) include interferon β and copolymer 1, which reduce relapse rate by 20-30% and only have a modest impact on disease progression. MS is also treated with immunosuppressive agents including methylprednisolone, other steroids, methotrexate, cladribine and cyclophosphamide. These immunosuppressive agents have minimal efficacy in treating MS. The introduction of the antibody Tysabri (natalizumab), an alpha 4-integrin antagonist, as treatment for MS has been overshadowed by incidences of progressive multifocal leucoencaphalopathy (PML) in patients receiving the therapy.

In the case of organ-specific autoimmunity, a number of different therapeutic approaches have been tried. Soluble protein antigens have been administered systemically to inhibit the subsequent immune response to that antigen. Such therapies include delivery of myelin basic protein, its dominant peptide, or a mixture of myelin proteins to animals with experimental autoimmune encephalomyelitis and humans with multiple sclerosis (Brocke et al., Nature 379: 343-6, 1996; Critchfield et al., Science 263: 1139-43, 1994; Weiner et al., Annu Rev Immunol 12: 809-37, 1994).

Another approach is the attempt to design rational therapeutic strategies for the systemic administration of a peptide antigen based on the specific interaction between the T- cell receptors and peptides bound to MHC molecules. One study using the peptide approach in an animal model of diabetes resulted in the development of antibody production to the peptide (Hurtenbach et al., J Exp Med 177:1499, 1993). Another approach is the administration of T cell receptor (TCR) peptide immunization (see e.g. Vandenbark et al., Nature 341:541, 1989). Still another approach is the induction of oral tolerance by ingestion of peptide or protein antigens (see e.g. Weiner, Immmunol Today 18:335, 1997).

Mucosal tolerance refers to the phenomenon of systemic tolerance to challenge with an antigen that has previously been administered via a mucosal route, usually oral, nasal or naso-respiratory, but also vaginal and rectal (Weiner et al., Annu Rev Immunol 12:809–837, 1994). Mucosal tolerance was discovered early in the 20th century in models of delayed-type and contact hypersensitivity reactions

in guinea pigs, but the mechanisms of tolerance remained ill-defined until the era of modern immunology. The use of cell separation techniques, tests for production of cytokines and transgenic models in which antigen-specific T cells can be tracked *in vivo* have gradually elucidated mechanisms of mucosal tolerance (Garside and Mowat., Crit Rev Immunol 17:119–137, 1997). It has become evident that antigen administration via mucosal routes can result in distinct types of tolerance, depending on the route of administration and dose of antigen. For example, a high dose of oral antigen induces T-cell activation followed by deletion or anergy of responding T cells (Chen et al., Nature 376:177–180, 1995), analogous to parenteral administration of high-dose soluble antigen. This results in extinction of T cells specific to that antigen and unresponsiveness to subsequent antigen challenge, i.e. passive tolerance. In contrast, a low dose of oral antigen does not induce deletion or anergy but, when given repeatedly, induces a distinct type of immune response characterized by the appearance of regulatory-protective T cells, Treg cells, that secrete anti-inflammatory cytokines, i.e. active tolerance (von Herrath, Res Immunol. 148:541–554, 1997). These Treg cells usually belong to the class of CD4 (helper) T cells. Instillation of intact protein antigen onto the nasopharyngeal mucosa also induces Treg cells that are protective. In this case, both CD4 and CD8 T cells may be induced. Regulatory Treg cells induced after oral or intranasal antigen administration produce anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β. To induce mucosal tolerance, antigen can also be given in the form of aerosol. Administration via these three routes, oral, intranasal and aerosol-inhalation, results in antigen uptake and presentation in different lymphoid compartments in each case. Accordingly, oral antigen is presented to T cells mostly in mesenteric lymph nodes and to some extent in Peyer's patches, intranasal antigen in deep cervical lymph nodes and inhaled antigen in mediastinal lymph nodes. Repeated exposure to antigen in each case is able to induce regulatory T cells, but the nature of these cells differs, depending on the route and form of antigen. While regulatory cells induced by oral antigen are CD4 T cells and express T cell receptors (TCR) consisting of αβ heterodimers, in the case of naso-respiratory antigen, the regulatory cells can also be CD8 T cells expressing a γδ heterodimer TCR (i.e. γδ T cells). Some of these cells may also have a CD8 receptor that is an αα homodimer instead of the conventional αβ-heterodimer TCR. A majority of cells that carry the CD8αα and γδ TCR reside in skin or mucosal tissues.

Immune responses are currently altered by delivering polypeptides, alone or in combination with adjuvants (immunomodulating agents). For example, the hepatitis B virus vaccine contains recombinant hepatitis B virus surface antigen, a non-self antigen, formulated in aluminum hydroxide, which serves as an adjuvant. This vaccine induces an immune response against hepatitis B virus surface antigen to protect against infection. An alternative approach involves delivery of an attenuated, replication deficient, and/or non-pathogenic form of a virus or bacterium, each a non-self antigen, to elicit a host protective immune response against the pathogen. For example, the oral polio vaccine is composed of a live attenuated virus, a non-self antigen, which infects cells and replicates in the

vaccinated individual to induce effective immunity against polio virus, a foreign or non-self antigen, without causing clinical disease. Alternatively, the inactivated polio vaccine contains an inactivated or 'killed' virus that is incapable of infecting or replicating and is administered subcutaneously to induce protective immunity against polio virus.

DNA therapies have been described for treatment of autoimmune diseases. Such DNA therapies include DNA molecules encoding the antigen-binding regions of the T cell receptor to alter levels of autoreactive T cells driving the autoimmune response (Waisman et al., Nat Med 2:899-905, 1996; U.S. Patent 5,939,400). DNA molecules encoding autoantigens were attached to particles and delivered by gene gun to the skin to prevent MS and collagen induced arthritis (WO 97/46253; Ramshaw et al., Immunol Cell Biol 75:409-413, 1997). DNA molecules encoding adhesion molecules, cytokines (e.g., TNFα), chemokines (e.g., C-C chemokines), and other immune molecules (e.g., Fas-ligand) have been used for treatment of autoimmune diseases in animal models (Youssef et al., J Clin Invest 106:361-371, 2000; Wildbaum et al., J Clin Invest 106:671-679, 2000; Wildbaum et al., J Immunol 165:5860-5866, 2000).

Methods for treating autoimmune disease by administering a nucleic acid encoding one or more autoantigens are described in WO 00/53019, WO 2003/045316, and WO 2004/047734. While these methods have been successful, further improvements are still needed.

Bacterial enterotoxins are used as immunostimulating adjuvants in vaccines for the prevention of infectious diseases. Cholera toxin (CT) and the closely related E.coli heat-labile toxin (LT) are perhaps the most powerful and best studied mucosal adjuvants in experimental use today (Rappuoli et al., Immunol Today 20:493-500), but when exploited in the clinic, their potential toxicity and association with cases of Bell's palsy (paralysis of the facial nerve) have led to their withdrawal from the market (Gluck et al., J Infect Dis 181: 1129-1132, 2000; Gluck et al., Vaccine 20 (Suppl.1): S42-44, 2001; Mutsch et al., N Engl J Med. 350: 896–903, 2004). The bacterial enterotoxins CT and LT have proven to be effective immunoenhancers in experimental animals as well as in humans (Freytag et al., Curr Top Microbiol Immunol 236: 215–236, 1999). Structurally, these enterotoxins are AB_5 complexes, and consist of one ADP-ribosyltransferase active A1 subunit and an A2 subunit that links the A1 to a pentamer of B subunits. The holotoxins bind to most mammalian cells via the B subunit (CTB), which specifically interacts with the GM1-ganglioside receptor in the cell membrane. Whereas the holotoxins have been found to enhance mucosal immune responses, conjugates between CTB and antigen have been used to specifically tolerize the immune system (Holmgren et al., Am J Trop Med Hyg 50: 42–54, 1994). Studies in mice have shown that CT and LT can accumulate in the olfactory nerve and bulb when given intranasally, a mechanism that is dependent on the ability of the B subunits of CT or LT to bind GM1-ganglioside receptors, present on all nucleated mammalian cells (Fujihashi et al., Vaccine

20: 2431–2438, 2002). Although less toxic mutants of CT and LT have been engineered with substantial adjuvant function, such molecules still carry a significant risk of causing adverse reactions (Giuliani et al., J Exp Med 187: 1123-1132, 1998; Yamamoto et al., J Exp Med 185: 1203–1210, 1997), especially when considering that the adjuvanticity of CT and LT appears to be a combination of the ADP-ribosyltransferase activity of the A subunit and the ability to bind ganglioside receptors on the target cells (Soriani et al., Microbiology 148: 667–676, 2002). These observations and others preclude the use of CT or LT holotoxins in vaccines for humans. On the other hand, recent observations have demonstrated that it is possible to retain adjuvant functions of these molecules with no toxicity or greatly reduced toxicity by introducing site-directed mutations in the gene coding for the A1 subunit. Examples of mutant molecules that have proven to be effective adjuvants are LTK63 and LTR72 (Giuliani et al., J Exp Med 187: 1123-1132, 1998), the former with no enzymatic activity and the latter with significantly reduced ADP-ribosylating ability. Notwithstanding this, the GM1-ganglioside receptor–dependent binding remains a problem in these mutants, and may therefore still cause nerve cell accumulation and neurotoxicity.

A better solution to this dilemma of efficacy versus toxicity is the CTA1-DD molecule that has proven to be a highly effective mucosal and systemic adjuvant (Ågren et al., J Immunol 158: 3936–3946, 1997; US 5,917,026). This unique adjuvant is based on the enzymatically active A1-subunit of CT, combined with a dimer of an immunoglobulin-binding element from Staphylococcus aureus protein A. The molecule thereby avoids binding to all nucleated cells, which could result in unwanted reactions, and exploits fully the CTA1-enzyme in the holotoxin. Accordingly, all studies to date have found that CTA1-DD is nontoxic and has retained excellent immunoenhancing functions. When given systemically, CTA1-DD provides comparable adjuvant effect to that of intact CT, greatly augmenting both cellular and humoral immunity against specific immunogens coadministered with the adjuvant. It also functions as a mucosal adjuvant and should be safe, as it is devoid of the B subunit that is a prerequisite of CT holotoxin toxicity. CTA1-DD cannot bind to ganglioside receptors; rather, it targets B cells, limiting the CTA1-DD adjuvant to a restricted repertoire of cells that it can interact with. However, the adjuvant effect is not completely dependent on B cells, as been shown in strong induction of specific CD4 T cell immunity following intranasal immunizations using the CTA1-DD adjuvant in B-cell deficient mice (Eliasson et al., Vaccine 25: 1243-52, 2008, Akhiani et al., Scand J. Immunol 63: 97-105,2006: Lycke, Annals of the New Youk Academy of Science, vol. 29, no. 1, p.193-208, 2004).

The adjuvant effect of CTA1-DD was absent in mutants CTA1(E112K)-DD and CTA1(R7K)-DD, which lack the ADP-ribosylating enzymatic activity (Lycke, Immunol Lett 97: 193-198, 2005).

WO 2009/078796 further describes immunomodulating complexes comprising the mutant CTA1(R7K)-DD, and more specifically the immunomodulating complexes comprising CTA1(R7K)-DD linked to the shared immunodominant collagen II peptide comprising amino acids 260-273 (CII260-273) (Hasselberg et al., The Journal of Immunolgy, vol. 184, no. 6, p. 2776-2784, 2010).

A conjugate of CTB and a peptide derived from bovine collagen II has been shown to be able to protect mice from developing collagen induced autoimmune ear disease as well as collagen-induced arthritis (Kim et al., Ann Otol Rhinol Laryngol 110: 646-654, 2001; Tarkowski et al., Arthritis Rheum 42: 1628-34, 1999). However, CTB may not be suited for human use due to its GM1-ganglioside-binding properties and potential neurotoxic effects, as discussed above.

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to new immunotolerizing fusion proteins and pharmaceutical compositions comprising them, as well as uses thereof for the prevention or treatment of autoimmune demyelinating diseases, in particular multiple sclerosis. The immunotolerizing fusion proteins according to the present invention comprise a mutant ADP-ribosylating A1-subunit of the cholera toxin (CTA1), one or more autoantigens associated with said diseases, and a peptide capable of binding to a specific cellular receptor. Administration of a therapeutically or prophylactically effective amount of the immunotolerizing fusion protein to a subject elicits suppression of an immune response against an antigen associated with the disease, thereby treating or preventing the disease.

The present invention provides an immunotolerizing fusion protein comprising:
(a) a mutant ADP-ribosylating A1-subunit of the cholera toxin (CTA1), wherein the mutant CTA1 subunit is the K-CTA1(R7K/C187A) mutant of SEQ ID NO:1,
(b) one or more autoantigens or epitopes thereof associated with an autoimmune demyelinating disease, and
(c) a peptide capable of binding to a specific cellular receptor, wherein the peptide specifically binds to a receptor expressed on an antigen presenting cell selected from the group consisting of lymphocytes, monocytes, macrophages, dendritic cells, and Langerhans cells.

Examples of autoimmune demyelinating diseases include multiple sclerosis and neuromyelitis optica (NMO, also known as Devic's disease or Devic's syndrome), optic neuritis, Guillain-Barré syndrome and Anti-MAG peripheral neuropathy.

Preferably, the one or more autoantigens or epitopes thereof are associated with multiple sclerosis (MS).

In one embodiment of the present invention, the one or more autoantigens are selected from the group of antigens consisting of myelin antigens, glial antigens, and neuronal antigens. Preferably, the one or more autoantigens are myelin antigens.

In one particular embodiment of the present invention, the myelin antigens are selected from the group of antigens consisting of antigens from myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated oligodendrocytic basic protein (MOBP), myelin-associated glycoprotein (MAG), oligodendrocyte-specific protein (OSP), myelin-associated neurite outgrowth inhibitor Nogo-A, and cyclic nucleotide phosphodiesterase (CNPase).

The present invention further relates to an isolated nucleic acid encoding an immunotolerizing fusion protein in accordance with the present invention. The nucleic acids of the invention can be DNA or RNA.

The present invention also relates to a pharmaceutical composition comprising one or more immunotolerizing fusion proteins or an isolated nucleic acid encoding such immunotolerizing fusion proteins in accordance with the present invention, and their use in the prevention or treatment of an autoimmune demyelinating disease, such as multiple sclerosis or neuromyelitis optica.

In one embodiment of the present invention, the pharmaceutical composition is made suitable for mucosal delivery, preferably intranasal delivery.

The present invention also relates to the use of an immunotolerizing fusion protein or a pharmaceutical composition in accordance with the present invention for the prevention or treatment of an autoimmune demyelinating disease, preferably multiple sclerosis.

In one embodiment of the present invention, a method is provided for the prevention or treatment, of multiple sclerosis (MS) comprising administering to a subject an immunotolerizing fusion protein in accordance with the present invention comprising one or more autoantigens or epitopes thereof associated with MS.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention belongs. As used

herein, the following terms and phrases have the meanings ascribed to them unless specified otherwise.

The amino acid sequence of the ADP-ribosylating A1-subunit of the cholera toxin (CTA1) can be found e.g. in GenBank Accesion Nos. AAM22586.1, ADG44926.1, AAM74170.1, CAE11218.1, or AAA27514.1. The term “a subunit of the ADP-ribosylating A1-subunit of the cholera toxin (CTA1)” refers to a polypeptide comprising at least a sequence corresponding to the sequence from amino acid 7, lysine, to amino acid 187, cysteine, of the sequence of the mature ADP-ribosylating A1-subunit of the cholera toxin (CTA1), such as a polypeptide comprising at least a sequence corresponding to the sequence from amino acid 1, aspargine, to amino acid 187, cysteine, of the sequence of the mature ADP-ribosylating A1-subunit of the cholera toxin (CTA1), or at least a sequence corresponding to the sequence from amino acid 1, aspargine, to amino acid 194, serine, of the sequence of the mature ADP-ribosylating A1-subunit of the cholera toxin (CTA1).

The terms "polynucleotide" and "nucleic acid" refer to a polymer composed of a multiplicity of nucleotide units (ribonucleotide or deoxyribonucleotide or related structural variants) linked via phosphodiester bonds. A polynucleotide or nucleic acid can be of substantially any length, typically from about six (6) nucleotides to about 10^9 nucleotides or larger. Polynucleotides and nucleic acids include RNA, DNA, synthetic forms, and mixed polymers, both sense and antisense strands, double- or single-stranded, and can also be chemically or biochemically modified or can contain non-natural or derivatized nucleotide bases, as will be readily appreciated by the skilled artisan.

"Antigen," as used herein, refers to any molecule that can be recognized by the immune system that is by B cells or T cells, or both.

"Autoantigen," as used herein, refers to an endogenous molecule, typically a polysaccharide or a protein or fragment thereof, that elicits a pathogenic immune response. Autoantigen includes glycosylated proteins and peptides as well as proteins and peptides carrying other forms of post-translational modifications, including citrullinated peptides. When referring to the autoantigen or epitope thereof as "associated with an autoimmune demyelinating disease," it is understood to mean that the autoantigen or epitope is involved in the pathophysiology of the disease either by inducing the pathophysiology (i.e., associated with the etiology of the disease), mediating or facilitating a pathophysiologic process; and/or by being the target of a pathophysiologic process. For example, in autoimmune disease, the immune system aberrantly targets autoantigens, causing damage and dysfunction of cells and tissues in which the autoantigen is expressed and/or present. Under normal physiological conditions, autoantigens are ignored by the host immune system through the elimination,

inactivation, or lack of activation of immune cells that have the capacity to recognize the autoantigen through a process designated "immune tolerance."

As used herein the term "epitope" is understood to mean a portion of a polysaccharide or polypeptide having a particular shape or structure that is recognized by either B-cells or T-cells of the animal's immune system. An epitope can include portions of both a polysaccharide and a polypeptide, e.g. a glycosylated peptide.

"Autoantigenic epitope" refers to an epitope of an autoantigen that elicits a pathogenic immune response.

The terms "polypeptide", "peptide", and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers.

"Self-protein", "self-polypeptide", or self-peptide" are used herein interchangeably and refer to any protein, polypeptide, or peptide, or fragment or derivative thereof that: is encoded within the genome of the animal; is produced or generated in the animal; may be modified posttranslationally at some time during the life of the animal; and, is present in the animal non-physiologically. The term "non-physiological" or "non-physiologically" when used to describe the self-protein(s), -polypeptide(s), or -peptide(s) of this invention means a departure or deviation from the normal role or process in the animal for that self-protein, - polypeptide, or -peptide. When referring to the self-protein, -polypeptide or -peptide as "associated with a disease" or "involved in a disease" it is understood to mean that the self- protein, -polypeptide, or -peptide may be modified in form or structure and thus be unable to perform its physiological role or process or may be involved in the pathophysiology of the condition or disease either by inducing the pathophysiology; mediating or facilitating a pathophysiologic process; and/or by being the target of a pathophysiologic process. For example, in autoimmune disease, the immune system aberrantly attacks self-proteins causing damage and dysfunction of cells and tissues in which the self-protein is expressed and/or present. Alternatively, the self-protein, -polypeptide or -peptide can itself be expressed at non-physiological levels and/or function non-physiologically. Examples of posttranslational modifications of self-protein(s), - polypeptide(s) or -peptide(s) are glycosylation, addition of lipid groups, reversible phosphorylation, addition of dimethylarginine residues, citrullination, and proteolysis, and more specifically citrullination of MBP. Immunologically, self-protein, -polypeptide or -peptide would all be considered host self-antigens and under normal physiological conditions are ignored by the host immune system through the elimination, inactivation, or lack of activation of immune cells that have the capacity to recognize self-

antigens through a process designated "immune tolerance". The immune system is the defence mechanism that provides the means to make rapid, highly specific, and protective responses against the myriad of potentially pathogenic microorganisms inhabiting the animal's world. Examples of immune protein(s), polypeptide(s) or peptide(s) are proteins comprising the T-cell receptor, immunoglobulins, cytokines, including the type I interleukins, and the type II cytokines, including the interferons and IL-10, TNF, lymphotoxin, and the chemokines, such as macrophage inflammatory protein -1 alpha and beta, monocyte-chemotactic protein and RANTES, and other molecules directly involved in immune function, such as Fas-ligand. There are certain immune protein(s), polypeptide(s) or peptide(s) that are included in the self-protein, -polypeptide or -peptide of the invention and they are: class I MHC membrane glycoproteins, class II MHC glycoproteins and osteopontin.

"Modulation of", "modulating", or "altering an immune response" as used herein refers to any alteration of an existing or potential immune responses against an autoimmune or allergy provoking epitope, including, e.g., nucleic acids, lipids, phospholipids, carbohydrates, self-polypeptides, protein complexes, or ribonucleoprotein complexes, that occurs as a result of administration of an immunomodulating complex or polynucleotide encoding an immunomodulating complex. Such modulation includes any alteration in presence, capacity, or function of any immune cell involved in, or capable of being involved in, an immune response. Immune cells include B cells, T cells, NK cells, NK T cells, professional antigen-presenting cells, non-professional antigen-presenting cells, inflammatory cells, or any other cell capable of being involved in or influencing an immune response. "Modulation" includes any change imparted on an existing immune response, a developing immune response, a potential immune response, or the capacity to induce, regulate, influence, or respond to an immune response. Modulation includes any alteration in the expression and/or function of genes, proteins and/or other molecules in immune cells as part of an immune response.

"Modulation of an immune response" includes, for example, the following: elimination, deletion, or sequestration of immune cells; induction or generation of immune cells that can modulate the functional capacity of other cells such as autoreactive lymphocytes, antigen presenting cells, or inflammatory cells; induction of an unresponsive state in immune cells (i.e., anergy); increasing, decreasing, or changing the activity or function of immune cells or the capacity to do so, including, but not limited to, altering the pattern of proteins expressed by these cells. Examples include altered production and/or secretion of certain classes of molecules such as cytokines, chemokines, growth factors, transcription factors, kinases, costimulatory molecules, or other cell surface receptors; or any combination of these modulatory events.

For example, administration of an immunomodulating complex or a polynucleotide encoding an immunomodulating complex can modulate an immune response by eliminating, sequestering, or

inactivating immune cells mediating or capable of mediating an undesired immune response; inducing, generating, or turning on immune cells that mediate or are capable of mediating a protective immune response; changing the physical or functional properties of immune cells; or a combination of these effects. Examples of measurements of the modulation of an immune response include, but are not limited to, examination of the presence or absence of immune cell populations (using flow cytometry, immunohistochemistry, histology, electron microscopy, polymerase chain reaction (PCR)); measurement of the functional capacity of immune cells, including ability or resistance to proliferate or divide in response to a signal (such as using T cell proliferation assays and pepscan analysis based on ^{3}H-thymidine incorporation following stimulation with anti-CD3 antibody, anti-T cell receptor antibody, anti-CD28 antibody, calcium ionophores, PMA, antigen presenting cells loaded with a peptide or protein antigen; B cell proliferation assays); measurement of the ability to kill or lyse other cells (such as cytotoxic T cell assays); measurements of the cytokines, chemokines, cell surface molecules, antibodies and other products of the cells (e.g., by flow cytometry, enzyme-linked immunosorbent assays, Western blot analysis, protein microarray analysis, immunoprecipitation analysis); measurement of biochemical markers of activation of immune cells or signaling pathways within immune cells (e.g., Western blot and immunoprecipitation analysis of tyrosine, serine or threonine phosphorylation, polypeptide cleavage, and formation or dissociation of protein complexes; protein array analysis; DNA transcriptional, profiling using DNA arrays or subtractive hybridization); measurements of cell death by apoptosis, necrosis, or other mechanisms (e.g., annexin V staining, TUNEL assays, gel electrophoresis to measure DNA laddering, histology; fluorogenic caspase assays, Western blot analysis of caspase substrates); measurement of the genes, proteins, and other molecules produced by immune cells (e.g., Northern blot analysis, polymerase chain reaction, DNA microarrays, protein microarrays, 2- dimentional gel electrophoresis, Western blot analysis, enzyme linked immunosorbent assays, flow cytometry); and measurement of clinical symptoms or outcomes, such as improvement of autoimmune, neurodegenerative, and other diseases involving self proteins or self polypeptides (clinical scores, requirements for use of additional therapies, functional status, imaging studies) for example, by measuring relapse rate or disease severity (using clinical scores known to the ordinarily skilled artisan) in the case of multiple sclerosis, measuring blood glucose in the case of type I diabetes, or joint inflammation in the case of rheumatoid arthritis.

”Immunotolerizing” shall mean ”immunomodulating” in which pro-inflammatory immune responses - that are elicited by autoantigens and that are targeting autoantigens - are downregulated. This downregulation may be accompanied by upregulation of regulatory T cells. The downregulation would suppress adaptive immunity (T and B effector cells) and innate immunity (macrophages, dendritic cells, neutrophils).

"Subjects" shall mean any animal, such as, for example, a human, non-human primate, horse, cow, dog, cat, mouse, rat, guinea pig or rabbit.

"Treating", "treatment", or "therapy" of a disease or disorder shall mean slowing, stopping or reversing the disease's progression, as evidenced by decreasing, cessation or elimination of either clinical or diagnostic symptoms, by administration of an immunotolerizing fusion protein or a polynucleotide encoding an immunotolerizing fusion protein, either alone or in combination with another compound as described herein. "Treating", "treatment", or "therapy" also means a decrease in the severity of symptoms in an acute or chronic disease or disorder or a decrease in the relapse rate as, for example, in the case of a relapsing or remitting autoimmune disease course or a decrease in inflammation in the case of an inflammatory aspect of an autoimmune disease. In the preferred embodiment, treating a disease means reversing or stopping or mitigating the disease's progression, ideally to the point of eliminating the disease itself. As used herein, ameliorating a disease and treating a disease are equivalent.

"Preventing", "prophylaxis", or "prevention" of a disease or disorder as used in the context of this invention refers to the administration of an immunotolerizing fusion protein or a polynucleotide encoding an immunotolerizing fusion protein, either alone or in combination with another compound as described herein, to prevent the occurrence or onset of a disease or disorder or some or all of the symptoms of a disease or disorder or to lessen the likelihood of the onset of a disease or disorder.

A "therapeutically or prophylactically effective amount" of an immunotolerizing fusion protein refers to an amount of the immunotolerizing fusion protein that is sufficient to treat or prevent the disease as, for example, by ameliorating or eliminating symptoms and/or the cause of the disease. For example, therapeutically effective amounts fall within broad range(s) and are determined through clinical trials, and for a particular patient is determined based upon factors known to the skilled clinician, including, e.g., the severity of the disease, weight of the patient, age, and other factors.

“Autoimmune demyelinating disease” as used herein refers to any autoimmune condition that results in damage to the myelin sheath surrounding nerve fibers, whether in relation to the central nervous system or in relation to the peripheral nervous system.

Thus, in particular “autoimmune demyelinating diseases” of the central nervous system would include, but are not limited to Multiple sclerosis, Neuromyelitis Optica (NMO, also known as Devic's disease or Devic's syndrome) and Optic neuritis. “Autoimmune demyelinating diseases” of the peripheral nervous system would include, but are not limited to Guillain-Barré syndrome and Anti-MAG peripheral neuropathy.

DESCRIPTION OF THE DRAWINGS

Figure 1. DNA construct encoding the immunotolerizing fusion protein K-CTA1(R7K/C187A)-MOG(35-55)-DD

Figure 2. DNA construct encoding the immunotolerizing fusion protein K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD

Figure 3. DNA construct encoding the immunotolerizing fusion protein K-CTA1(R7K/C187A)-PLP(178-191)-DD

Figure 4. DNA construct encoding the immunotolerizing fusion protein K-CTA1(R7K/C187A)-human MOG(10-60)-DD

Figure 5. Comparison of the therapeutic effects of CTA1(R7K)-MOG(35-55)-DD and K-CTA1-(R7K/C187A)-MOG(35-55)-DD in the mouse EAE model.

Figure 6. Therapeutic effects of K-CTA1(R7K/C187A)-MOG(35-55)-DD and K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD in the mouse EAE model.

Figure 7. The therapeutic effect of K-CTA1(R7K/C187A)-MOG(35-55)-DD is peptide specific in the mouse EAE model.

Figure 8. The therapeutic effect of K-CTA1(R7K/C187A)-MOG(35-55)-DD on the incidence of disease is peptide specific in the mouse EAE model.

Figure 9. The therapeutic effects of K-CTA1(R7K/C187A)-MOG(35-55)-DD and K-CTA1(R7K/C187A)-PLP(178-191)-DD in the mouse EAE model.

Figure 10. The therapeutic effects of K-CTA1(R7K/C187A)-MOG(35-55)-DD, K-CTA1(R7K/C187A)-PLP(178-191)-DD, K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD and K-CTA1(R7K/C187A)-human MOG(10-60)-DD in the mouse EAE model.

SEQUENCE LISTING

SEQ ID NO:1 K-CTA1(R7K/C187A) mutant
SEQ ID NO:2 K-CTA1(R7K/C187A)-MOG(35-55)-DD fusion protein
SEQ ID NO:3 K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD fusion protein
SEQ ID NO:4 K-CTA1(R7K/C187A)-PLP(178-191)-DD fusion protein
SEQID NO:5 K-CTA1(R7K/C187A)-human MOG(10-60)-DD fusion protein

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to new immunotolerizing fusion proteins and pharmaceutical compositions comprising them, as well as uses thereof for the prevention or treatment of autoimmune demyelinating diseases, in particular multiple sclerosis. The immunotolerizing fusion proteins according to the present invention comprise a mutant ADP-ribosylating A1-subunit of the cholera toxin (CTA1), one or more autoantigens or epitopes thereof associated with said diseases, and a peptide capable of binding to a specific cellular receptor. Administration of a therapeutically or prophylactically effective amount of the immunotolerizing fusion protein to a subject elicits suppression of an immune response against an antigen associated with the disease, thereby treating or preventing the disease.

The present invention provides an immunotolerizing fusion protein comprising:
(a) a mutant ADP-ribosylating A1-subunit of the cholera toxin (CTA1), wherein the mutant CTA1 subunit is the K-CTA1(R7K/C187A) mutant of SEQ ID NO:1,
(b) one or more autoantigens or epitopes thereof associated with an autoimmune demyelinating disease, and
(c) a peptide capable of binding to a specific cellular receptor, wherein the peptide specifically binds to a receptor expressed on an antigen presenting cell selected from the group consisting of lymphocytes, monocytes, macrophages, dendritic cells, and Langerhans cells.

In said mutant subunit of SEQ ID NO:1, the amino acid lysine (K) has been added to the N-terminus of CTA1(1-194), amino acid 7, arginine (R), has been replaced by lysine (K), and amino acid 187 cysteine (C), has been replaced by alanine (A), and in the context of the present invention this sequence is abbreviated as K-CTA1(R7K/C187A).

The replacement of amino acid 7, arginine, by lysine abolishes the ADP-ribosylating activity, replacement of amino acid 187, cysteine, by alanine prevents the formation of dimers, and the addition of a lysine to the N-terminus of CTA1(1-194) drastically increases the expression and production of

the fusion protein. The inventors of the present invention further have surprisingly found that replacement of amino acid 187, cysteine, by alanine provides a significantly improved therapeutic effect in experimental autoimmune encephalomyelitis (EAE) in mice.

Thus, the fusion proteins according to the present invention comprising K-CTA1(R7K/C187A) provide surprising and advantageous effects as compared to CTA1(R7K)-containing fusion proteins according to the prior art WO 2009/078796. Notably, the therapeutic effect of K-CTA1(R7K/C187A)-MOG(35-55)-DD has surprisingly been found to be significantly better than the therapeutic effect of CTA1(R7K)-MOG(35-55)-DD, as shown in the Examples below. In another EAE experiment, shown in the examples below, it was shown that the therapeutic effect of the fusion protein is peptide specific since no effect on either disease score or incidence of disease was found when the mice were treated with the empty vector alone, without an inserted disease specific peptide, that is K-CTA1R7K(C187A)-DD. Interestingly it was also found that K-CTA1(R7K/C187A)-MOG(35-55)-DD can effectively and significantly reduce disease scores in an EAE model induced by another epitope, PLP(178-191), indicating that the MOG(35-55) epitope is central to the EAE disease and may thus be central to MS in humans suggesting that K-CTA1(R7K/C187A)-MOG(35-55)-DD may be effective in therapeutically treating MS in human subjects.
Also shown in the examples below, in yet another variant of the EAE model in C57Bl/6 mice where disease was induced by a combination of two myelin antigens involved in human MS, that is MOG(35-55) together with PLP(178-191), it was shown that a fusion protein containing a combination of the two epitopes, K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD, used for disease induction was significantly better than the fusion proteins containing the two different epitopes alone in lowering disease scores suggesting that the combination of several myelin epitopes inserted into the fusion proteins might have a better therapeutic effect in MS in humans where several autoantigens are involved in the disease.

Examples of autoimmune demyelinating diseases include multiple sclerosis and neuromyelitis optica (NMO, also known as Devic's disease or Devic's syndrome), optic neuritis, Guillain-Barré syndrome and Anti-MAG peripheral neuropathy.

Preferably, the one or more autoantigens or epitopes thereof are associated with multiple sclerosis (MS).

Multiple sclerosis (MS) is the most common demyelinating disorder of the CNS and affects 350,000 Americans and one million people worldwide. Onset of symptoms typically occurs between 20 and 40 years of age and manifests as an acute or sub-acute attack of unilateral visual impairment, muscle weakness, paresthesias, ataxia, vertigo, urinary incontinence, dysarthria, or mental disturbance (in

order of decreasing frequency). Such symptoms result from focal lesions of demyelination which cause both negative conduction abnormalities due to slowed axonal conduction, and positive conduction abnormalities due to ectopic impulse generation (e.g., Lhermitte's symptom). Diagnosis of MS is based upon a history including at least two distinct attacks of neurologic dysfunction that are separated in time, produce objective clinical evidence of neurologic dysfunction, and involve separate areas of the CNS white matter. Laboratory studies providing additional objective evidence supporting the diagnosis of MS include magnetic resonance imaging (MRI) of CNS white matter lesions, cerebral spinal fluid (CSF) oligoclonal banding of IgG, and abnormal evoked responses. Although most patients experience a gradually progressive relapsing remitting disease course, the clinical course of MS varies greatly between individuals and can range from being limited to several mild attacks over a lifetime to fulminant chronic progressive disease. A quantitative increase in myelin- autoreactive T cells with the capacity to secrete IFN- gamma is associated with the pathogenesis of MS and EAE.

The autoantigen targets of the autoimmune response in autoimmune demyelinating diseases, such as multiple sclerosis and experimental autoimmune encephalomyelitis (EAE), may comprise epitopes from proteolipid protein (PLP); myelin basic protein (MBP); myelin oligodendrocyte glycoprotein (MOG); cyclic nucleotide phosphodiesterase (CNPase); myelin-associated glycoprotein (MAG) and myelin-associated oligodendrocytic basic protein (MBOP); alpha-B-crystalin (a heat shock protein); viral and bacterial mimicry peptides, e.g. , influenza, herpes viruses, hepatitis B virus, etc.; OSP (oligodendrocyte specific-protein); citrulline-modified MBP (the C8 isoform of MBP in which 6 arginines have been de-imminated to citrulline), etc. The integral membrane protein PLP is a dominant autoantigen of myelin. Determinants of PLP antigenicity have been identified in several mouse strains, and include residues 139-151, 103-116, 215-232, 43-64 and 178-191. At least 26 MBP epitopes have been reported (Meinl et al., J Clin Invest 92, 2633-43, 1993). Notable are residues 1-11, 59-76 and 87-99. Immunodominant MOG epitopes that have been identified in several mouse strains include residues 1-22, 35-55, 64-96.

Experimental autoimmune encephalomyelitis (EAE) in the common marmoset *(Callithrix jacchus)* is a non-human primate model for multiple sclerosis (MS) in humans. The disease course is characterized by lesions with inflammation and demyelination within the central nervous system (CNS) white and grey matter. This new and highly refined model shares essential clinical, radiological and pathological similarities with human MS. T-cells specific for MOG34-56 have been implicated in the disease progression to clinically evident EAE (Jagessar et al., J Neuropathol Exp Neurol 69:372-385, 2010; Kap et al., J Immunol 180:1326-1337, 2008). These T-cells have an effector memory phenotype and cytotoxic function triggered by MOG40-48 epitope presented by Caja-E molecules (Jagessar et al., Eur J Immunol 42:217-227, 2012).

In human MS patients, the following myelin proteins and epitopes were identified as targets of the autoimmune T and B cell response. Antibody eluted from MS brain plaques recognized myelin basic protein (MBP) peptide 83-97 (Wucherpfennig et al., J Clin Invest 100:1114-1122, 1997). Another study found approximately 50% of MS patients having peripheral blood lymphocyte (PBL) T cell reactivity against myelin oligodendrocyte glycoprotein (MOG) (6-10% control), 20% reactive against MBP (8-12% control), 8% reactive against PLP (0% control), 0% reactive against MAG (0% control). In this study, 7 of 10 MOG reactive patients had T cell proliferative responses focused on one of 3 peptide epitopes, including MOG 1-22, MOG 34-56, MOG 64-96 (Kerlero de Rosbo et al., Eur J Immunol 27: 3059-69, 1997). T and B cell (brain lesion-eluted Ab) response focused on MBP 87-99 (Oksenberg et al., Nature 362: 68-70, 1993). In MBP 87-99, the amino acid motif HFFK is a dominant target of both the T and B cell response (Wucherpfennig et al., J Clin Invest 100: 1114-22, 1997). Another study observed lymphocyte reactivity against myelin-associated oligodendrocytic basic protein (MOBP), including residues MOBP 21-39 and MOBP 37-60 (Holz et al., J Immunol 164: 1103-9, 2000). Using immunogold conjugates of MOG and MBP peptides to stain MS and control brains, both MBP and MOG peptides were recognized by MS plaque-bound Abs (Genain and Hauser, Methods 10: 420-34, 1996).

The shared immunodominant epitope may be selected from any suitable autoantigen known to be associated with an autoimmune demyelinating disease. The epitope may, for instance, be selected from any of the autoantigens associated with the disease. However, epitopes with a high content of cysteine may counteract the advantageous effect provided by the replacement of amino acid 187, cysteine, by an alanine in K-CTA1(R7K/C187A) of the immunotolerizing fusion protein according to the present invention as compared to CTA1(R7K). Therefore, it is preferable that epitopes according to the present invention are choosen in such a way as to avoid high contents of cysteine.

In the context of the present invention, autoantigens and epitopes thereof associated with an autoimmune demyelinating disease, in particular multiple sclerosis, can be found using antigen microarrays as described by Quintana et al. in Proc Natl Acad Sci USA 105: 18889-18894, 2008.

In one embodiment of the present invention, the one or more autoantigens are selected from the group of antigens consisting of myelin antigens (MBP, PLP, MOG, MOBP, MAG, OSP, Nogo-A, CNPasw), glial antigens (GFAP, S100β, αβ-crystallin), and neuronal antigens (Neurofilament-L, Neurofilament-M, β-Synuclein, Contactin-2, Neurofascin), as described by Krishnamoorthy and Wekerle in Eur J Immunol 39: 1991-2058, 2009. Preferably, the one or more autoantigens are myelin antigens. Autoantigens or epitopes thereof of different species, including mice, rats, primates, and humans can be used in accordance with the present invention. Preferably, human autoantigens or epitopes thereof

are used in accordance with the present invention. A description of suitable, including human, autoantigens/epitopes can be found, for example, in the articles by Krishnamoorthy and Wekerle in Eur J Immunol 39: 1991-2058, 2009, and Kaushansky et al. in PLoS ONE 6(11): e27860 (November 2011).

In one particular embodiment of the present invention, the myelin antigens are selected from the group of antigens consisting of antigens from myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated oligodendrocytic basic protein (MOBP), myelin-associated glycoprotein (MAG), oligodendrocyte-specific protein (OSP), myelin-associated neurite outgrowth inhibitor Nogo-A, and cyclic nucleotide phosphodiesterase (CNPase).

In another embodiment of the present invention, the myelin antigens are selected from the group of antigens consisting of antigens from myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated oligodendrocytic basic protein (MOBP), myelin-associated glycoprotein (MAG), and oligodendrocyte-specific protein (OSP).

In a particular embodiment in accordance with the present invention, the myelin antigens are selected from the group of autoantigenic epitopes consisting of PLP(41-60), PLP(43-64), PLP(45-53), PLP(56-70), PLP(103-116), PLP(104-117), PLP(139-151), PLP(175-194), PLP(178-191), PLP(215-232), PLP(215-235), PLP(258-276), MBP(1-11), MBP(11-30), MBP(21-35), MBP(29-84), MBP(59-76), MBP(61-82), MBP(80-105), MBP(84-102), MBP(85-99), MBP(87-99), MBP(89-101), MBP(89-104), MBP(147-162), MBP(170-186), MAG(97-112), MOG(1-22), MOG(10-60), MOG(14-36), MOG(24-36), MOG(34-56), MOG(35-55), MOG(40-48), MOG(43-57), MOG(64-96), MOG(92-106), MOG(94-116), MOG(134-148), MOG(202-218), MOBP(15-36), MOBP(37-60), MOBP(55-77), MOBP(158-181), OSP(22-46), OSP(55-71), OSP(55-74), OSP(55-80), OSP(57-72), OSP(103-123), OSP(142-161), OSP(179-201), and OSP(179-207).

These epitopes and their amino acid sequences are known in the art and have been described, e.g., by Krishnamoorthy and Wekerle in Eur J Immunol 39: 1991-2058, 2009, and Kaushansky et al. in PLoS ONE 6(11): e27860 (November 2011), and in references cited therein. As can be seen from the above selection of epitopes, the epitope amino acid sequences vary in length and sometimes overlap. In any case, these amino acid sequences contain one or more autoantigenic epitopes, of which the exact amino acid sequence may not always be known. In the context of the present invention, peptides comprising one or more of the above epitope amino acid sequences can be used in accordance with the present invention. Typically, the length of the peptides does not matter as long as it contains one or more autoantigenic epitope amino acid sequences. On the basis of the disclosure in the present specification, the person skilled in the art of MS-related autoantigens is able to select one or more

suitable, preferably human, epitopes for incorporation into one or more immunotolerizing fusion proteins to be used in accordance with the present invention.

In a preferred embodiment in accordance with the present invention, the myelin antigens are selected from the group of autoantigenic epitopes consisting of hPLP(45-53), hPLP(139-151), hPLP(175-194), hMBP(84-102), hMBP(85-99), hMBP(89-104), hMOG(10-60), hMOG(34-56), hMOBP(15-36), hMOBP(55-77), hOSP(55-74), and hOSP(55-80).

In another embodiment, the immunotolerizing fusion protein in accordance with the present invention is selected from the group consisting of K-CTA1-R7K/C187A-MOG(35-55)-DD (SEQ ID NO:2), K-CTA1-R7K/C187A-MOG(35-55)-PLP(178-191)-DD (SEQ ID NO:3), K-CTA1-R7K/C187A-PLP(178-191)-DD (SEQ ID NO:4) and K-CTA1(R7K/C187A)-human MOG(10-60)-DD.

In accordance with the present invention, the fusion protein comprises a peptide that specifically binds to a receptor expressed on a cell capable of antigen presentation, especially cells expressing MHC class I or MHC class II antigen. The antigen presenting cell is selected from the group consisting of lymphocytes, such as B-lymphocytes, monocytes, macrophages, dendritic cells, and Langerhans cells.

The peptide is a peptide that binds to receptors of the above cells, preferably to an Ig or Fc receptor expressed by said antigen presenting cell and most preferably to receptors of B-lymphocytes and dendritic cells.

According to a particularly preferred embodiment of the present invention, said peptide is constituted by protein A or a fragment thereof in single or multiple copies, such as one or more D subunits thereof. According to another particularly preferred embodiment of the invention, said peptide is constituted by an antibody fragment, such as a single chain antibody fragment, that specifically binds to a receptor expressed on a cell capable of antigen presentation.

Examples of specific peptides are peptides capable of binding to receptors such as:

(i) different isotypes of the Ig heavy chain constant regions which interact with a number of high or low affinity Fc receptors present on mast cells, basophils, eosinophils, platelets, dendritic cells, macrophages, NK cells and B cells,

(ii) complement receptors (CRs), CR1, CR2 and CR3, expressed on B-cells and follicular dendritic cells have been shown to be important in the generation of normal humoral immune responses, and they likely also participate in the development of autoimmunity,

(iii) C-type lectin receptors (CLRs), like the Dectin-1 expressed on dendritic cells,

(iv) DEC205, an endocytic receptor for antigen uptake and processing expressed at high levels on a subset of dendritic cells,

(v) CD11c, a cell surface receptor for numerous soluble factors and proteins (LPS, fibrinogen, iC3b) found primarily on myeloid cells,

(vi) the mannose receptor, present on dendritic cells, macrophages and other antigen presenting cells,

(vii) the specific HSP60 receptor, present on macrophages,

(viii) CD103, an integrin alpha chain expressed by a subset of dendritic cells, and

(ix) the 33D1 antigen, present on dendritic cells.

The peptide is preferably such that the resulting fusion protein is in possession of water solubility and capability of targeting the fusion protein to a specific cell receptor different from receptors binding to the native toxin, thereby mediating intracellular uptake of at least said subunit.

The present invention further relates to an isolated nucleic acid encoding an immunotolerizing fusion protein in accordance with the present invention. The nucleic acids of the invention can be DNA or RNA.

In one embodiment, the present invention provides recombinant plasmids, vectors and expression systems comprising a nucleic acid according to the invention. The recombinant expression systems can be adapted for eukaryotic or bacterial expression. The invention further provides transformed cells containing a plasmid, vector or an expression system according to the invention. The transformed cells can be transformed eukaryotic or bacterial cells.

The present invention also relates to a pharmaceutical composition comprising one or more immunotolerizing fusion proteins or an isolated nucleic acid encoding such immunotolerizing fusion proteins in accordance with the present invention, and their use in the prevention or treatment of an autoimmune demyelinating disease, in particular multiple sclerosis.

In one embodiment of the present invention, the pharmaceutical composition is made suitable for mucosal delivery, preferably intranasal delivery. In other variations of the present invention, the immunotolerizing fusion protein is delivered orally, sublingually, subcutaneously, transcutaneously, intradermally, intravenously, or intramuscularly.

The present invention also relates to the use of an immunotolerizing fusion protein or a pharmaceutical composition in accordance with the present invention for the prevention or treatment of an autoimmune demyelinating disease, in particular multiple sclerosis.

In one embodiment of the present invention, a method is provided for the prevention or treatment of an autoimmune demyelinating disease, preferably multiple sclerosis (MS), comprising administering to a subject one or more immunotolerizing fusion proteins in accordance with the present invention comprising one or more autoantigens or epitopes thereof associated with MS.

Multiple immunotolerizing fusion proteins comprising different autoantigens or autoantigenic epitopes may be administered as a mixture or cocktail of fusion proteins, and each individual immunotolerizing fusion protein may comprise multiple autoantigens or autoantigenic epitopes.

In certain variations, the methods and compositions for the treatment, prophylaxis and/or prevention of an autoimmune disease further comprise the administration of the immunotolerizing fusion protein according to the invention in combination with other substances which may enhance tissue penetration, uptake into dendritic cells or stimulate tolerance response, such as, for example, polynucleotides comprising an immune modulatory sequence, immune stimulating complexes (ISCOMS), other pharmacological agents, adjuvants, cytokines, or vectors encoding cytokines.

The immunotolerizing fusion proteins according to the invention can be produced by conventional recombinant DNA technology.

Techniques for construction of plasmids, vectors and expression systems and transfection of cells are well-known in the art, and the skilled artisan will be familiar with the standard resource materials that describe specific conditions and procedures.

Construction of the plasmids, vectors and expression systems of the invention employs standard ligation and restriction techniques that are well-known in the art (see generally, e.g., Ausubel et al., Current Protocols in Molecular Biology, Wiley Interscience, 1989; Sambrook and Russell, Molecular Cloning, A Laboratory Manual 3rd ed. 2001). Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired. Sequences of DNA constructs

can be confirmed using, e.g., standard methods for DNA sequence analysis (see, e.g., Sanger et al. (1977), Proc. Natl. Acad. Sci., 74, 5463-5467).

Yet another convenient method for isolating specific nucleic acid molecules is by the polymerase chain reaction (PCR) (Mullis et al., *Methods Enzymol* 155:335-350, 1987) or reverse transcription PCR (RT-PCR). Specific nucleic acid sequences can be isolated from RNA by RT-PCR. RNA is isolated from, for example, cells, tissues, or whole organisms by techniques known to one skilled in the art. Complementary DNA (cDNA) is then generated using poly-dT or random hexamer primers, deoxynucleotides, and a suitable reverse transcriptase enzyme. The desired polynucleotide can then be amplified from the generated cDNA by PCR. Alternatively, the polynucleotide of interest can be directly amplified from an appropriate cDNA library. Primers that hybridize with both the 5' and 3' ends of the polynucleotide sequence of interest are synthesized and used for the PCR. The primers may also contain specific restriction enzyme sites at the 5' end for easy digestion and ligation of amplified sequence into a similarly restriction digested plasmid vector.

Therapeutically and prophylactically effective amounts of the one or more immunotolerizing fusion proteins in accordance with the present invention are in the range of about 1 μg to about 10 mg. In one embodiment of the present invention, the therapeutic or prophylactically effective amount of an immunotolerizing fusion protein is in the range of about 5 μg to about 1 mg. In certain embodiments, the immunotolerizing fusion protein of the present invention is administered weekly or monthly, for example monthly for 6-12 months, and then every 3-12 months as a maintenance dose. Alternative treatment regimens may be developed and may range from daily, to weekly, to every other month, to yearly, to a one-time administration, depending upon the severity of the disease, the age of the patient, the immunotolerizing fusion protein being administered, and such other factors as would be considered by the ordinary treating physician.

EXAMPLES

The following examples are specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the claims of the present invention in any way.

Example 1. Immunotolerizing fusion protein K-CTA1(R7K/C187A)-MOG(35-55)-DD (Figure 1)

MOG(35-55): MEVGWYRSPFSRVVHLYRNGK

5 Construction of CTA1-DD mutants, expression and purification of fusion proteins were performed essentially as described by Ågren (J Immunol 1999, 162: 2432-2440). Construction of CTA1(R7K)-DD mutants, expression and purification of fusion proteins were performed essentially as described in WO 2009/078796.

10 The amino acid sequences for the peptides A-B were used in BLAST searches on the NCBI homepage to confirm each nucleotide sequence in Mus Musculus.

The cloning was performed in competent DH5α cells. That is, annealed and phosphorylated oligonucleotides were ligated into BamHI cleaved and SAP (Shrimp Alkaline Phosphatase) treated
15 vector and then transformed into competent DH5α cells. Correct nucleotide sequences were confirmed before each vector was transformed, into either competent DH5 bacteria for the K-CTA1(R7K/C187A)-DD-based vectors or competent BL21 bacteria for the CTA1(R7K)-DD-based vector, for corresponding protein expression and production.

20 ”BglI-MOG-BamHI-FP”
5’- GATCT atg gag gtg ggt tgg tac cgt tct ccc ttc tca aga gtg gtt cac ctc tac cga aat ggc aag G
”BglI-MOG-BamHI-RP”
5’- GATCC ctt gcc att tcg gta gag gtg aac cac tct tga gaa ggg aga acg gta cca acc cac ctc cat A

25 The oligonucleotides were ordered with the highest purity “Hypur” from Eurofins MWG Operon.
In the cloning of A. pTrc-K-CTA1(R7K/C187A)-MOG(35-55)-DD the MOG oligos were inserted in the BamHI site on the vector pTrc-K-CTA1(R7K/C187A)-DD.
In the cloning of B. pSY-nCTA1(R7K)-MOG(35-55)-DD the same MOG oligos were inserted in the BamHI site on the vector pSY-nCTA1(R7K)-DD.
30

Following sequencing the plasmids were then either transformed into *E.coli*, BL21 (for the CTA1(R7K)-DD-based vector) or DH5 (for the K-CTA1(R7K/C187A)-DD-based vectors).
The transformation mixture was transferred onto agar/LB plates with antibiotics, (ampicillin or Kanamycin depending on the vector) and after overnight incubation five colonies from each construct
35 were collected and cultivated in LB media, containing antibiotics, for eight hours.
The cultures were frozen and stored at -80°C as glycerol stock solutions.

The frozen glycerol stock solutions were thawed and cultivated in 2xYT media, containing antibiotics. The protein expression levels were analyzed four hours after induction (IPTG).

The pellets and respective supernatants of the five different clones were lysed, separated by SDS-PAGE electrophoresis and Coomassie stained. Western blot was performed on the selected clones to confirm the identity of the primarily expressed protein from the respective expression vector and the fusion protein was purified from the pellet using Q Sepharose FF and SEC Superdex 200 pg on the Akta FPLC system.

Antibody utilized in the Western blot: Polyclonal Chicken Anti CTA1-DD, alkaline phosphatase (AP)-labelled antibody (Agrisera/MDAB).
The total protein concentration was measured using BCA assay (Pierce).
Samples were sent to the Laboratory for Bacteriology, Sahlgrenska University Hospital, for determination of endotoxin levels.
The purity was analyzed using Quantity One, Bio-Rad.
Enzymatic activity was determined in an ADP-ribosylation assay where the fusionproteins were compared to CT.
The Ig-binding capacity of the fusionproteins was determined using ELISA assay.

Results for K-CTA1(R7K/C187A)-MOG(35-55)-DD:

Yield:	1.8 mg/ml, 8.13 ml, totally 14.6 mg
Endotoxin:	0.11 EU/mg
Purity:	more than 95-96% (single peak)
Enzymatic activity:	none
Ig-binding:	good

Results for CTA1(R7K)-MOG(35-55)-DD:

Yield:	1.5 mg/ml, 6.7 ml, totally 10.05 mg
Endotoxin:	< 0.03 EU/mg
Purity:	more than 98-99% (single peak)
Enzymatic activity:	none
Ig-binding:	good

Example 2. Immunotolerizing fusion protein K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD (Figure 2)

MOG(35-55): MEVGWYRSPFSRVVHLYRNGK
PLP(178-191): NTWTTCQSIAFPSK

The cloning was performed in a manner analogous to the method described in Example 1.

Results for K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD:

Yield:	1.97 mg/ml, 1.15 ml, 2.26 mg in total
Endotoxin:	0.15 EU/mg
Purity:	>90 % (single peak)
Enzymatic activity:	none
Ig-binding:	good
Sequence identity:	confirmed

Example 3. Immunotolerizing fusion protein K-CTA1(R7K/C187A)-PLP(178-191)-DD (Figure 3)

PLP(178-191): NTWTTCQSIAFPSK

The cloning was performed in a manner analogous to the method described in Example 1.

Results for K-CTA1(R7K/C187A)-PLP(178-191)-DD:

Yield:	2.57 mg/ml, 12.05 ml, 30.96 mg in total
Endotoxin:	0.21 EU/mg
Purity:	>95 % (single peak)
Enzymatic activity:	none
Ig-binding:	good
Sequence identity:	confirmed

Example 4. Immunotolerizing fusion protein K-CTA1(R7K/C187A)-human MOG(10-60)-DD (Figure 4)

human MOG(10-60):
HPIRALVGDEVELPCRISPGKNATGMEVGWYRPPFSRVVHLYRNGKDQDGD

The cloning was performed in a manner analogous to the method described in Example 1.

Results for K-CTA1(R7K/C187A)-human MOG(10-60)-DD:

Yield:	2.96 mg/mL, 12.63 mL, 37.38 mg in total
Endotoxin:	<0.02 EU/mg
Purity:	>98 %
Enzymatic activity:	none
Ig-binding:	good
Sequence identity:	confirmed

Example 5. Comparison of the therapeutic effects of CTA1(R7K)-MOG(35-55)-DD and K-CTA1(R7K/C187A)-MOG(35-55)-DD in the mouse EAE model. (Figure 5)

C57Bl/6 mice were obtained from Harlan and were 7-9 weeks of age at the start of the experiment. One hundred microliter induction mixture containing 100 microgram MOG(35-55) was mixed 1:1 with Complete Freund's Adjuvant containing 10 mg/ml Mycobacterium tuberculosis and the induction mixture was given by subcutaneous injection at the base of the tail at Day 0. Two hundred nanogram Pertussis toxin was given intraperitoneally on Day 0 and on Day 2. Treatment was performed by intranasal immunization with the fusion proteins at indicated concentrations in a volume of 20 microliter per nostril on Days -2, 0, 2, 10, 12, 14. Dexamethasone at a dose of 1 mg/kg was given intraperitoneally 5 Days per week (not weekends), starting on Day 0. Each animal was evaluated from Day 5 until Day 28 for EAE severity using the disability scoring system; 0, no disease; 0.5, tail paresis or partial paralysis; 1, complete tail paralysis or limb weakness; 1.5, limb weakness with partial tail paralysis; 2, paraparesis: limb weakness and tail paralysis; 2.5, partial limb paralysis; 3, complete hind- or front limb paralysis; 3.5, paraplegia; 4, quadriplegia, moribund; 5, death due to EAE. The highest clinical score of each mouse during the complete experimental period was determined. The experiment was terminated on Day 28.

PBS	intranasally, d -2, 0, 2, 10, 12, 14
Dexamethasone,	1 mg/kg, intraperitoneally 5 times/w (mon-fri)
K-CTA1(R7K/C187A)-MOG-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14
CTA1(R7K)-MOG-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14

Mean Maximum Score (MMS) was calculated:

	MMS	Reduction compared to PBS (%)
PBS	3,29	--
Dexamethasone	2,29	30%
K-CTA1(R7K/C187A)-MOG(35-55)-DD	2,22	33%
CTA1(R7K)-MOG(35-55)-DD	3,00	9%

Accumulated Mean Score (AMS), representing treatment effects over time:

	AMS	Reduction compared to PBS (%)
PBS	39,44	--
Dexamethasone	18,14	54%
K-CTA1(R7K/C187A)-MOG(35-55)-DD	19,99	49%
CTA1(R7K)-MOG(35-55)-DD	30,91	22%

The therapeutic effect of K-CTA1(R7K/C187A)-MOG(35-55)-DD was significantly better than the therapeutic effect of CTA1(R7K)-MOG(35-55)-DD, as seen in the decrease of the EAE disease score as compared with the control group (PBS).

Example 6. Therapeutic effects of K-CTA1(R7K/C187A)-MOG(35-55)-DD and K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD in the mouse EAE model. (Figure 6)

Following a protocol similar to the one described in Example 4 above, the following experiments were carried out:

PBS	intranasally, d -2, 0, 2, 10, 12, 14
Dexamethasone,	1 mg/kg, intraperitoneally 7 times/w
K-CTA1(R7K/C187A)-MOG(35-55)-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14
K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14

Mean Maximum Score (MMS) was calculated:

	MMS	Reduction compared to PBS (%)
PBS	2.89	--
Dexamethasone	0	100%
K-CTA1(R7K/C187A)-MOG(35-55)-DD	1.22	58%

K-CTA1(R7K/C187)-MOG(35-55)-PLP(178-191)-DD	1.33	54%

Accumulated Mean Score (AMS), representing treatment effects over time:

	AMS	Reduction compared to PBS (%)
PBS	30.6	--
Dexamethasone	0	100%
K-CTA1(R7K/C187A)-MOG(35-55)-DD	9.5	69%
K-CTA1(R7K/C187)-MOG(35-55)-PLP(178-191)-DD	9.7	68%

The therapeutic effects of K-CTA1(R7K/C187A)-MOG(35-55)-DD and K-CTA1(R7K/C187)-MOG(35-55)-PLP(178-191)-DD on EAE disease were significant as seen in the decrease of the EAE disease score as compared with the control group (PBS).

Example 7. The therapeutic effect of K-CTA1(R7K/C187A)-MOG(35-55)-DD is peptide specific in the mouse EAE model. (Figure 7)

EAE was induced in C57Bl/6 mice in the same way as described in example 5 above.

PBS	intranasally, d -2, 0, 2, 10, 12, 14
Dexamethasone,	1 mg/kg, intraperitoneally 5 times/w (mon-fri)
K-CTA1(R7K/C187A)-MOG(35-55)-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14
K-CTA1(R7K/C187A)-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14

Mean Maximum Score (MMS) was calculated:

	MMS	Reduction compared to PBS (%)
PBS	3,33	--
Dexamethasone	0,44	77%
K-CTA1R7K(C187A)-MOG(35-55)-DD	1,56	53%
K-CTA1R7K(C187A)-DD	3,67	0%

Accumulated Mean Score (AMS), representing treatment effects over time:

	AMS	Reduction compared to PBS (%)
PBS	54,3	--
Dexamethasone	2,3	96%
K-CTA1R7K(C187A)-MOG(35-55)-DD	26,1	52%
K-CTA1R7K(C187A)-DD	63,7	0%

The therapeutic effect of K-CTA1R7K(C187A)-MOG(35-55)-DD was significantly better than treatment with the empty vector, K-CTA1R7K(C187A)-DD, which had no therapeutic effect indicating that the disease specific epitope, in this case MOG(35-55) is needed to achieve any therapeutic effect.

Example 8. The therapeutic effect of K-CTA1(R7K/C187A)-MOG(35-55)-DD on the incidence of disease is peptide specific in the mouse EAE model. (Figure 8)

EAE was induced in C57Bl/6 mice in the same way as described in example 5 above.

Maximum frequency(MF) of animal showing signs of disease/Maximum incidence of disease:

	MF(%)	Reduction compared to PBS (%)
PBS	100	--
Dexamethasone	11	89%
K-CTA1R7K(C187A)-MOG(35-55)-DD	56	44%
K-CTA1R7K(C187A)-DD	100	0%

Significantly fewer animals showed sign of disease following treatment with K-CTA1R7K(C187A)-MOG(35-55)-DD compared to control animals treated with PBS. Treatment with the empty vector alone, K-CTA1R7K(C187A)-DD had no effect on the number of animals showing signs of disease.

Example 9. The therapeutic effects of K-CTA1(R7K/C187A)-MOG(35-55)-DD and K-CTA1(R7K/C187A)-PLP(178-191)-DD in the mouse EAE model. (Figure 9)

EAE was induced in C57Bl/6 mice in the same way as described in example 5 above with the exception that 100 microgram PLP(178-191) was used to induce EAE instead of 100 microgram MOG(35-55).

PBS	intranasally, d -2, 0, 2, 10, 12, 14
K-CTA1R7K(C187A)-MOG(35-55)-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14
K-CTA1R7K(C187A)-PLP(178-191)-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14

Mean Maximum Score (MMS) was calculated:

	MMS	Reduction compared to PBS (%)
PBS	1,56	--
K-CTA1R7K(C187A)-MOG(35-55)-DD	1,11	29%
K-CTA1R7K(C187A)-PLP(178-191)-DD	1,11	29%

Accumulated Mean Score (AMS), representing treatment effects over time:

	AMS	Reduction compared to PBS (%)
PBS	15,9	--
K-CTA1R7K(C187A)-MOG(35-55)-DD	8,9	44%
K-CTA1R7K(C187A)-PLP(178-191)-DD	11,3	29%

The therapeutic effect of K-CTA1R7K(C187A)-MOG(35-55)-DD as well as of K-CTA1R7K(C187A)-PLP(178-191)-DD was significant on EAE disease as seen in the decrease of the disease score as compared with the control group (PBS). K-CTA1R7K(C187A)-MOG(35-55)-DD had a significant therapeutic effect on EAE disease induced by PLP(178-191) indicating that this MOG-epitope is central to the EAE disease and thus potentially also in the human MS disease.

Example 10. The therapeutic effects of K-CTA1(R7K/C187A)-MOG(35-55)-DD, K-CTA1(R7K/C187A)-PLP(178-191)-DD, K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD and K-CTA1(R7K/C187A)-human MOG(10-60)-DD in the mouse EAE model. (Figure 10)

EAE was induced in C57Bl/6 mice in the same way as described in example 5 above with the exception that 100 microgram PLP(178-191) together with 100 microgram MOG(35-55) was used to induce EAE instead of only 100 microgram MOG(35-55) alone.

PBS	intranasally, d -2, 0, 2, 10, 12, 14
K-CTA1R7K(C187A)-MOG(35-55)-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14
K-CTA1R7K(C187A)-PLP(178-191)-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14
K-CTA1R7K(C187A)-MOG(35-55) -PLP(178-191)-DD,	5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14

K-CTA1R7K(C187A)-humanMOG(10-60)-DD, 5 µg/dose intranasally, d -2, 0, 2, 10, 12, 14

Mean Maximum Score (MMS) was calculated:

	MMS	Reduction compared to PBS (%)
PBS	3,8	--
K-CTA1R7K(C187A)-MOG(35-55)-DD	2,89	24%
K-CTA1R7K(C187A)-PLP(178-191)-DD	3,1	18%
K-CTA1R7K(C187A)-MOG(35-55) -PLP(178-191)-DD	2,4	37%
K-CTA1R7K(C187A) -humanMOG(10-60)-DD	2,4	37%

Accumulated Mean Score (AMS), representing treatment effects over time:

	AMS	Reduction compared to PBS (%)
PBS	40,4	--
K-CTA1R7K(C187A)-MOG(35-55)-DD	26,6	34%
K-CTA1R7K(C187A)-PLP(178-191)-DD	33,9	16%
K-CTA1R7K(C187A)-MOG(35-55) -PLP(178-191)-DD	22,0	46%
K-CTA1R7K(C187A) -humanMOG(10-60)-DD	24,5	39%

The therapeutic effect of K-CTA1R7K(C187A)-MOG(35-55)-DD, CTA1R7K(C187A)-PLP(178-191)-DD as well as of K-CTA1R7K(C187A)-MOG(35-55)-PLP(178-191)-DD was significant on EAE disease as seen in the decrease of the disease score as compared with the control group (PBS). Further the fusion protein containing the combination of two epitopes, the K-CTA1R7K(C187A)-MOG(35-55)-PLP(178-191)-DD had a significant better therapeutic effect compared to either K-CTA1R7K(C187A)-MOG(35-55)-DD or CTA1R7K(C187A)-PLP(178-191)-DD alone. The fusion protein K-CTA1R7K(C187A)-humanMOG(10-60)-DD, containing two potential human epitopes, amino acids 24-36 and amino acids 35-55, also had a significant therapeutic effect on EAE disease induced by a combination of PLP(178-191) and MOG(35-55) indicating that this humanized fusion protein have the potential to be effective in the human MS disease.

This could also imply that a longer stretch containing several epitopes can be effective. Further, the inclusion of additional amino acids besides the potential epitopes does not appear to affect the effect negatively.

SEQUENCE LISTING

SEQ ID NO:1 K-CTA1(R7K/C187A) mutant

Lys Asn Asp Asp Lys Leu Tyr Lys Ala Asp Ser Arg Pro Pro Asp Glu
1 5 0 15

Ile Lys Gln Ser Gly Gly Leu Met Pro Arg Gly Gln Ser Glu Tyr Phe
20 25 30

Asp Arg Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg Gly
35 40 45

Thr Gln Thr Gly Phe Val Arg His Asp Asp Gly Tyr Val Ser Thr Ser
50 55 60

Ile Ser Leu Arg Ser Ala His Leu Val Gly Gln Thr Ile Leu Ser Gly
65 70 75 80

His Ser Thr Tyr Tyr Ile Tyr Val Ile Ala Thr Ala Pro Asn Met Phe
85 90 95

Asn Val Asn Asp Val Leu Gly Ala Tyr Ser Pro His Pro Asp Glu Gln
100 105 110

Glu Val Ser Ala Leu Gly Gly Ile Pro Tyr Ser Gln Ile Tyr Gly Trp
115 120 125

Tyr Arg Val His Phe Gly Val Leu Asp Glu Gln Leu His Arg Asn Arg
130 135 140

Gly Tyr Arg Asp Arg Tyr Tyr Ser Asn Leu Asp Ile Ala Pro Ala Ala
145 150 155 160

Asp Gly Tyr Gly Leu Ala Gly Phe Pro Pro Glu His Arg Ala Trp Arg
165 170 175

Glu Glu Pro Trp Ile His His Ala Pro Pro Gly Ala Gly Asn Ala Pro
180 185 190

Arg Ser Ser
195

or (in one-letter amino acid code):

1 KNDDKLYKA DSRPPDEIKQ SGGLMPRGQS EYFDRGTQMN INLYDHARGT
51 QTGFVRHDDG YVSTSISLRS AHLVGQTILS GHSTYYIYVI ATAPNMFNVN
101 DVLGAYSPHP DEQEVSALGG IPYSQIYGWY RVHFGVLDEQ LHRNRGYRDR
151 YYSNLDIAPA ADGYGLAGFP PEHRAWREEP WIHHAPPGAG NAPRSS

SEQ ID NO:2 K-CTA1(R7K/C187A)-MOG(35-55)-DD fusion protein

1 MKNDDKLYKA DSRPPDEIKQ SGGLMPRGQS EYFDRGTQMN INLYDHARGT
51 QTGFVRHDDG YVSTSISLRS AHLVGQTILS GHSTYYIYVI ATAPNMFNVN
101 DVLGAYSPHP DEQEVSALGG IPYSQIYGWY RVHFGVLDEQ LHRNRGYRDR
151 YYSNLDIAPA ADGYGLAGFP PEHRAWREEP WIHHAPPGAG NAPRSSGSME
201 VGWYRSPFSR VVHLYRNGKG SGKTPEADAQ QNNFNKDQQS AFYEILNMPN
251 LNEAQRNGFI QSLKDDPSQS TNVLGEAKKL NESQAPKPEA DAQQNNFNKD
301 QQSAFYEILN MPNLNEAQRN GFIQSLKDDP SQSTNVLGEA KKLNESQAPK
351 PEVAGQN

SEQ ID NO:3 K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD fusion protein

1 MKNDDKLYKA DSRPPDEIKQ SGGLMPRGQS EYFDRGTQMN INLYDHARGT
51 QTGFVRHDDG YVSTSISLRS AHLVGQTILS GHSTYYIYVI ATAPNMFNVN
101 DVLGAYSPHP DEQEVSALGG IPYSQIYGWY RVHFGVLDEQ LHRNRGYRDR
151 YYSNLDIAPA ADGYGLAGFP PEHRAWREEP WIHHAPPGAG NAPRSSGSME
201 VGWYRSPFSR VVHLYRNGKG SNTWTTCQSI AFPSKGSGKT PEADAQQNNF
251 NKDQQSAFYE ILNMPNLNEA QRNGFIQSLK DDPSQSTNVL GEAKKLNESQ
301 APKPEADAQQ NNFNKDQQSA FYEILNMPNL NEAQRNGFIQ SLKDDPSQST
351 NVLGEAKKLN ESQAPKPEVA GQN

SEQ ID NO:4 K-CTA1(R7K/C187A)-PLP(178-191)-DD fusion protein

1 MKNDDKLYKA DSRPPDEIKQ SGGLMPRGQS EYFDRGTQMN INLYDHARGT
51 QTGFVRHDDG YVSTSISLRS AHLVGQTILS GHSTYYIYVI ATAPNMFNVN
101 DVLGAYSPHP DEQEVSALGG IPYSQIYGWY RVHFGVLDEQ LHRNRGYRDR
151 YYSNLDIAPA ADGYGLAGFP PEHRAWREEP WIHHAPPGAG NAPRSSGSNT
201 WTTCQSIAFP SKGSGKTPEA DAQQNNFNKD QQSAFYEILN MPNLNEAQRN
251 GFIQSLKDDP SQSTNVLGEA KKLNESQAPK PEADAQQNNF NKDQQSAFYE

301 ILNMPNLNEA QRNGFIQSLK DDPSQSTNVL GEAKKLNESQ APKPEVAGQN

SEQ ID NO 5: K-CTA1(R7K/C187A)-human MOG(10-60)-DD

1 MKNDDKLYKA DSRPPDEIKQ SGGLMPRGQS EYFDRGTQMN INLYDHARGT
51 QTGFVRHDDG YVSTSISLRS AHLVGQTILS GHSTYYIYVI ATAPNMFNVN
101 DVLGAYSPHP DEQEVSALGG IPYSQIYGWY RVHFGVLDEQ LHRNRGYRDR
151 YYSNLDIAPA ADGYGLAGFP PEHRAWREEP WIHHAPPGAG NAPRSSGSHP
201 IRALVGDEVE LPCRISPGKN ATGMEVGWYR PPFSRVVHLY RNGKDQDGDG
251 SGKTPEADAQ QNNFNKDQQS AFYEILNMPN LNEAQRNGFI QSLKDDPSQS
301 TNVLGEAKKL NESQAPKPEA DAQQNNFNKD QQSAFYEILN MPNLNEAQRN
351 GFIQSLKDDP SQSTNVLGEA KKLNESQAPK PEVAGQN

Claims

1. An immunotolerizing fusion protein comprising:
(a) a mutant subunit of the ADP-ribosylating A1-subunit of the cholera toxin (CTA1), wherein the mutant CTA1 subunit is the K-CTA1(R7K/C187A) mutant of SEQ ID NO:1
(b) one or more autoantigens or epitopes thereof associated with an autoimmune demyelinating disease, and
(c) a peptide capable of binding to a specific cellular receptor, wherein the peptide specifically binds to a receptor expressed on an antigen presenting cell selected from the group consisting of lymphocytes, monocytes, macrophages, dendritic cells, and Langerhans cells.

2. The immunotolerizing fusion protein according to claim 1, wherein the autoimmune demyelinating disease is multiple sclerosis.

3. The immunotolerizing fusion protein according to claim 1 or 2, wherein the one or more autoantigens are selected from the group of antigens consisting of myelin antigens, glial antigens, and neuronal antigens.

4. The immunotolerizing fusion protein according to claim 3, wherein the myelin antigens are selected from the group of antigens consisting of antigens from myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated oligodendrocytic basic protein (MOBP), myelin-associated glycoprotein (MAG), oligodendrocyte-specific protein (OSP), myelin-associated neurite outgrowth inhibitor Nogo-A, and cyclic nucleotide phosphodiesterase (CNPase).

5. The immunotolerizing fusion protein according to claim 4, wherein the myelin antigens are selected from the group of autoantigenic epitopes consisting of PLP(41-60), PLP(43-64), PLP(45-53), PLP(56-70), PLP(103-116), PLP(104-117), PLP(139-151), PLP(175-194), PLP(178-191), PLP(215-232), PLP(215-235), PLP(258-276), MBP(1-11), MBP(11-30), MBP(21-35), MBP(29-84), MBP(59-76), MBP(61-82), MBP(80-105), MBP(84-102), MBP(85-99), MBP(87-99), MBP(89-101), MBP(89-104), MBP(147-162), MBP(170-186), MAG(97-112), MOG(1-22), MOG(10-60), MOG(14-

36), MOG(24-36), MOG(34-56), MOG(35-55), MOG(40-48), MOG(43-57), MOG(64-96), MOG(92-106), MOG(94-116), MOG(134-148), MOG(202-218), MOBP(15-36), MOBP(37-60), MOBP(55-77), MOBP(158-181), OSP(22-46), OSP(55-71), OSP(55-74), OSP(55-80), OSP(57-72), OSP(103-123), OSP(142-161), OSP(179-201), and OSP(179-207).

6. The immunotolerizing fusion protein according to any one of claims 1-5, wherein said peptide is constituted by protein A or a fragment thereof in single or multiple copies, such as one or more D subunits thereof.

7. The immunotolerizing fusion protein according to any one of claims 1-6 selected from the group consisting of K-CTA1(R7K/C187A)-MOG(35-55)-DD (SEQ ID NO:2), K-CTA1(R7K/C187A)-MOG(35-55)-PLP(178-191)-DD (SEQ ID NO:3), K-CTA1(R7K/C187A)-PLP(178-191)-DD (SEQ ID NO:4) and K-CTA1(R7K/C187A)-human MOG(10-60)-DD (SEQ ID NO:5).

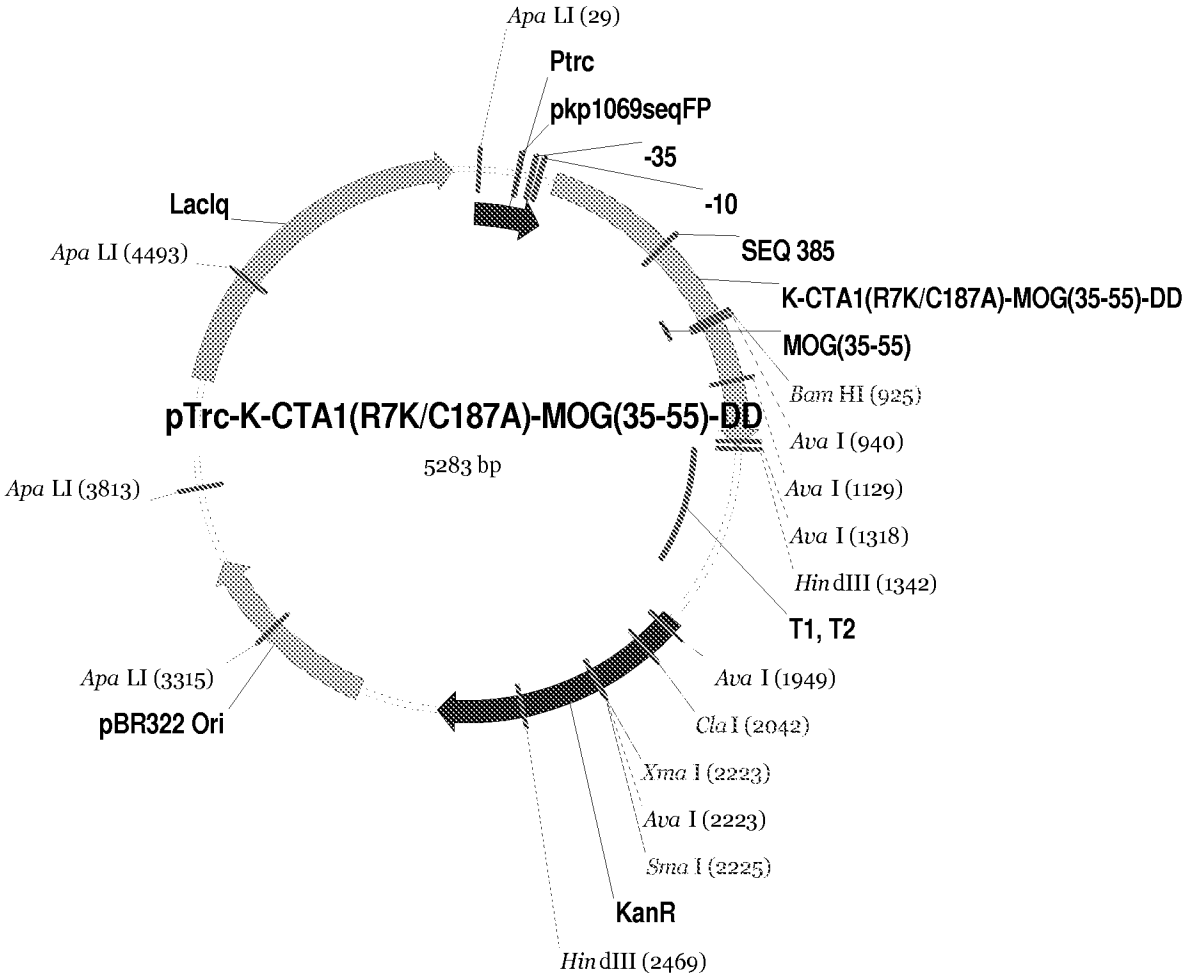
8. An isolated nucleic acid encoding an immunotolerizing fusion protein according to any of claims 1 to 7.

9. A pharmaceutical composition comprising one or more immunotolerizing fusion proteins according to any of the claims 1 to 7 or an isolated nucleic acid encoding an immunotolerizing fusion protein according to claim 8.

10. The pharmaceutical composition according to claim 9 for use in the prevention or treatment of an autoimmune demyelinating disease, preferably multiple sclerosis.

11. The pharmaceutical composition according to claim 9 or 10, wherein the composition is made suitable for mucosal delivery, preferably intranasal delivery.

12. Use of an immunotolerizing fusion protein according to any one of claims 1 to 7 or a pharmaceutical composition according to claim 9 or 10 for the prevention or treatment of an autoimmune demyelinating disease, preferably multiple sclerosis.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MOG(35-55)
Bam HI (925)
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5283 bp
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Ava I (1318)
Hin dIII (1342)
T1, T2
Apa LI (3315)
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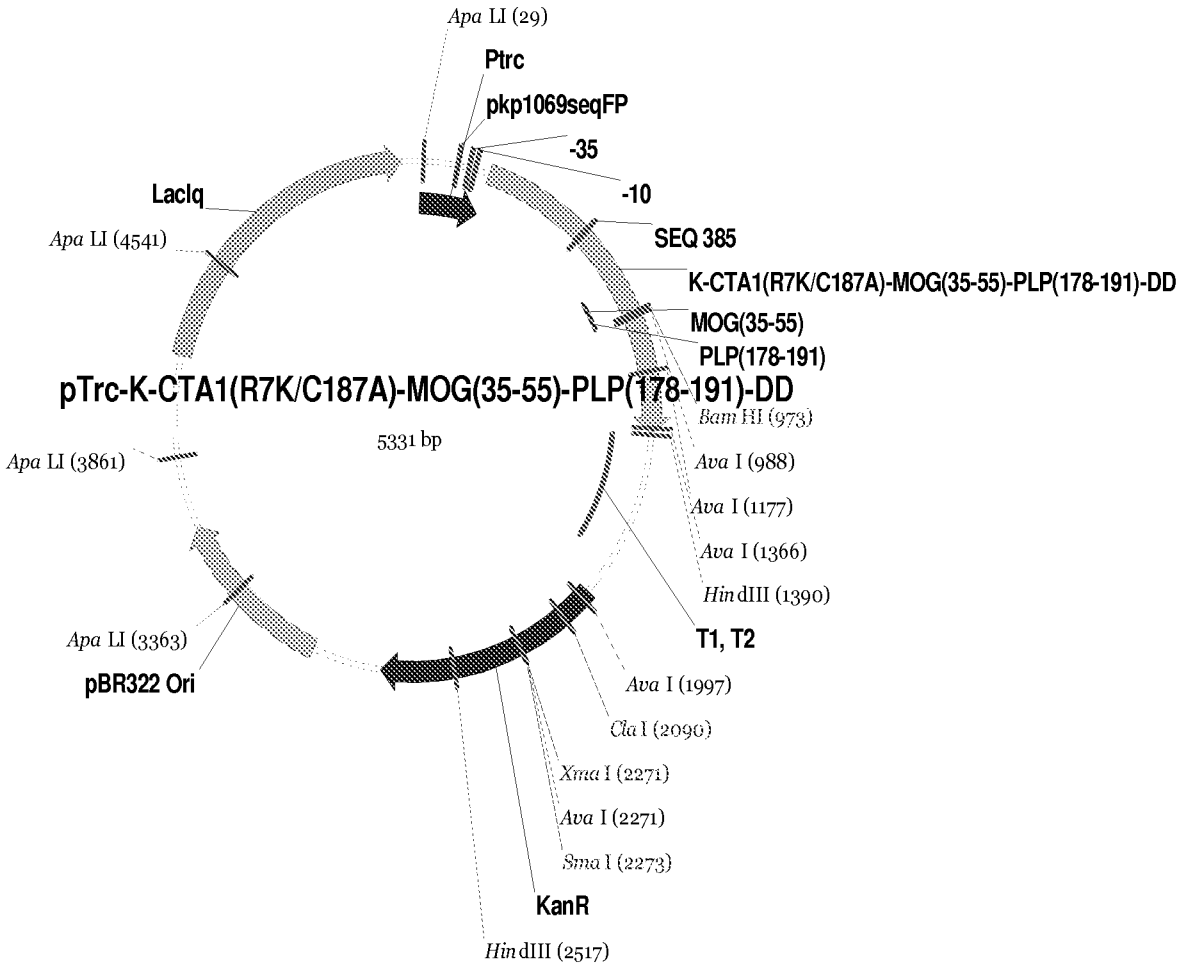
KanR
Hin dIII (2469)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Ava I (1177)
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Hin dIII (1390)
Apa LI (3861)
T1, T2
Apa LI (3363)
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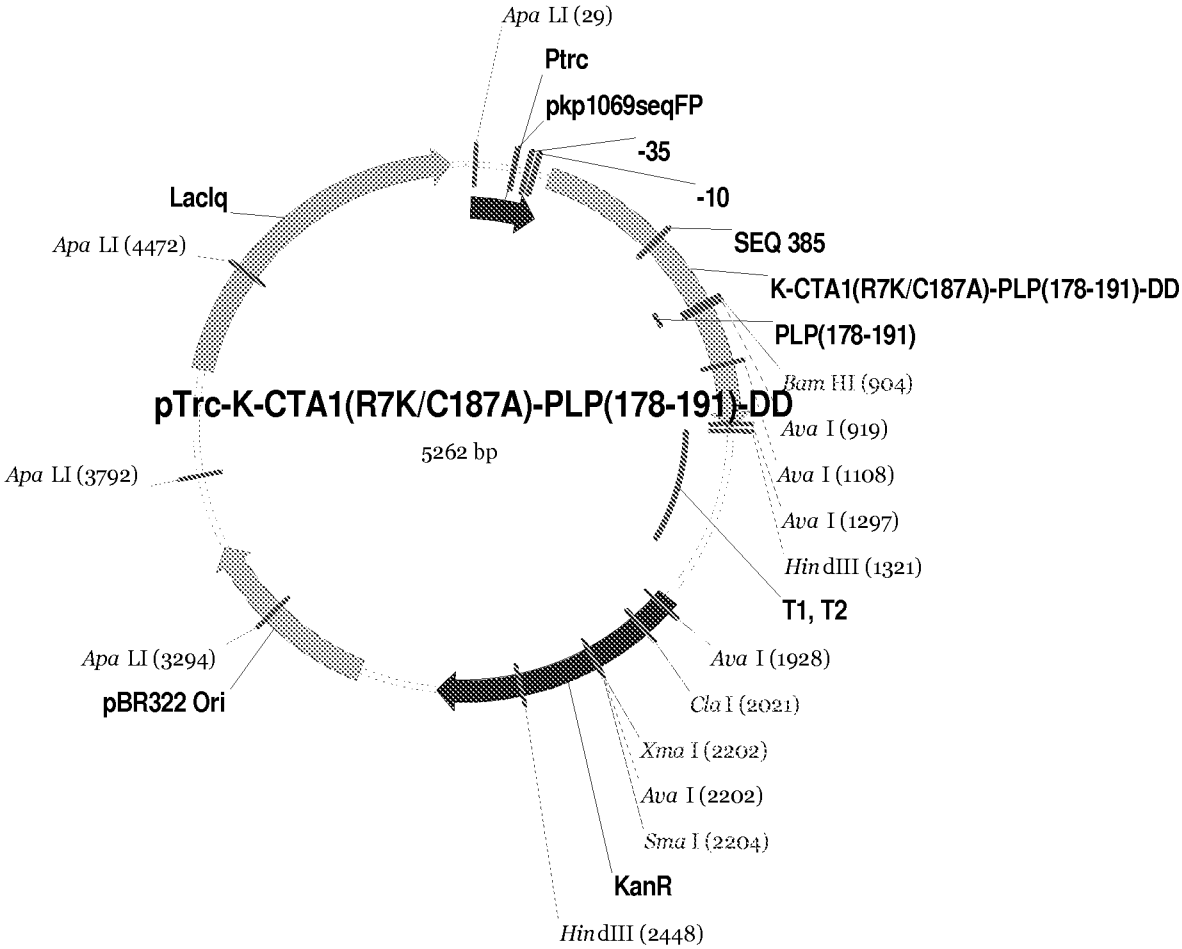
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Bam HI (904)
pTrc-K-CTA1(R7K/C187A)-PLP(178-191)-DD
Ava I (919)
5262 bp
Apa LI (3792)
Ava I (1108)
Ava I (1297)
Hin dIII (1321)
T1, T2
Apa LI (3294)
Ava I (1928)
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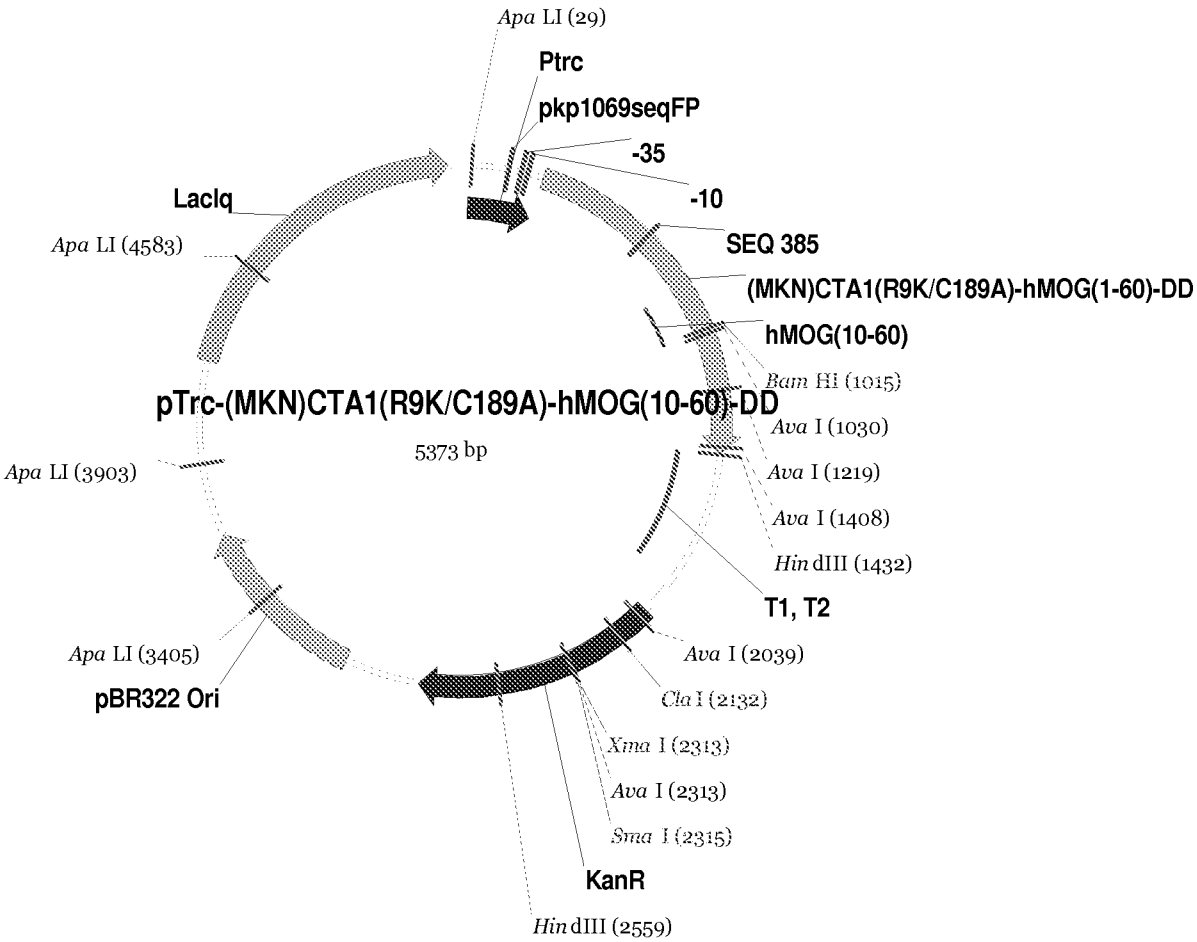
Cla I (2021)
Xma I (2202)
Ava I (2202)
Sma I (2204)
KanR
Hin dIII (2448)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Ptrc
pkp1069seqFP
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Bam HI (1015)
Ava I (1030)
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Ava I (1408)
Hin dIII (1432)
T1, T2
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Cla I (2132)
Xma I (2313)
Ava I (2313)
Sma I (2315)
KanR
Hin dIII (2559)
Apa LI (3405)
pBR322 Ori
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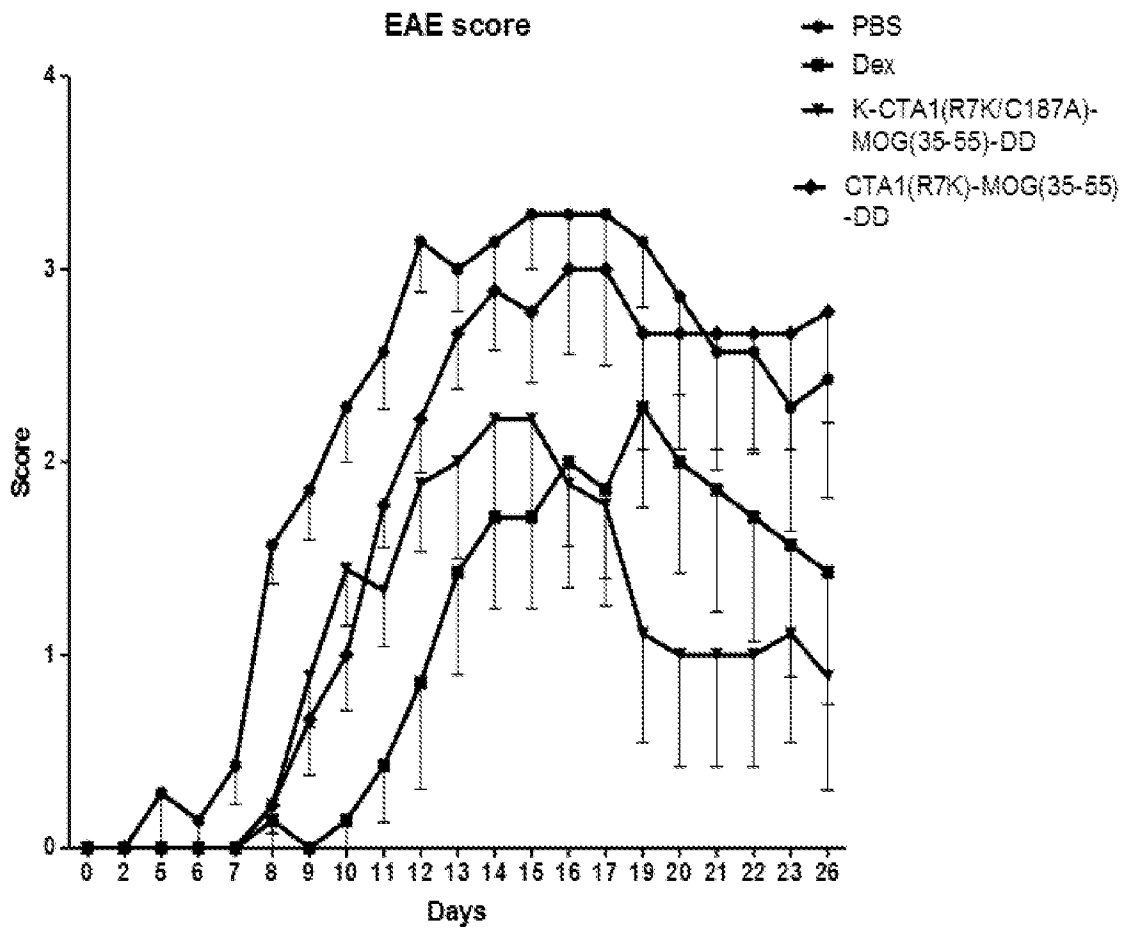
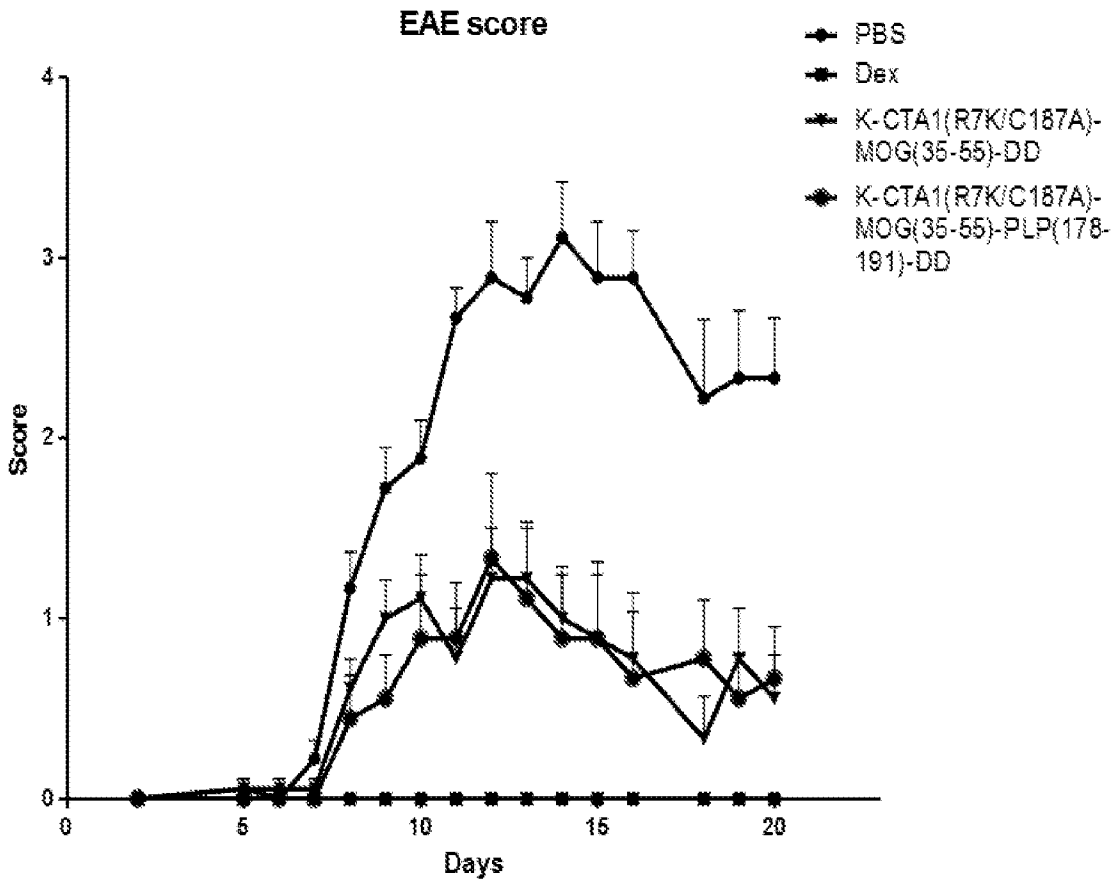
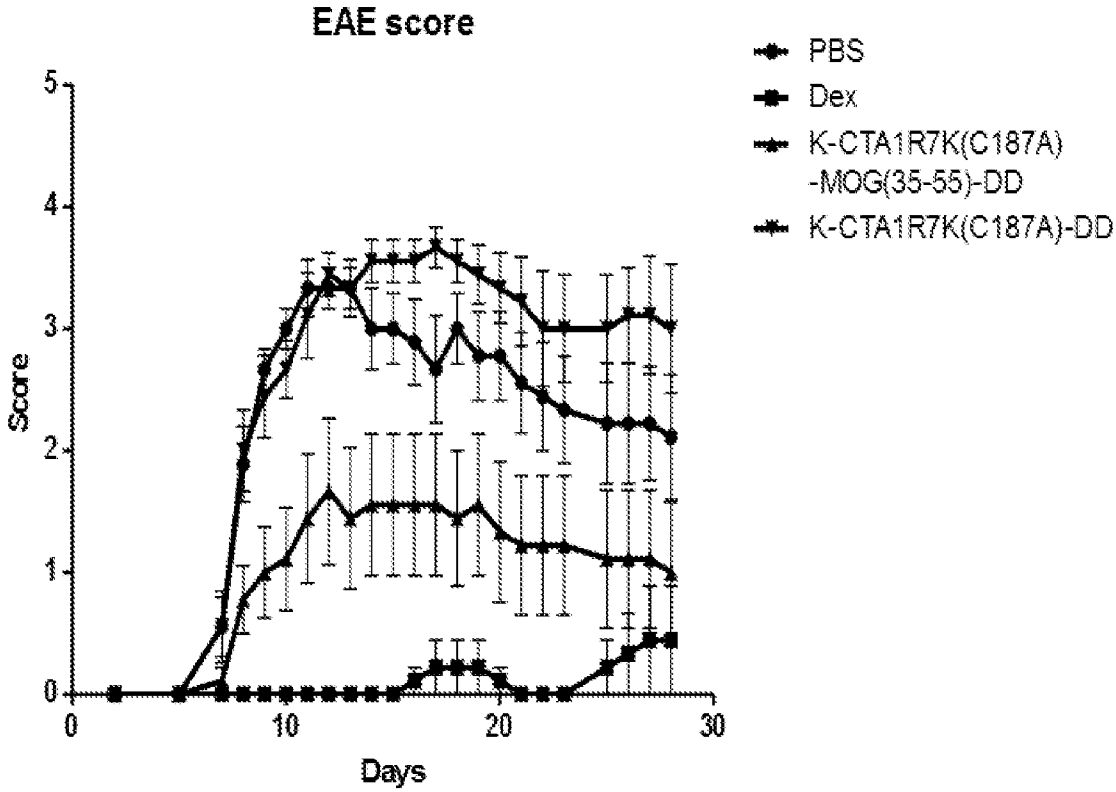
LacIq
pTrc-(MKN)CTA1(R9K/C189A)-hMOG(10-60)-DD
5373 bp



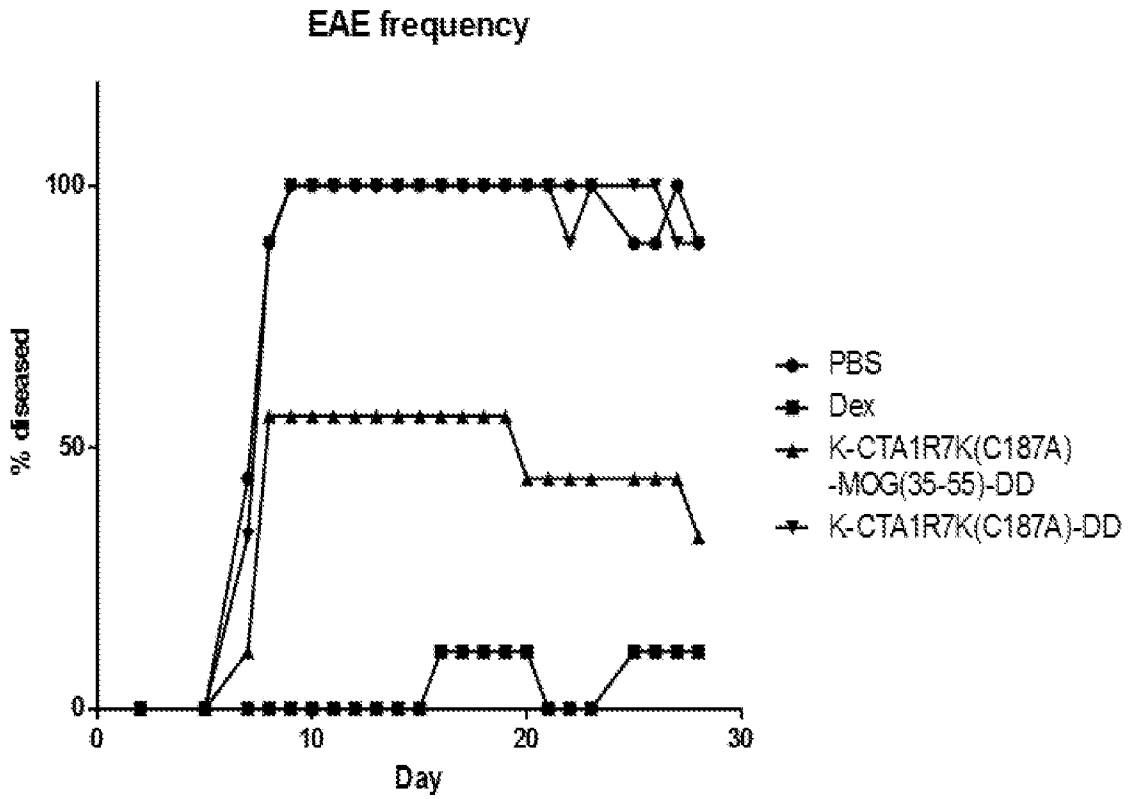
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary
PBS vs Dex	1.095	7.941	Yes	***
PBS vs K-CTA1(R7K/C187A)-MOG(35-55)DD	1.054	7.645	Yes	***
PBS vs CTA1(R7K)-MOG(35-55)-DD	0.3719	2.696	No	ns
Dex vs K-CTA1(R7K/C187A)-MOG(35-55)DD	-0.04082	0.2959	No	ns
Dex vs CTA1(R7K)-MOG(35-55)-DD	-0.7234	5.245	Yes	***
K-CTA1(R7K/C187A)-MOG(35-55)DD vs CTA1(R7K)-MOG(35-55)-DD	-0.6825	4.949	Yes	***



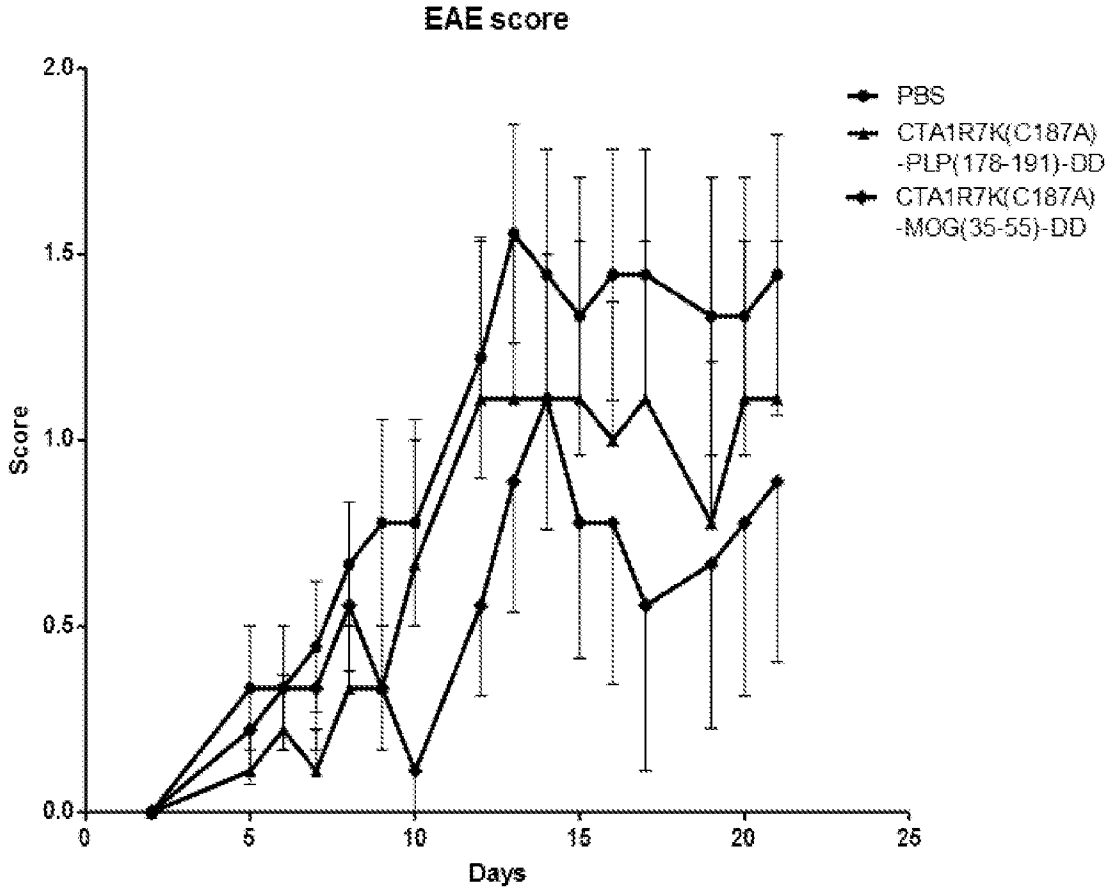
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary
PBS vs Dex	1.823	10.20	Yes	****
PBS vs K-CTA1(R7K/C187A)-MOG(35-55)DD	1.170	6.548	Yes	****
PBS vs K-CTA1(R7K/C187A)-MOG-PLP-DD	1.219	6.820	Yes	****
Dex vs K-CTA1(R7K/C187A)-MOG(35-55)DD	-0.6528	3.653	Yes	**
Dex vs K-CTA1(R7K/C187A)-MOG-PLP-DD	-0.6042	3.381	Yes	**
K-CTA1(R7K/C187A)-MOG(35-55)DD vs K-CTA1(R7K/C187A)-MOG-PLP-DD	0.04861	0.2720	No	ns



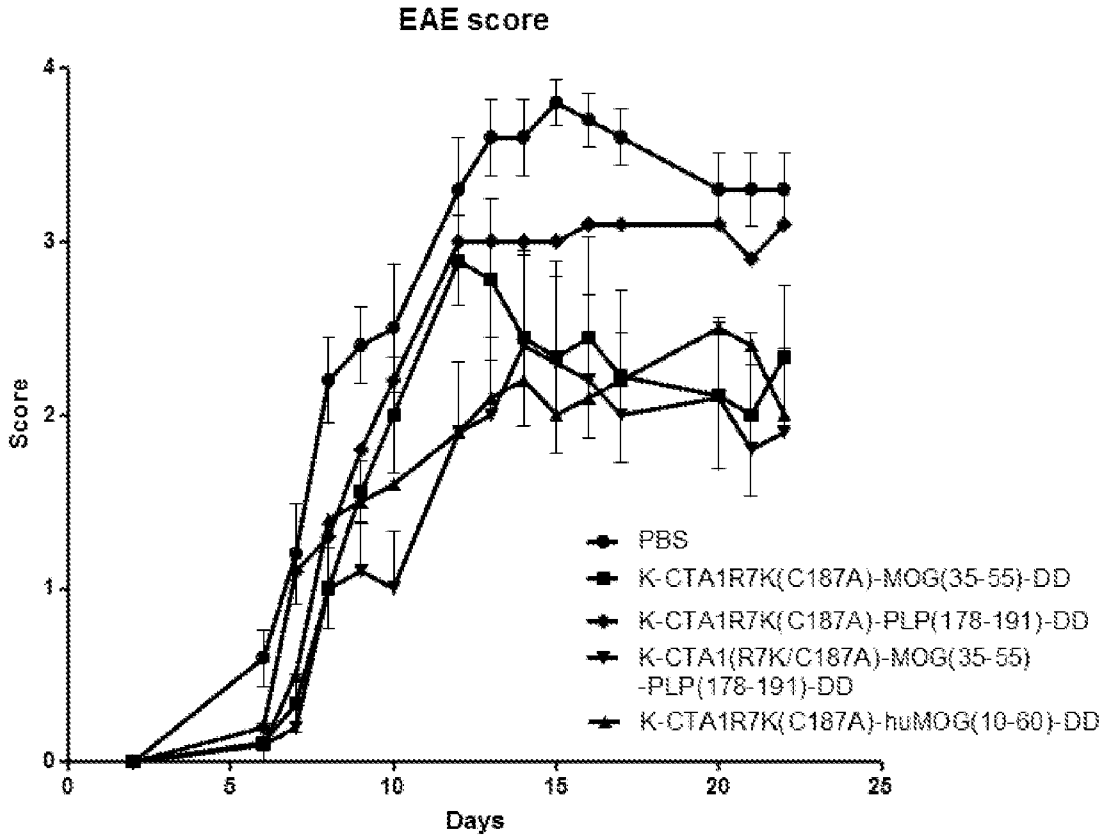
Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary
PBS vs Dex	2.261	15.04	Yes	***
PBS vs K-CTA1R7K(C187A)-MOG(35-55)-DD	1.227	8.163	Yes	***
PBS vs K-CTA1R7K(C187A)-DD	-0.4059	2.699	No	ns
Dex vs K-CTA1R7K(C187A)-MOG(35-55)-DD	-1.034	6.877	Yes	***
Dex vs K-CTA1R7K(C187A)-DD	-2.667	17.74	Yes	***
K-CTA1R7K(C187A)-MOG(35-55)-DD vs K-CTA1R7K(C187A)-DD	-1.633	10.86	Yes	***



Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary
PBS vs Dex	82.65	19.37	Yes	****
PBS vs K-CTA1R7K(C187A)-MOG(35-55)-DD	42.43	9.945	Yes	****
PBS vs K-CTA1R7K(C187A)-DD	0.4783	0.1121	No	ns
Dex vs K-CTA1R7K(C187A)-MOG(35-55)-DD	-40.22	9.425	Yes	****
Dex vs K-CTA1R7K(C187A)-DD	-82.17	19.26	Yes	****
K-CTA1R7K(C187A)-MOG(35-55)-DD vs K-CTA1R7K(C187A)-DD	-41.96	9.833	Yes	****



Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary
PBS vs CTA1R7K(C187A)-PLP(178-191)-DD	0.2547	4.715	Yes	***
PBS vs CTA1R7K(C187A)-MOG(35-55)-DD	0.4375	7.248	Yes	***
CTA1R7K(C187A)-PLP(178-191)-DD vs CTA1R7K(C187A)-MOG(35-55)-DD	0.1828	2.530	No	ns



Bonferroni's Multiple Comparison Test	Mean Diff.	t	Significant? P < 0.05?	Summary
PBS vs K-CTA1R7K(C187A)-MOG(35-55)-DD	0.9230	9.384	Yes	****
PBS vs K-CTA1R7K(C187A)-PLP(178-1)-DD	0.4333	4.406	Yes	***
PBS vs K-CTA1R7K(C187A)-MOG-PLP-DD	1.227	12.47	Yes	****
PBS vs K-CTA1R7K(C187A)-huMOG(10-60)-	1.060	10.78	Yes	****
K-CTA1R7K(C187A)-MOG(35-55)-DD vs K-CTA1R7K(C187A)-PLP(178-1)-DD	-0.4896	4.978	Yes	****
K-CTA1R7K(C187A)-MOG(35-55)-DD vs K-CTA1R7K(C187A)-MOG-PLP-DD	0.3037	3.088	Yes	*
K-CTA1R7K(C187A)-MOG(35-55)-DD vs K-CTA1R7K(C187A)-huMOG(10-60)-	0.1370	1.393	No	ns
K-CTA1R7K(C187A)-PLP(178-1)-DD vs K-CTA1R7K(C187A)-MOG-PLP-DD	0.7933	8.066	Yes	****

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2013/053278

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 A61K39/35 A61K39/39 C12N15/62
ADD.

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)
A61K C12N

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 practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/078796 A1 (MIVAC DEV AKTIEBOLAG [SE]; LYCKE NILS [SE]) 25 June 2009 (2009-06-25) cited in the application the whole document	1-12

X,P	WO 2012/057671 A1 (MIVAC DEV AKTIEBOLAG [SE]; LYCKE NILS [SE]) 3 May 2012 (2012-05-03) the whole document	1-12

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family 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 application or patent 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

"T" 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

"X" 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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 August 2013	05/09/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Valcárcel, Rafael

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	A. HASSELBERG ET AL: "ADP-Ribosylation Controls the Outcome of Tolerance or Enhanced Priming Following Mucosal Immunization", THE JOURNAL OF IMMUNOLOGY, vol. 184, no. 6, 15 March 2010 (2010-03-15), pages 2776-2784, XP055049150, ISSN: 0022-1767, DOI: 10.4049/jimmunol.0901445 the whole document	1-12

Y	AGREN L C ET AL: "Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit", THE JOURNAL OF IMMUNOLOGY, THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS, US, vol. 158, no. 8, 15 April 1997 (1997-04-15), pages 3936-3946, XP002628512, ISSN: 0022-1767 cited in the application the whole document	1-12

Y	US 5 917 026 A (LOEWENADLER BJOERN [SE] ET AL) 29 June 1999 (1999-06-29) cited in the application the whole document	1-12

Y	FRANCISCO J QUINTANA ET AL: "Antigen microarrays identify unique serum autoantibody signatures in clinical and pathologic subtypes of multiple sclerosis", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 105, no. 48, 12 February 2008 (2008-02-12), pages 18889-18894, XP008147701, ISSN: 0027-8424, DOI: 10.1073/PNAS.0806310105 [retrieved on 2008-11-21] cited in the application the whole document	1-12

	-/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LYCKE NILS: "ADP-ribosylating bacterial enzymes for the targeted control of mucosal tolerance and immunity", ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, WILEY-BLACKWELL PUBLISHING, INC, US, vol. 29, no. 1, 1 December 2004 (2004-12-01), pages 193-208, XP008131531, ISSN: 0077-8923, DOI: 10.1196/ANNALS.1309.036 the whole document -----	1-12
Y	LYCKE ET AL: "From toxin to adjuvant: basic mechanisms for the control of mucosal IgA immunity and tolerance", IMMUNOLOGY LETTERS, ELSEVIER BV, NL, vol. 97, no. 2, 15 March 2005 (2005-03-15) , pages 193-198, XP027672186, ISSN: 0165-2478 [retrieved on 2005-03-15] the whole document -----	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2013/053278

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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