Title: SOLUBLE COMPOSITIONS FOR THE TREATMENT OF CXCR3-LIGAND ASSOCIATED DISEASES

Abstract: Isolated soluble CXCR3 molecules binding to CXCR3 ligands are provided. Thus, for example, provided is a soluble polypeptide comprising a heterologous amino acid sequence conjugated to a CXCR3 amino acid sequence comprising an amino acid sequence of an extracellular loop 3 (E3) domain of CXCR3 having a substitution mutation at the amino acid corresponding to aspartic acid (D) at amino acid coordinate 278 of CXCR3, wherein said soluble polypeptide binds at least one CXCR3 ligand. Use of such molecules in the treatment of CXCR3-associated diseases in general and Multiple Sclerosis in particular is also envisaged.
SOLUBLE COMPOSITIONS FOR THE TREATMENT OF CXCR3-LIGAND ASSOCIATED DISEASES

FIELD OF THE INVENTION

The present invention relates to soluble CXCR3 molecules and, more particularly, to methods of using same for treating CXCR3- and CXCR3- associated diseases, such as inflammatory diseases.

BACKGROUND OF THE INVENTION

Immunological diseases/disorders, like autoimmune diseases, inflammation disorders as well as infectious diseases are not only increasing but represent substantial threats to global health. Currently, three groups of drugs—non-steroidal anti-rheumatics, cortisone preparations and second-line agents—and TNF-α blocking agents are used for treating inflammatory joint diseases. Non-steroidal anti-rheumatics have many side effects when applied frequently (e.g. gastric ulcers, nephroses). In high dosages, cortisone preparations lead to a quick relapse after discontinuation of the therapy, and entail severe side effects. Second-line agents entail considerable toxicity (e.g., allergies, infections, malignant diseases, renal insufficiency, etc). In many cases no sufficient reduction of disease activity is achieved with current drugs, such that even surgical intervention is sometimes necessary.

Chemokines are small (~8-14 kDa), structurally cytokine-like, secreted proteins that regulate cell trafficking. They are produced and secreted by a wide variety of cell types in response to early inflammatory mediators, such as IL-1β or TNF-α, and in response to bacterial or viral infection. Chemokines function mainly as chemoattractants for leukocytes, recruiting monocytes, neutrophils and other effector cells from the blood to sites of infection or damage. They can be released by many different cell types (e.g. macrophages) and can mediate a range of pro-inflammatory effects on leukocytes, such as triggering of chemotaxis, degranulation, synthesis of lipid mediators, and integrin activation.

Chemokines can be subdivided into four classes, the C-C, C-X-C, C and C-X3-C chemokines, depending on the location of the first two cysteines in their protein sequence. The interaction of these soluble proteins with their specific receptors, which
belong to the superfamily of seven-transmembrane domain G-protein-coupled receptors (GPCRs), mediate their biological effects resulting in, among other responses, rapid increase in intracellular calcium concentration, changes in cell shape, increased expression of cellular adhesion molecules, degranulation and promotion of cell migration.

In the last several years, the key role of chemokines as important mediators in inflammatory, autoimmune, infectious and cancerous disorders and diseases has been well established.

The chemokine receptor CXCR3, also referred to as G protein-coupled receptor 9 (GPR9) and CD183, is predominantly expressed on inflammatory effector T cells, including Th1 as well as the newly defined IL-17 producing Th_{17} cells, CD4+, CD8+ cells, plasmacytoid dendritic cells, but is also expressed on other lymphocytes, including B cells and NK-T cells. CXCR3 is highly induced following cell activation. Three chemokine ligands compete for binding to this receptor: CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC) [Colvin et al., J Biol Chem (2004) 279:30219-27]. These ligands bind different epitopes on CXCR3, yet CXCL11 binds CXCR3 with higher affinity than CXCL9 and CXCL10. CXCL11 may antagonize the function of the other two CXCR3 ligands since it rapidly leads to receptor internalization, which thus becomes inaccessible to the other CXCR3 ligands (Colvin et al., supra). It has been suggested that CXCR3 ligands bind to different sites on the extracellular domains of the receptor, and that activation of CXCR3 signaling pathways may require additional interactions with its ligands following initial binding (Colvin et al., supra).

CXCL9 and CXCL10 represent a sub-group of the CXC chemokines, more closely related to each other (37% amino acid identity) than to other CXC chemokines and their expression by keratinocytes, endothelial cells, lymphocytes, monocytes and neutrophils is induced by IFN-γ. CXCL9 and CXCL10 also differ from other CXC chemokines in that they do not attract neutrophils but instead stimulate migration of monocytes and activated T lymphocytes. The genes encoding CXCL9 and CXCL10 are close together on chromosome 4 at a locus distinct from the cluster of other CXC chemokine gene. These chemokines have been implicated in the recruitment of such cells in delayed-type hypersensitivity (DTH) cutaneous lesions in tuberculoid leprosy, in cutaneous Leishmaniasis, and in autoimmune inflammatory diseases such as
psoriasis, in which high CXCR10 expression levels were detected. Mig and IP-10 are implicated in the recruitment of activated T cells following virus or protozoal infection and in some situations can contribute to pathology e.g. in chronic infection with hepatitis C virus. Other conditions in which IP-10 may play a causative role in disease are in adult respiratory distress syndrome, Lyme disease, atherosclerosis and restenosis, breast cancer cell migration, invasion and metastasis, cutaneous T cell lymphomas and tubulointerstitial nephritis associated with glomerular disease. IP-10 and Mig also suppress production of haemopoietic progenitor cells which might be disadvantageous following bone marrow transplants.

CXCR3 and CXCR3 ligands have been implicated in the pathogenesis of multiple sclerosis (MS), inflammatory bowel disease (IBD), periodontal disease, optic neuritis, pre-eclampsia, cystitis, cerebral malaria, allergic responses, COPD and asthma, atherosclerosis, glomerulonephritis, pancreatitis, restenosis, rheumatoid arthritis (RA), diabetic nephropathy, pulmonary fibrosis, transplant rejection, ischemia-reperfusion injury and cancer. CXCR3 ligands have been differentially indicated as important mediators in many diseases: elevated levels of CXCL9 and CXCL10 are found in multiple sclerosis, COPD and asthma, chronic HCV infection, acute cystitis, optic neuritis, metastatic disease and many inflammatory and auto-immune diseases. Blocking CXCL9 and CXCL10 has been shown effective in suppressing or alleviating symptoms of multiple sclerosis, allograft rejection, ischemia-perfusion injury, acute cystitis, smoke-induced lung injury, metastatic melanoma and other CXCR3-associated conditions. Recent studies have shown that administering CXCL11 is effective in altering the cytokine profile of activated T-cells and suppressing clinical symptoms in animal models of multiple sclerosis (see PCT publication WO2008/149354 to Karin et al), and in preventing pulmonary fibrosis (see Burdick et al, Am Thorac Soc 2005;171:261-68) and tumorogenesis (Hensbergen et al, J Immunotherapy, 2005;28:343-51).

Taken together, these studies support a pivotal role for the CXCR3/CXCL10/CXCL9 axis in the pathogenesis of numerous diseases, and for therapeutic applications of selective inhibition of CXCR3 ligand effects.

Various approaches for blocking CXCR3/CXCL9/CXCL10 activation have been attempted, some are summarized infra.
U.S. Publication No. 20050191293 to Deshpande et al. discloses anti CXCL10 antibodies, antibody conjugates and and bispecific antibodies which bind the chemokine and an antibody receptor, causing effector cell mediated activity (phagocytosis, etc), leading to depletion of CXCL10-producing cells.

U.S. Patent No. 6,723,538, and US Publications 20050191702 and 20030017979, all to Mack et al, disclose chimeric polypeptides having first moieties binding chemokine receptors and second moieties binding to a T-cell surface polypeptide or a cell toxin. The inventors demonstrated effective depletion of CXCR3-specific target cells from inflamed tissue using a fusion protein having a CXCR3 ligand moiety fused to a cell toxin.

US Patent No. 6,843,991 to Efstathiou et al. teaches the use of an isolated Herpesvirus protein (M3 of MHV68) with broad non-specific chemokine binding properties for blocking inflammation. US Patent No. 6,355,252 and US Publication 20020071849 to Smith et al. disclose a soluble poxvirus chemokine receptor (from Vaccinia) fragment (the A41L protein), which has specific CXCL9 and CXCL10 binding properties.

US Patent Nos. 7,259,000, 6,686,175 and 6,140,064, all to Loetscher et al., disclose the cloning and recombinant expression of the human CXCR3 receptor, and anti-CXCR3 monoclonal antibodies recognizing epitopes found within the region of amino acid coordinates 1-45 of CXCR3. Cloned CXCR3, expressed in cultured lymphocytes, demonstrated effective chemokine binding. The use of the receptor protein, soluble receptors, fragments thereof and the monoclonal antibodies for diagnostics and therapeutics of inflammatory disease was contemplated.

PCT Publication No. WO05049799 discloses chimeric chemokine receptors. This invention teaches chimeric chemokine receptors comprising N terminal and TM regions of a first chemokine receptor, such as CCR3, and the intracellular C terminus of a second chemokine receptor, such as CCR2. The first or second chemokine receptors may comprise CXCR3 domains. PCT Publication No. WO05049799 specifically states that GPCRs retain all of their known ligand binding regions within the extracellular regions and TM domains 2 through 7.

Colvin et al. (MoI Cell Biol 2006;26:5838-49) have created a number of point mutations and amino acid substitutions in CXCR3 domains in order to analyze the
extracellular domains and identify residues important for CXCR3 ligand binding and receptor activation, contemplating inhibitors specifically targeted to CXCR3-mediated inflammation. Mutations in N-terminal tyrosine residues were found to prevent binding and signal induction with all three CXCR3 ligands. Deletion of proximal N-terminal amino acids was found to be important to CXCL10 and CXCL11, but not CXCL9 binding and activation. Mutations of basic residues R197 and R212, but not R216 in the second extracellular loop domain prevented binding and activation for all three CXCR3 ligands. Mutations of charged residues D112 and D278 in the first and third extracellular loop domains were found to prevent binding and activation for all three CXCR3 ligands, while mutations in D282 and E293 inhibited activation by CXCL9 and CXCL10, but not CXCL11 activation. The authors concluded that all four extracellular domains are important for binding, that CXCR3 binding requires ligand interactions with at least one sulfated tyrosine in the N-terminus, that charged residues R197, R212, D112 and D278 are essential for binding all three CXCR3 ligands and that CXCL10 and CXCL11 require the proximal terminus for stable binding. No residues critical to CXCL11-specific binding were uncovered.

PCT publication WO2005/103722 discloses diagnostic and therapeutic use of purified native or recombinant CXCR3 protein. CXCR3 fusion proteins having C or N terminal sequences, such as signal sequences for secretion, increased solubility, ease of separation in solution and purification are contemplated.

Dr. Christian Engwerda (see website of Queensland Institute of Medical Research, QIRM, Australia) has proposed developing soluble CXCR3 receptors for studying the role of CXCR3 ligands in infectious diseases such as cerebral malaria and visceral Leishmania, and perhaps provide a means of blocking the chemokines recruitment of leukocytes into infected tissue.

There is thus a widely recognized need and it would be highly advantageous to have therapeutic modalities which target the CXCR3/CXCL9/CXCL10 axis and which can be used in the treatment of the many and varied CXCR3/CXCL9/CXCL10-associated inflammatory, autoimmune, infectious and metastatic disease.
SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided an isolated soluble polypeptide comprising an amino acid sequence of extracellular loop 3 (E3) domain of CXCR3 (SEQ ID NO: 9), the amino acid sequence having a substitution mutation at the amino acid corresponding to aspartic acid (D) at amino acid coordinate 278 of CXCR3 (SEQ ID NO: 11), wherein the soluble polypeptide binds at least one CXCR3 ligand.

According to one aspect of the present invention there is provided a method of treating a CXCR3-associated disease in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the isolated soluble polypeptide, thereby treating the CXCR3-associated disease in the subject.

According to another aspect of the present invention there is provided a use of the isolated soluble polypeptide for the manufacture of a medicament identified for treating a CXCR3-associated disease.

According to further features in preferred embodiments of the invention described below the isolated soluble polypeptide is devoid of the N-terminal domain (SEQ ID NO: 8) of CXCR3.

According to yet further features in preferred embodiments of the invention described below the aspartic acid at amino acid coordinate 278 is substituted with a non-charged amino acid.

According to still further features in preferred embodiments of the invention described below the non-charged amino acid sequence is alanine.

According to further features in preferred embodiments of the invention described below the amino acid sequence of the soluble polypeptide is as set forth in SEQ ID NO: 10.

According to another aspect of the present invention there is provided an isolated soluble polypeptide comprising an amino acid sequence of CXCR3 (SEQ ID NO: 11), wherein the soluble polypeptide binds at least one CXCR3 ligand and is devoid of the N-terminal domain of CXCR3 (SEQ ID NO: 8).
According to another aspect of the present invention there is provided a pharmaceutical composition comprising the isolated soluble polypeptide and a pharmaceutically acceptable carrier.

According to yet another aspect of the present invention there is provided an isolated polynucleotide encoding the isolated soluble polypeptide.

According to still another aspect of the present invention there is provided a nucleic acid construct comprising the isolated polynucleotide encoding the isolated soluble polypeptide.

According to still further features in preferred embodiments of the invention described below the amino acid sequence is a CXCR3 extracellular loop domain.

According to yet further features in preferred embodiments of the invention described below the isolated soluble polypeptide is as set forth in SEQ ID NO: 11 (native E3).

According to further features in preferred embodiments of the invention described below the amino acid sequence is a mutated CXCR3 extracellular (E3) loop domain having a substitution mutation at the amino acid corresponding to aspartic acid (D) at amino acid coordinate 278 of CXCR3 (SEQ ID NO: 11).

According to still further features in preferred embodiments of the invention described below the isolated soluble polypeptide further comprising a heterologous amino acid sequence contiguously attached to the amino acid sequence.

According to yet further features in preferred embodiments of the invention described below the heterologous sequence is an immunoglobulin amino acid sequence.

According to still further features in preferred embodiments of the invention described below the immunoglobulin sequence is an IgG Fc amino acid sequence.

According to still further features in preferred embodiments of the invention described below the isolated soluble polypeptide comprises the amino acid sequence as set forth in SEQ ID NO: 9 or 10 (non-mutated or mutated E3).

According to still further features in preferred embodiments of the invention described below the isolated polynucleotide comprises the nucleic acid sequence as set forth in SEQ ID NOs: 23, 24, 25 or 26.
According to further features in preferred embodiments of the invention described below the CXCR3-associated disease is a disease associated with increased CXCL9 or CXCL10.

According to yet further features in preferred embodiments of the invention described below the CXCR3-associated disease is a disease associated with decreased CXCL11.

According to yet further features in preferred embodiments of the invention described below the CXCR3-associated disease is selected from the group consisting of multiple sclerosis, rheumatoid arthritis, hematological diseases, cardiovascular diseases, disorders of the peripheral and central nervous system, genitourinary diseases, respiratory diseases, parasitic infection, viral infection, bacterial infection, respiratory diseases, ischemia-reperfusion injury, allograft rejection, auto-immune disease, inflammatory disease and cancer.

According to still further features in preferred embodiments of the invention described below the CXCR3-associated disease is Multiple Sclerosis.

According to further features in preferred embodiments of the invention described below the method comprises administering to the subject a therapeutically effective amount of the isolated soluble polypeptide and further comprises concomitantly administering to said subject an anti-Multiple Sclerosis drug selected from the group consisting of Interferon Beta Ia, Interferon Beta Ib, Glatiramer Acetate, Mitoxantrone, Methylprednisolone, Prednisone, Prednisolone, Dexamethasone, Adrenocorticotropic Hormone (ACTH) and Corticotropin.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with
the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1A-1B are schematics showing the construction of the CXCR3-3(\textsubscript{mut})-Ig fusion protein (SEQ ID NO:1) nucleic acid construct. FIG. 1A shows the nucleotide sequence encoding the mutated CXCR3-3 D278A fragment (SEQ ID NO:2), with the arrow indicating the single nucleotide mutation (G → A), resulting in the D278A amino acid replacement. FIG. 1B shows the construction of the CXCR3-3(\textsubscript{mut})-Ig expression vector, with the inset showing the mutated CXCR3-3 fragment amino acid sequence (MUT) (SEQ ID NO:3), compared with the wild type (E-3) CXCR3-3 fragment sequence.

FIG. 2A is a histogram depicting the selective binding of CXCL9 and CXCL10, but not CXCL12 by CXCR3-3(\textsubscript{mut})-Ig (SEQ ID NO:1). Binding of soluble chemokine receptor fusion proteins representing CXCR3-3-2-Ig fusion protein (E-2) (SEQ ID NO:4), CXCR3-N-terminal-Ig (NT) (SEQ ID NO:5), native CXCR3-3-3-Ig (E-3) (SEQ ID NO:6) or mutant CXCR3-3(\textsubscript{mut})-3-Ig (MUT) fusion protein (SEQ ID NO:1) to CXCL9, CXCL10 and CXCL12 (Peprotec, Rocky Hill, NJ) was detected by an ELISA assay, developed and visualized by goat anti-hlgG-HRP (Jackson ImmunoResearch, Inc., West Grove, PA). O.D. was determined at 450nm with the reference filter set to 620nm. Note the highly specific binding of CXCL9, CXCL10, but not CXCL12.

FIG. 2B is a histogram depicting the selective binding of CXCL9 and CXCL10 to CXCR3-3(\textsubscript{mut})-3-Ig(SEQ ID NO:1) compared to different CC and CXC chemokines. Binding of the mutant CXCR3-3(\textsubscript{mut})-3-Ig (MUT) fusion protein (SEQ ID NO:1) to different CC and CXC chemokines (CXCL9, CXCL10, CXCL12, CXCL16 and CCL3; R&D Systems, Minneapolis, MN) was detected by an ELISA assay, developed and visualized by goat anti-hlgG-HRP (Jackson ImmunoResearch, Inc., West Grove, PA). O.D. was determined at 450 nm with the reference filter set to 620 nm. Note the highly specific binding of CXCL9 and CXCL10.

FIG. 3 is a histogram depicting selective inhibition of CXCL9 and CXCL10-induced chemotaxis/migration of activated CD4+ T cells by CXCR3-3(\textsubscript{mut})-3-Ig (SEQ ID NO:1). CXCL9, CXCL10 and CXCL12-induced migration of activated CD4+ T cells (IXIO\textsuperscript{6}) were performed in the presence of 50 µg/ml of either mutant CXCR3-3(\textsubscript{mut})-
Ig fusion protein (SEQ ID NO:1) (white columns) or control isotype-matched IgG (grey columns), or without added protein (PBS, black columns). Migrating cells were collected, counted by FACS, and chemotaxis index calculated. Note the absence of inhibition of CXCL1-induced migration by CXCR3-E3(mut)-Ig (SEQ ID NO:1);

FIGs. 4A-4F are histograms illustrating the anti-inflammatory effect of CXCR3-E3(mut)-Ig fusion protein (SEQ ID NO:1) on *in-vitro* cytokine production in antigen-activated primary T cells. Pre-EAE primary splenocytes isolated from EAE induced C57BL/6 mice (day 9) were cultured and stimulated with their target autoimmune antigen, and then exposed to 50 ng/ml of either recombinant CXCR3(mut)-Ig fusion protein or a control isotype IgGl. Cytokines IL-17, IL-10, IL-12, IFNγ, IL-2 and IL-4 secreted by the cells were then measured by commercially available ELISA kits. Note the increase in anti-inflammatory cytokines IL4 (FIG. 4A) and IL10 (FIG. 4B), and the decrease in pro-inflammatory cytokines (IL-17, FIG 4C; IFNγ, FIG. 4E; IL-12, FIG. 4D and IL-2, FIG 4F) induced by the CXCR3(mut)-IgG fusion protein (SEQ ID NO:1);

FIGs. 5A and 5B are graphs illustrating effective *in-vivo* suppression of EAE in mice by administration of CXCR3-E3(mut)-Ig fusion protein (SEQ ID NO:1). EAE-induced mice (day 13) were treated with repeated intra-peritoneal (IP) administration (every other day) of 300 μg/mouse of either CXCR3(mut)-IgG (SEQ ID NO:1), isotype IgGl control, or PBS, and monitored daily for clinical symptoms. Note the decline (day 18) and total resolution (day 22) of symptoms in both representative experiments (5A and 5B);

FIG. 6 is a photomicrograph of hematoxylin-eosin stained histological sections from spinal cords of EAE-induced mice following treatment with CXCR3(mut)-IgG (SEQ ID NO:1), isotype IgGl control, or PBS, as indicated. Magnification X10 or X40 as indicated. Note the significant inhibition of demyelination in CXCR3-E3(mut)-Ig treated mice.

FIGs. 7A-7D are histograms illustrating the anti-inflammatory effect of CXCR3-E3(mut)-Ig fusion protein (SEQ ID NO:1) on *in-vitro* cytokine production in antigen-activated primary T cells. PLP139-151/CFA specific primary T cells from sick SJL mice were cultured and stimulated with their target autoimmune antigen (PLP) and then exposed to 50 ng/ml of either recombinant CXCR3(mut)-IgG fusion protein (right column) or a control isotype IgGl (middle column). Cytokines IL-10 (FIG. 7A), IL-4
(FIG. 7B), IFN-γ (FIG. 7C) and IL-17 (FIG. 7D) secreted by the cells were then measured by commercially available ELISA kits. The results shown depict the results of one out of three independent experiments with similar observations. Note the significant increase in anti-inflammatory cytokines IL-4 and IL-10 (FIGs. 7B and 7A, respectively, p<0.001) and the decrease in pro-inflammatory cytokines IL-17 and IFN-γ (FIGs. 7D and 7C, respectively, P<0.001) induced by the CXCR3(mut)-IgG fusion protein (SEQ ID NO:1);

FIG. 8 is a graph illustrating effective in-vivo suppression of EAE in SJL mice by administration of CXCR3-E3(mut)-Ig fusion protein (SEQ ID NO:1). EAE-induced SJL mice (12 per group) were treated with repeated intra-peritoneal (IP) administration (every other day) of 300 µg/mouse of either CXCR3(mut)-IgG (SEQ ID NO:1), isotype IgGl control, or PBS, and monitored daily for clinical symptoms. Results are shown as mean maximal score ± SE. Note the decline (day 13) and total resolution (day 16) of symptoms.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of compositions for modulating signaling through CXCR3, particularly of CXCL9/CXCL10-specific binding proteins and uses of same for treating CXCR3-associated conditions such as inflammation, infection and respiratory disease.

The principles and operation of the method according to the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

CXCR3 and CXCR3 ligands have been implicated in the pathogenesis of multiple sclerosis (MS), inflammatory bowel disease (IBD), periodontal disease, optic neuritis, pre-eclampsia, cystitis, cerebral malaria, allergic responses, COPD and asthma, atherosclerosis, glomerulonephritis, pancreatitis, restenosis, rheumatoid arthritis (RA),
diabetic nephropathy, pulmonary fibrosis, transplant rejection, ischemia-reperfusion injury and cancer. Elevated levels of CXCL9 and CXCL10 are found in multiple sclerosis, COPD and asthma, chronic HCV infection, acute cystitis, optic neuritis, metastatic disease and many inflammatory and auto-immune diseases. Blocking CXCL9 and CXCL10 has been shown effective in suppressing or alleviating symptoms of multiple sclerosis, allograft rejection, ischemia-perfusion injury, acute cystitis, smoke-induced lung injury, metastatic melanoma and other CXCR3-associated conditions, while administering CXCL11 is effective in suppressing clinical symptoms of multiple sclerosis and preventing pulmonary fibrosis and tumorogenesis.

However, agents capable of effectively inhibiting CXCR3 binding and activation by CXCL9 and CXCL10, but not CXCL11, have not been available.

While reducing the present invention to practice, the present inventors have surprisingly discovered that residue D278 of the E3 extracellular loop domain of CXCR3 is critical for binding and activation of CXCL11, but not CXCL9 and CXCL10, and that an isolated CXCR3 E3 polypeptide bearing the amino acid substitution D278A selectively binds and inhibits signaling function of CXCL9 and CXCL10, but not CXCL11. Providing such a CXCL9/CXCL10-specific binding molecule is critical to the development of new therapeutic modalities for CXCR3/CXCL9/CXCL10- associated diseases, such as multiple sclerosis.

The present inventors have constructed mutant CXCR3 E3(mut) and soluble CXCR3 E3(mut)-Ig fusion polypeptides, expressed them in mammalian cell systems (see Example I of the Examples section which follows). Specificity of binding affinity of the fusion polypeptides, and the resultant inhibition of CXCL9 and CXCL10 effects on T cells was demonstrated (see Examples II and III of the Examples section which follows).

Thus, the present inventors have surprisingly shown, for the first time, that binding of CXCR3 ligands CXCL9 and CXCL10 to an extracellular loop domain is not dependent on binding or the presence of all or any of the N-terminal domain (see Example II of the Examples hereinbelow), thus providing for the first time a soluble CXCR3 which can be short enough for in-vivo therapeutic applications (as was well demonstrated in in-vivo treatment of multiple sclerosis in both C57BL/6 and SJL murine models, see Examples IV and VI, respectively, of the Examples hereinbelow). Such short polypeptides are advantageous as they allow economy of dosage, produce fewer extraneous and
undesirable effects, may be easily produced by chemical synthesis, and are easier to purify.

Thus, according to one aspect of the present invention, there is provided an isolated soluble polypeptide comprising an amino acid sequence of CXCR3 (SEQ ID NO: 11), wherein said soluble polypeptide binds at least one CXCR3 ligand and is devoid of the N-terminal domain of CXCR3 (SEQ ID NO:8). It will be appreciated that the isolated soluble CXCR3 E3 domain polypeptide is devoid of CXCR3 N-terminal domain amino acid sequences. Thus, for example, the soluble polypeptide of the present invention can be devoid of an amino acid sequence representing any or all of amino acid coordinates 1-58 of murine CXCR3 NT domain (SEQ ID NO: 8), as described above. In one embodiment, the isolated soluble CXCR3 polypeptide devoid of the CXCR3 N-terminal sequence can be any CXCR3 portion or fragment having CXCR3 ligand binding properties, for example, an extracellular loop domain such as native CXCR3 E3, such as SEQ ID NO:9, or a mutated CXCR3 E3 loop domain, such as SEQ ID NO: 10, having a D278A substitution mutation.

Thus, according to yet another aspect of one exemplary embodiment of the present invention there is provided an isolated soluble polypeptide comprising an amino acid sequence of extracellular loop 3 (E3) domain of CXCR3 (SEQ ID NO: 9), said amino acid sequence having a substitution mutation at the amino acid corresponding to aspartic acid (D) at amino acid coordinate 278 of CXCR3 (SEQ ID NO: 10), wherein said soluble polypeptide binds at least one CXCR3 ligand.

As used herein the term "CXCR3" refers to naturally occurring or endogenous mammalian CXCR3 proteins and to proteins having an amino acid sequence which is the same as that of a naturally occurring or endogenous corresponding mammalian CXCR3 protein (e.g., recombinant proteins), including mature protein, polymorphic or allelic variants, and other isoforms of mammalian CXCR3 (e.g., produced by alternative splicing or other cellular processes), and modified or unmodified forms of the foregoing (e.g., glycosylated, unglycosylated, phosphorylated or unphosphorylated CXCR3 proteins), such as set forth in GenBank Accession Nos. NP_001495 (SEQ ID NO: 11) or NP_001136269.1 (SEQ ID NO: 12).
CXCR3 proteins are characterized by seven transmembrane domains and four extracellular domains, the N-terminal "CXCR3-NT" domain, and three extracellular loop domains designated "CXCR3-E1", "CXCR3-E2" and "CXCR3-E3" loop domains.

As used herein, the term "extracellular domain" refers to a portion or fragment of a CXCR3 protein naturally found on the external surface, and not within the membrane, of a mammalian cell expressing CXCR3, functional variants and fragments thereof. The extracellular domains of human CXCR3 are as follows: human CXCR3-NT (SEQ ID NO:8), human CXCR3-E1 (SEQ ID NO: 13), human CXCR3-E2 (SEQ ID NO: 14) and human CXCR3-E3 (SEQ ID NO: 9). According to an exemplary embodiment of this aspect of the present invention, the soluble CXCR3 polypeptide does not comprise at least a portion of CXCR3-NT. According to further exemplary embodiment the isolated soluble polypeptide does not comprise Y27 and Y29 of CXCR3-NT (SEQ ID NO: 8).

According to further exemplary embodiment the isolated polypeptide is devoid of at least CXCR3-E1 and CXCR3-E2.

According to an exemplary embodiment, the CXCR3 polypeptide of the invention is a mutant polypeptide, having a mutation at the amino acid corresponding to aspartic acid (D) at amino acid coordinate 278 of CXCR3. As used herein, the term "mutation" refers to a deletion or substitution, for example, the substitution for aspartic acid at coordinate D278 of any amino acid that is not aspartic acid, such as, for example, alanine (A). Such a mutation is designated D278A. As generally described herein, a single amino acid residue substitution can be indicated as follows: the original amino acid residue (expressed as a single-letter abbreviation), followed by the position of the original amino acid residue (i.e., a numerical expression), followed by the new amino acid residue (expressed as a single-letter abbreviation) to be inserted in place of the original amino acid residue. For example, "D278A" means that the original aspartic acid (D) residue at position 278 is to be replaced by the new alanine (A) residue. For multiple substitutions (e.g., double-substitutions, triple-substitutions, and quadruple-substitutions), the various substitutions are separated by either a slash (/) or by a space. An example of a double-substitution may be expressed as either "D278A/E293A" or as "D278A E293A."
Inasmuch as D278 is a charged amino acid, suitable amino acids for substitution may be optionally chosen from non-charged or non-polar, or slightly polar amino acids such as alanine, glutamine, glycine, isoleucine, valine, etc.

As used herein, the term "soluble" refers to the ability of the polypeptides and proteins of the present invention to dissolve in a physiological aqueous solution (pH about 7, e.g., solubility level in aqueous media of >100 µg/ml) without substantial aggregation. Thus, it is readily understood that soluble CXCR3 extracellular loop proteins, such as CXCR3-E3, are preferably devoid of any hydrophobic transmembrane CXCR3 domains.

The soluble polypeptides of the present invention bind at least one CXCR3 ligand. As used herein the phrase "bind at least one CXCR3 ligand" refers to the extracellular loop domain polypeptide having binding affinity for at least one of CXCR3 ligands (e.g., CXCL9, CXCL10 and CXCL11). Binding affinity refers to a minimal \( K_d \) value of at least \( 10^{-6} \) M, \( 10^{-7} \) M, \( 10^{-8} \) M, \( 10^{-9} \) M, \( 10^{-10} \) M. Methods of assaying ligands for qualified affinity are well known in the art and include Scatchard plotting. It should be noted that a single CXCR3 amino acid sequence may be included in the molecules of the present invention, but inclusion of at least two CXCR3 amino acid sequences (e.g., of similar affinity), each being capable of binding CXCR3 ligand (preferably with high affinity) may be preferred. Due to increased avidity, these polypeptides may be used as potent inhibitors of CXCR3 ligand activity and lower dosages may be administered. An example of a mutant CXCR amino acid sequence according to the teachings of the present invention is set forth in SEQ ID NO: 10. Such CXCR3 amino acid sequences may be encoded, by way of example, by nucleic acid sequences as set forth in SEQ ID NO. 15, SEQ ID NO. 16, SEQ ID NO. 17 and SEQ ID NO. 18 (E2E3, E3E2, E3E3, E2E3E3, respectively).

As shown in the Examples section hereinbelow, the soluble CXCR3 E3 domain polypeptide having a D278A substitution mutation selectively binds CXCL9 and CXCL10, but not CXCL11. Thus, according to one embodiment of the present invention, the soluble CXCR3 E3 domain polypeptide has reduced binding to CXCL11, as compared to that of native CXCR3 E3 domain. According to yet another embodiment of the present invention, the soluble CXCR3 E3 domain polypeptide is capable of binding CXCL9 and CXCL10 with similar affinity as native CXCR E3 domain.
Reduced or similar binding, as compared with native CXCR3 E3 domain, can be assayed using methods known in the art, directly or in competition binding assays using both native and soluble mutant CXCR3 E3 polypeptides. In one exemplary embodiment, reduced binding is determined as having a $K_D$ value greater than $10^{-5}$M, $10^{-4}$M, $10^{-3}$M.

Native murine CXCR3-E3 polypeptide is 25 amino acids in length. The soluble mutant CXCR3-E3 polypeptide of the present invention can comprise an amino acid sequence having fewer or greater than 25 amino acids, but retaining the CXCR3 ligand binding properties described herein. Due to the soluble nature of the molecules of the present invention, the soluble polypeptide is no longer than 50 amino acids in length.

It will be noted that the isolated soluble polypeptide of the present invention can be non-immunogenic in a human subject (for maximizing therapeutic efficacy). Such molecules can be devoid of CXCR3 extracellular domain sequences which are not necessary for ligand binding.

The term "polypeptide" as used herein encompasses native peptides (either degradation products, synthetically synthesized peptides, or recombinant peptides), peptidomimetics (typically, synthetically synthesized peptides), and the peptide analogues peptoids and semipeptoids, and may have, for example, modifications rendering the peptides more stable while in a body or more capable of penetrating into cells. Such modifications include, but are not limited to: N-terminus modifications; C-terminus modifications; peptide bond modifications, including but not limited to CH$_2$-NH, CH$_2$-S, CH$_2$-S=O, O=C-NH, CH$_2$-O, CH$_2$-CH$_2$, S=C-NH, CH=CH, and CF=CH; backbone modifications; and residue modifications. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in Ramsden, C. A., ed. (1992), Quantitative Drug Design, Chapter 17.2, F. Choplin Pergamon Press, which is incorporated by reference as if fully set forth herein. Further details in this respect are provided hereinbelow.

Peptide bonds (-C0-NH-) within the peptide may be substituted, for example, by N-methylated bonds (-N(CHS)-CO-); ester bonds (-C(R)H-C-O-O-C(R)-N-); ketomethylene bonds (-CO-CH2-); $\alpha$-aza bonds (-NH-N(R)-CO-), wherein R is any alkyl group, e.g., methyl; carba bonds (-CH2-NH-); hydroxyethylene bonds (-CH(OH)-CH2-); thioamide bonds (-CS-NH-); olefinic double bonds (-CH=CH-); retro amide
bonds (-NH-CO-); and peptide derivatives (-N(R)-CH2-CO~), wherein R is the "normal" side chain, naturally presented on the carbon atom. These modifications can occur at any of the bonds along the peptide chain and even at several (2-3) at the same time.

Natural aromatic amino acids, Trp, Tyr, and Phe, may be substituted for synthetic non-natural acids such as, for instance, tetrahydroisoquinoline-3-carboxylic acid (TIC), naphthylelanine (NoI), ring-methylated derivatives of Phe, halogenated derivatives of Phe, and o-methyl-Tyr.

In addition to the above, the polypeptides of the present invention may also include one or more modified amino acids or one or more non-amino acid monomers (e.g., fatty acids, complex carbohydrates, etc.).

The term "amino acid" or "amino acids" is understood to include the 20 naturally occurring amino acids; those amino acids often modified post-translationally in vivo, including, for example, hydroxyproline, phosphoserine, and phosphothreonine; and other less common amino acids, including but not limited to 2-amino adipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine, and ornithine. Furthermore, the term "amino acid" includes both D- and L-amino acids.

Tables 1 and 2 below list naturally occurring amino acids (Table 1) and non-conventional or modified amino acids (Table 2) which can be used with the present invention.

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Generation of peptide mimetics (e.g., which comprise a CXCR3 E3 amino acid sequence with various natural and/or synthetic alterations but which still display dominant negative activity), as described hereinabove, can be effected using various approaches, including, for example, display techniques.

Thus, the present invention contemplates a display library comprising a plurality of display vehicles (such as phages, viruses or bacteria) each displaying at least 5, at least 7, at least 11, at least 15, at least 20, at least 25 consecutive amino acids derived from polypeptide sequences of the E3 domain of CXCR3 (e.g., SEQ ID NO: 9).

Peptide mimetics can also be uncovered using computational biology.

As mentioned herein, the soluble CXCR3 polypeptides of the present invention can be attached to a heterologous amino acid sequence.

As used herein the phrase "heterologous amino acid sequence" refers to a non-immunogenic amino acid sequence which does not form a part of the CXCR3 or CXCR3-domain amino acid sequence. This sequence can confer solubility to the molecule of this embodiment of the present invention, thereby increasing the half-life of such a fusion polypeptide molecule in the serum.

The heterologous amino acid sequence is generally localized at the amino- or carboxyl- terminus of the CXCR3 or CXCR3 extracellular domain polypeptide of the present invention.

As mentioned, the at least one heterologous amino acid sequence can be contiguously attached to the CXCR3 amino acid sequence of the present invention. For
example, the at least one CXCR3 amino acid sequence may be embedded between two heterologous sequences, such as described Hoogenboom (1991) Mol. Immunol. 28:1027-1037. The heterologous amino acid sequence may be attached to the CXCR3 amino acid sequence by any of peptide or non-peptide bond. Attachment of the CXCR3 amino acid sequence to the heterologous amino acid sequence may be effected by direct covalent bonding (peptide bond or a substituted peptide bond) or indirect binding such as by the use of a linker having functional groups. Functional groups include, without limitation, a free carboxylic acid (C(=O)OH), a free amino group (NH2), an ester group (C(=O)OR, where R is alkyl, cycloalkyl or aryl), an acyl halide group (C(=O)A, where A is fluoride, chloride, bromide or iodide), a halide (fluoride, chloride, bromide or iodide), a hydroxyl group (OH), a thiol group (SH), a nitrile group (C≡N), a free C-carbamic group (NR"-C(=O)-OR\ where each of R' and R" is independently hydrogen, alkyl, cycloalkyl or aryl).

An example of a heterologous amino acid sequence which may be used in accordance with this aspect of the present invention is an immunoglobulin amino acid sequence, such as the hinge and Fc regions of an immunoglobulin heavy domain (see U.S. Pat. No. 6,777,196). The immunoglobulin moiety in the chimeras of this aspect of the present invention may be obtained from IgG1, IgG2, IgG3 or IgG4 subtypes, IgA, IgE, IgD or IgM, as further discussed hereinbelow.

Chimeras constructed from a receptor sequence linked to an appropriate immunoglobulin constant domain sequence (immunoadhesins) are known in the art. Immunoadhesins reported in the literature include fusions of the T cell receptor; CD4; L-selectin (homing receptor); CD44; CD28-and B7; CTLA-4; CD22; TNF receptor; NP receptors; and IgE receptor α.

Typically, in such fusions the chimeric molecule will retain at least functionally active hinge and CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions can also be generated to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain.

The exact site at which fusion (conjugation) between the heterologous sequence and the CXCR3 amino acid sequence is not critical. Particular sites are well known in the art and may be selected in order to optimize the biological activity, secretion or
binding characteristics of the chimeric molecules of this aspect of the present invention (see Example I of the Example section which follows).

Though it may be possible to conjugate the entire heavy chain constant region to the CXCR3 amino acid sequence of the present invention, it is preferable to fuse shorter sequences. For example, a sequence beginning in the hinge region just upstream of the papain cleavage site, which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114, or analogous sites of other immunoglobulins, is used in the fusion. In a particularly preferred embodiment, the CXCR3 amino acid sequence is fused to the hinge region and CH2 and CH3, or to the CH1, hinge, CH2 and CH3 domains of an IgGl, IgG2, or IgG3 heavy chain (see U.S. Pat. No. 6,777,196). The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

As mentioned, the immunoglobulin sequences used in the construction of the chimeric molecules of this aspect of the present invention may be from an IgG immunoglobulin heavy chain constant domain. The use of human IgGl immunoglobulin sequences (e.g., as set forth in SEQ ID NOs. 19) is preferred. A major advantage of using IgGl is that IgGl can be purified efficiently on immobilized protein A. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular chimera construction. For example, the IgG3 hinge is longer and more flexible, so it can accommodate larger CXCR3 amino acid sequences that may not fold or function properly when fused to IgGl. Another consideration may be valency; IgG are bivalent homodimers, whereas Ig subtypes like IgA and IgM may give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit. Other considerations in selecting the immunoglobulin portion of the chimeric molecules of this aspect of the present invention are described in U.S. Pat. No. 6,777,196.

Further examples of heterologous amino acid sequences commonly used in fusion protein construction include, but are not limited to galactosidase, glucuronidase, glutathione-S-transferase (GST), carboxy terminal peptide (CTP) from chorionic gonadotrophin (CGβ) and chloramphenicol acetyltransferase (CAT).
According to one embodiment of this aspect of the present invention, the isolated soluble polypeptide of this aspect of the present invention is as set forth in SEQ ID NOs: 21 (non-mutated E3-Ig) or 22(mutated E3-Ig).

The isolated soluble molecule of this aspect of the present invention is encoded by a nucleic acid sequences as set forth in SEQ ID NO: 23(non-mutated E3-Ig) or 24 (mutated E3-Ig).

Thus, molecules of this aspect of the present invention may comprise heterologous amino acid sequences, as described above.

Additionally or alternatively as mentioned hereinabove CXCR3 amino acid sequences of the present invention may be attached to a non-proteinaceous moiety, such molecules are preferably selected non-immunogenic in a subject.

Thus, according to a preferred embodiment of this aspect of the present invention, there is provided an isolated soluble molecule comprising a CXCR3 amino acid sequence (as described above) attached to a non-proteinaceous moiety.

Such a molecule is highly stable (resistant to in-vivo proteolytic activity probably due to steric hindrance conferred by the non-proteinaceous moiety) and may be produced using common solid phase synthesis methods which are inexpensive and highly efficient, as further described hereinbelow. However, it will be appreciated that recombinant techniques may still be used, whereby the recombinant peptide product is subjected to in-vitro modification (e.g., PEGylation as further described hereinbelow).

The phrase "non-proteinaceous moiety" as used herein refers to a molecule not including peptide bonded amino acids that is attached to the above-described CXCR3 amino acid sequence.

It will be appreciated that such non-proteinaceous moieties may be also attached to the above mentioned fusion molecules (i.e., which comprise a heterologous amino acid sequence) to promote stability and possibly solubility of the molecules.

Bioconjugation of such a non-proteinaceous moiety (such as PEGylation) can confer the CXCR3 amino acid sequence with stability (e.g., against protease activities) and/or solubility (e.g., within a biological fluid such as blood, digestive fluid) while preserving its biological activity and prolonging its half-life. Methods of PEGylation, for example, are well known in the art.
Molecules of this aspect of present invention can be biochemically synthesized such as by using standard solid phase techniques. These methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation and classical solution synthesis. These methods are preferably used when the peptide is relatively short (i.e., 10 kDa) and/or when it cannot be produced by recombinant techniques (i.e., not encoded by a nucleic acid sequence, such as a "Tag" further described hereinbelow) and therefore involve different chemistry.

Solid phase peptide synthesis procedures are well known in the art and further described by John Morrow Stewart and Janis Dillaha Young, Solid Phase Peptide Syntheses (2nd Ed., Pierce Chemical Company, 1984).

Synthetic peptides can be purified by preparative high performance liquid chromatography and the composition of which can be confirmed via amino acid sequencing.


Briefly, an expression construct (i.e., expression vector), which includes an isolated polynucleotide (e.g., SEQ ID NO: 23 or SEQ ID NO: 24) which comprises a nucleic acid sequence encoding the CXCR3 amino acid sequence binding at least one CXCR3 ligand, such as SEQ ID NO: 25 (mutated E3) or SEQ ID NO: 26 (non-mutated E3) (optionally in frame fused to a nucleic acid sequence encoding the heterologous amino acid sequence e.g., SEQ ID NO: 27) of the present invention positioned under the transcriptional control of a regulatory element, such as a promoter, is introduced into host cells.

The nucleotide sequence encoding a mutant CXCR3 amino acid sequence may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent CXCR3 portion, such as the CXCR3 extracellular loop domain E3 with the amino acid
sequence shown in SEQ ID NO: 9 (E3 non-mutant). The nucleotide sequence may then
be changed so as to affect the substitution or insertion of the relevant amino acid
residues. The nucleotide sequence can be modified by site directed mutagenesis, as
described in detail in Example I of the Examples section below, and illustrated in FIGs.
IA and IB. In the alternative, the nucleotide sequence may be prepared by chemical
synthesis, wherein oligonucleotides are designed based on the desired specific amino
acid sequence of the CXCR3 mutant. Methods for producing mutant polynucleotides
are well known in the art (see, for example, US Patent No. 7,403, 383).

For example, a nucleic acid sequence encoding a soluble CXCR3 polypeptide of
the present invention (e.g., SEQ ID NO: 9 or 10) is ligated in frame to an
immunoglobulin cDNA sequence (e.g., SEQ ID NO: 27), resulting in a nucleic acid
sequence (e.g. SEQ ID NO: 24) encoding the desired fusion protein (e.g. SEQ ID NO:
22). An exemplary nucleotide construct suitable for expressing such a soluble fusion
protein is detailed in SEQ ID NO: 28. It will be appreciated that, ligation of genomic
immunoglobulin fragments can also be used. In this case, fusion requires the presence
of immunoglobulin regulatory sequences for expression. cDNAs encoding IgG heavy-
chain constant regions can be isolated based on published sequence from cDNA libraries
derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase
chain reaction (PCR) techniques. The nucleic acid sequences encoding the CXCR3
amino acid sequence and immunoglobulin can be ligated in tandem into an expression
construct (vector) that directs efficient expression in the selected host cells, further
described hereinbelow. For expression in mammalian cells, pRK5-based vectors [Schall
(1989)] can be used. The exact junction can be created by removing the extra sequences
between the designed junction codons using oligonucleotide-directed deletional
337:525-531 (1989)]. Synthetic oligonucleotides can be used, in which each half is
complementary to the sequence on either side of the desired junction; ideally, these are
11 to 48-mers. Alternatively, PCR techniques can be used to join the two parts of the
molecule in-frame with an appropriate vector.
Methods of introducing the expression construct into a host cell are well known in the art and include, electroporation, lipofection and chemical transformation (e.g., calcium phosphate). See also Example I of the Examples section which follows.

The "transformed" cells are cultured under suitable conditions, which allow the expression of the chimeric molecule encoded by the nucleic acid sequence.

Following a predetermined time period, the expressed chimeric molecule is recovered from the cell or cell culture, and purification is effected according to the end use of the recombinant polypeptide.

Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, and the like, can be used in the expression vector [see, e.g., Bitter et al., (1987) Methods in Enzymol. 153:516-544].

Other than containing the necessary elements for the transcription and translation of the inserted coding sequence (encoding the chimera), the expression construct of the present invention can also include sequences engineered to optimize stability, production, purification, yield or toxicity of the expressed fusion protein.

A variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the fusion protein coding sequence. These include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the chimera coding sequence; yeast transformed with recombinant yeast expression vectors containing the chimera coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors, such as Ti plasmid, containing the chimera coding sequence. Mammalian expression systems are preferably used to express the chimera of the present invention.

The choice of host cell line for the expression of the molecules depends mainly on the expression vector. Eukaryotic expression systems are preferred (e.g., mammalian and insects) since they allow post translational modifications (e.g., glycosylation). Another consideration is the amount of protein that is required. Milligram quantities often can be produced by transient transfections. For example, the adenovirus EIA-transformed 293 human embryonic kidney cell line can be transfected transiently with
pRK5-based vectors by a modification of the calcium phosphate method to allow efficient expression.- CDM8-based vectors can be used to transfect COS cells by the DEAE-dextran method. If larger amounts of protein are desired, the molecules can be expressed after stable transfection of a host cell line (see Example I of the Examples section). It will be appreciated that the presence of a hydrophobic leader sequence at the N-terminus of the molecule will ensure processing and secretion of the molecule by the transfected cells.

It will be appreciated that the use of bacterial or yeast host systems may be preferable to reduce cost of production. However since bacterial host systems are devoid of protein glycosylation mechanisms, a post production glycosylation may be needed.

In any case, transformed cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant polypeptide. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce the recombinant chimera molecule of the present invention. Such a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Depending on the vector and host system used for production, resultant proteins of the present invention may either remain within the recombinant cell, secreted into the fermentation medium, secreted into a space between two cellular membranes, such as the periplasmic space in *E. coli*; or retained on the outer surface of a cell or viral membrane.

Following a predetermined time in culture, recovery of the recombinant protein is effected. The phrase "recovering the recombinant protein" refers to collecting the whole fermentation medium containing the protein and need not imply additional steps of separation or purification. Proteins of the present invention can be purified using a
variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

Molecules of the present invention are preferably retrieved in "substantially pure" form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the protein in the applications, described hereinbelow.

Recombinant molecules of the present invention can be conveniently purified by affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of the immunoglobulin Fc domain that is used in the chimera. Protein A can be used to purify chimeric molecules that are based on human γ1, γ2, or γ4 heavy chains. Protein G can be used for all mouse isotypes and for human γ3. The solid support to which the affinity ligand is attached is most often agarose, but other solid supports are also available. Mechanically stable solid supports such as controlled pore glass or poly(styrene-divinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. The conditions for binding the chimeric molecules to the protein A or G affinity column are dictated entirely by the characteristics of the Fc domain; that is, its species and isotype. Generally, when the proper ligand is chosen, efficient binding occurs directly from unconditioned culture fluid. One distinguishing feature of chimeric molecules of this aspect of the present invention is that, for human γ1 molecules, the binding capacity for protein A is somewhat diminished relative to an antibody of the same Fc type. Bound chimeric molecules of this aspect of the present invention can be efficiently eluted either at acidic pH (at or above 3.0), or in a neutral pH buffer containing a mildly chaotropic salt. This affinity chromatography step can result in a chimeric molecule preparation that is > 95% pure. Medical grade purity is essential for therapeutic applications.

Other methods known in the art can be used in place of, or in addition to, affinity chromatography on protein A or G to purify chimeric molecules which include an immunoglobulin portion. Such chimeric molecules behave similarly to antibodies in thiophilic gel chromatography and immobilized metal chelate chromatography. In contrast to antibodies, however, their behavior on ion exchange columns is dictated not
only by their isoelectric points, but also by a charge dipole that may exist in the molecules due to their chimeric nature.

Thus, the present invention provides for numerous configurations of soluble molecules which are capable of binding CXCR3 ligands, for example, CXCL9 and CXCL10.

While reducing the present invention to practice, the inventors have shown that incubation of activated T cells with an effective amount of an isolated soluble CXCR3 E3 polypeptide according to the present invention, having a substitution mutation at D278 and attached to IgG heterologous amino acid sequence, effectively supresses CXCL9 and CXCL10, but not CXCL11-mediated CXCR3 biological activity (see Example II of the Examples section hereinbelow). As used herein CXCR3 activity refers to cell signaling activity (e.g., G protein signaling, NF-kappa B signaling), chemokine binding activity (CXCL9, CXCL10, CXCL11), viral replication and/or co-receptor for SIM and HIV, cell adhesion, cell proliferation or chemotaxis. Thus, in one embodiment, the isolated soluble CXCR3 polypeptide according to the present invention can be used to suppress CXCL9/CXCL10-associated activity, and/or enhance CXCL11-associated activity of CXCR3, such as modulating the polarity of T cell cytokine secretion from a pro-inflammatory to anti-inflammatory profile.

Administration of the mutant CXCR3-E3\textsubscript{(mut)} fusion polypeptide in the murine encephalomyelitis (EAE) was proven therapeutic for the treatment of the clinical symptoms (see FIGs. 5A-5B and 8) and suppression of neuropathology (e.g. demyelination) characteristic of ongoing multiple sclerosis \textit{in-vivo}.

Thus, according to one aspect of the present invention there is provided a method of treating a CXCR3-associated disease in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an isolated soluble polypeptide comprising an amino acid sequence of extracellular loop 3 (E3) domain of CXCR3, said amino acid sequence having a substitution mutation at the amino acid corresponding to aspartic acid (D) at amino acid coordinate 278 of CXCR3, or an isolated soluble polypeptide comprising an amino acid sequence of CXCR3 devoid of the N-terminal sequence, wherein the soluble polypeptide binds at least one CXCR3 ligand, thereby treating the CXCR3-associated disease in the subject.
As used herein the term "treating" refers to preventing, curing, reversing, attenuating, alleviating, minimizing, suppressing or halting the deleterious effects of a CXCR3-associated disease.

CXCR3-associated diseases include, but are not limited to, multiple sclerosis, rheumatoid arthritis, hematological diseases, cardiovascular diseases, disorders of the peripheral and central nervous system, genitourinary diseases, respiratory diseases, parasitic infection, viral infection, bacterial infection, respiratory diseases, ischemia-reperfusion injury, allograft rejection, autoimmune disease, inflammatory disease and cancer. In one embodiment of the present invention, the CXCR3-associated disease is a disease associated with increased CXCL9 or CXCL10. In another embodiment of the present invention the CXCR3-associated disease is a disease associated with decreased CXCL11.

Yet further, it will also be appreciated that CXCR3 ligands such as CXCL9, CXCL10 and CXCL11 can exert their biological activity via non-CXCR3 signaling pathways, such as the binding and activation by CXCL10 of the CCR3 receptor (see Booth et al, Biochemistry, 2002;41:10418) and the CXCL10 activation of TLR4 signaling in diabetes (Schulthess et al, Cell Metab 2009;9:125-39). Thus, the isolated soluble polypeptides of the present invention can be useful in treating or prevention of CXCL9/CXCL10/CXCL11-associated diseases or conditions not associated with CXCR3.

It will be appreciated that treatment of CXCR3-associated diseases according to the present invention may be combined with other treatment methods known in the art (i.e., combination therapy). Thus for example, Multiple Sclerosis may be treated with the isolated soluble CXCR3 polypeptides of the present invention in conjunction with other agents including but are not limited to, Interferon Beta 1a, Interferon Beta 1b, Glatiramer Acetate, Mitoxantrone, MethylPrednisolone, Prednisone, Prednisolone, Dexamethasone, Adreno-corticotropic Hormone (ACTH) and Corticotrophin. The present invention therefore contemplates articles of manufacture comprising the isolated soluble CXCR3 polypeptides of the present invention and an anti-Multiple Sclerosis agent being packaged in a packaging material and identified in print, in or on the packaging material for use in the treatment of Multiple Sclerosis.
As mentioned, the isolated soluble CXCR3 polypeptides of the present invention may be used to treat inflammatory, infectious, cancerous and other diseases. Some representative inflammatory and other diseases and disorders are summarized infra.

Inflammatory diseases associated with hypersensitivity

Examples of hypersensitivity include, but are not limited to, Type I hypersensitivity, Type II hypersensitivity, Type III hypersensitivity, Type IV hypersensitivity, immediate hypersensitivity, antibody mediated hypersensitivity, immune complex mediated hypersensitivity, T lymphocyte mediated hypersensitivity and DTH.

Type I or immediate hypersensitivity, such as asthma.

Type II hypersensitivity include, but are not limited to, rheumatoid diseases, rheumatoid autoimmune diseases, rheumatoid arthritis, spondylitis, ankylosing spondylitis, systemic diseases, systemic autoimmune diseases, systemic lupus erythematosus, sclerosis, systemic sclerosis, glandular diseases, glandular autoimmune diseases, pancreatic autoimmune diseases, diabetes, Type I diabetes, thyroid diseases, autoimmune thyroid diseases, Graves' disease, thyroiditis, spontaneous autoimmune thyroiditis, Hashimoto's thyroiditis, myxedema, idiopathic myxedema; autoimmune reproductive diseases, ovarian diseases, ovarian autoimmunity, autoimmune anti-sperm infertility, repeated fetal loss, neurodegenerative diseases, neurological diseases, neurological autoimmune diseases, multiple sclerosis, Alzheimer's disease, myasthenia gravis, motor neuropathies, Guillain-Barre syndrome, neuropathies and autoimmune neuropathies, myasthenic diseases, Lambert-Eaton myasthenic syndrome, paraneoplastic neurological diseases, cerebellar atrophy, paraneoplastic cerebellar atrophy, non-paraneoplastic stiff man syndrome, cerebellar atrophies, progressive cerebellar atrophies, encephalitis, Rasmussen's encephalitis, amyotrophic lateral sclerosis, Sydeham chorea, Gilles de la Tourette syndrome, polyendocrinopathies, autoimmune polyendocrinopathies; neuropathies, dysimmune neuropathies; neuromyotonia, acquired neuromyotonia, arthrogryposis multiplex congenita, cardiovascular diseases, cardiovascular autoimmune diseases, atherosclerosis, myocardial infarction, thrombosis, granulomatosis, Wegener's granulomatosis, arteritis, Takayasu's arteritis and Kawasaki syndrome; anti-factor VIII autoimmune disease; vasculitides, necrotizing small vessel

Autoimmune diseases

Examples of autoimmune diseases include, but are not limited to, cardiovascular diseases, rheumatoid diseases, glandular diseases, gastrointestinal diseases, cutaneous diseases, hepatic diseases, neurological diseases, muscular diseases, nephric diseases, diseases related to reproduction, connective tissue diseases and systemic diseases.


Examples of autoimmune gastrointestinal diseases include, but are not limited to, chronic inflammatory intestinal diseases (Garcia Herola A. et al, Gastroenterol Hepatol. 2000 Jan;23 (1):16), celiac disease (Landau YE. and Shoenfeld Y. Harefuah 2000 Jan 16;138 (2):122), colitis, ileitis and Crohn's disease.

Examples of autoimmune cutaneous diseases include, but are not limited to, autoimmune bullous skin diseases, such as, but are not limited to, pemphigus vulgaris, bullous pemphigoid and pemphigus foliaceus.


Examples of autoimmune muscular diseases include, but are not limited to, myositis, autoimmune myositis and primary Sjogren's syndrome (Feist E. et al, Int Arch Allergy Immunol 2000 Sep;123 (1):92) and smooth muscle autoimmune disease (Zauli D. et al, Biomed Pharmacother 1999 Jun;53 (5-6):234).

Examples of autoimmune nephric diseases include, but are not limited to, nephritis and autoimmune interstitial nephritis (Kelly CJ. J Am Soc Nephrol 1990 Aug;1 (2):140).

Examples of autoimmune diseases related to reproduction include, but are not limited to, repeated fetal loss (Tincani A. et al, Lupus 1998;7 Suppl 2:S107-9).

Examples of autoimmune connective tissue diseases include, but are not limited to, ear diseases, autoimmune ear diseases (Yoo TJ. et al, Cell Immunol 1994 Aug;157 (1):249) and autoimmune diseases of the inner ear (Gloddek B. et al, Ann N Y Acad Sci 1997 Dec 29;830:266).


Infectious diseases
Examples of infectious diseases include, but are not limited to, chronic infectious diseases, subacute infectious diseases, acute infectious diseases, viral diseases, bacterial diseases, protozoan diseases, parasitic diseases, fungal diseases, mycoplasma diseases and prion diseases.

Graft rejection diseases
Examples of diseases associated with transplantation of a graft include, but are not limited to, graft rejection, chronic graft rejection, subacute graft rejection, hyperacute graft rejection, acute graft rejection and graft versus host disease.

Allergic diseases
Examples of allergic diseases include, but are not limited to, asthma, hives, urticaria, pollen allergy, dust mite allergy, venom allergy, cosmetics allergy, latex allergy, chemical allergy, drug allergy, insect bite allergy, animal dander allergy, stinging plant allergy, poison ivy allergy and food allergy.
Cancerous diseases

Examples of cancer include but are not limited to carcinoma, lymphoma, blastoma, sarcoma, and leukemia. Particular examples of cancerous diseases but are not limited to: Myeloid leukemia such as Chronic myelogenous leukemia. Acute myelogenous leukemia with maturation. Acute promyelocytic leukemia. Acute nonlymphocytic leukemia with increased basophils. Acute monocytic leukemia. Acute myelomonocytic leukemia with eosinophilia; Malignant lymphoma, such as Birkitt's Non-Hodgkin's; Lymphocytic leukemia, such as Acute lymphoblastic leukemia. Chronic lymphocytic leukemia; Myeloproliferative diseases, such as Solids tumors

Benign Meningioma, Mixed tumors of salivary gland, Colonic adenomas; Adenocarcinomas, such as Small cell lung cancer, Kidney, Uterus, Prostate, Bladder, Ovary, Colon, Sarcomas, Liposarcoma, myxoid, Synovial sarcoma, Rhabdomyosarcoma (alveolar), Extraskeletal myxoid chondrosarcoma, Ewing's tumor; other include Testicular and ovarian dysgerminoma, Retinoblastoma, Wilms' tumor, Neuroblastoma, Malignant melanoma, Mesothelioma, breast, skin, prostate, and ovarian.

The isolated soluble CXCR3 polypeptides of the present invention can be administered to the subject per se, or as part of a pharmaceutical composition, which also includes a physiologically acceptable carrier. The purpose of a pharmaceutical composition is to facilitate administration of the active ingredient to an organism.

As used herein, a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

As used herein, the term "active ingredient" refers to the preparation accountable for the intended biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmacologically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases. One of the ingredients included in the pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a
biocompatible polymer with a wide range of solubility in both organic and aqueous media [Mutter et al. (1979)].

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, intertapitoneal, intranasal, or intraocular injections.

Alternately, one may administer a preparation in a local rather than systemic manner, for example, via injection of the preparation directly into a specific region of a patient's body.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.
For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries as desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, and sodium carboxymethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate, may be added.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.
For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The preparations described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., a sterile, pyrogen-free, water-based solution, before use.

The pharmaceutical composition of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, for example, conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in the context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a "therapeutically
effective amount" means an amount of active ingredients (e.g., a nucleic acid construct) effective to prevent, alleviate, or ameliorate symptoms of a disorder (e.g., ischemia) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the dosage or the therapeutically effective amount can be estimated initially from in vitro and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration, and dosage can be chosen by the individual physician in view of the patient's condition. (See, e.g., Fingl, E. et al. (1975), "The Pharmacological Basis of Therapeutics," Ch. 1, p.1.)

Dosage amount and administration intervals may be adjusted individually to provide sufficient plasma or brain levels of the active ingredient to induce or suppress the biological effect (i.e., minimally effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks, or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.
Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA-approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser device may also be accompanied by a notice in a form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions for human or veterinary administration. Such notice, for example, may include labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a pharmaceutically acceptable carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as further detailed above.

As used herein the phrase "Multiple Sclerosis" refers to the inflammatory, demyelinating disease of the central nervous system (CNS) which is typically characterized by various symptoms of neurological dysfunction. Multiple sclerosis according to the present teachings refers to any type of multiple sclerosis (e.g., stage, severity) as outlined infra.

Relapsing-remitting - Relapsing-remitting describes the initial course of 85 % to 90 % of individuals with MS. This subtype is characterized by unpredictable attacks (relapses) followed by periods of months to years of relative quiet (remission) with no new signs of disease activity. Deficits suffered during the attacks may either resolve or may be permanent. When deficits always resolve between attacks, this is referred to as "benign" MS.

Secondary progressive - Secondary progressive describes around 80 % of those with initial relapsing-remitting MS, who then begin to have neurological decline between their acute attacks without any definite periods of remission. This decline may include new neurological symptoms, worsening cognitive function, or other deficits. Secondary progressive is the most common type of MS and causes the greatest amount of disability.

Primary progressive - Primary progressive describes the approximately 10 % of individuals who never have remission after their initial MS symptoms. Decline occurs
continuously without clear attacks. The primary progressive subtype tends to affect people who are older at disease onset.

Progressive relapsing - Progressive relapsing describes those individuals who, from the onset of their MS, have a steady neurological decline but also suffer superimposed attacks; and is the least common of all subtypes.

Special cases of the disease with non-standard behavior have also been described although many researchers believe they are different diseases. These cases are sometimes referred to as borderline forms of multiple sclerosis and are: Neuromyelitis optica (NMO), BaIo concentric sclerosis, Schilder disease, Marburg multiple sclerosis, acute disseminated encephalomyelitis (ADEM) and autoimmune variants of peripheral neuropathies.

As used herein the phrase "a subject in need thereof" refers to a mammal, preferably a human subject who has been diagnosed with probable or definite multiple sclerosis, e.g., a subject who experienced one neurological attack affecting the CNS and accompanied by demyelinating lesions on brain magnetic resonance imaging (MRI). The neurological attack can involve acute or sub-acute neurological symptomatology (attack) manifested by various clinical presentations like unilateral loss of vision, vertigo, ataxia, incoordination, gait difficulties, sensory impairment characterized by paresthesia, dysesthesia, sensory loss, urinary disturbances until incontinence, diplopia, dysarthria, various degrees of motor weakness until paralysis, cognitive decline either as a monosymptomatic or in combination. The symptoms usually remain for several days to few weeks, and then partially or completely resolve.

The diagnosis MS can also include laboratory tests involving evaluation of IgG synthesis and oligoclonal bands (immunoglobulins found in 85-95 % of subjects diagnosed with definite MS) in the cerebrospinal fluid (CSF, obtained by e.g., lumbar puncture) which provide evidence of chronic inflammation of the central nervous system. Combined with MRI of the brain and spinal cord and clinical data, the presence of oligoclonal bands can help make a definite diagnosis of MS.

The affinity of the isolated soluble CXCR3 polypeptide of the present invention to CXCR3 ligands (e.g., CXCL9 or CXCL10) allows use thereof in purification and detection of CXCR3 ligands.
According to an embodiment of this aspect of the present invention, there is provided an isolated soluble polypeptide comprising a tag attached to a CXCR3 amino acid sequence comprising an amino acid sequence of extracellular loop 3 (E3) domain of CXCR3, said amino acid sequence having a substitution mutation at the amino acid corresponding to aspartic acid (D) at amino acid coordinate 278 of CXCR3, or an isolated soluble polypeptide comprising an amino acid sequence of CXCR3, said soluble polypeptide being devoid of the N-terminal domain of CXCR3, and wherein said soluble polypeptide binds at least one CXCR3 ligand (as described above).

As used herein the term "tag" refers to a moiety which is specifically recognized by a binding partner such as an antibody, a chelator or an avidin (biotin) molecule. The tag can be placed C-terminally or N-terminally of the CXCR3 peptide, as long as it does not interfere with a biological activity thereof (e.g., ligand binding).

For example, a tag polypeptide has enough residues to provide an epitope (i.e., epitope tag) against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with biological activity of the CXCR3 peptide. The epitope tag preferably also is fairly unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Preferred are poly-histidine sequences, which bind nickel, allowing isolation of the tagged protein by Ni-NTA chromatography as described (Lindsay et al. Neuron 17:571-574 (1996)), for example (see Example I of the Example section hereinbelow, and FIG.1B).

Such epitope-tagged forms of the CXCR3 are desirable, as the presence thereof can be detected using a labeled antibody against the tag polypeptide. Also, provision of the epitope tag enables the CXCR3 peptide of the present invention to be readily purified by affinity purification using the anti-tag antibody. Affinity purification techniques and diagnostic assays involving antibodies are described later herein.

Tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field et al., MoL Cell. Biol., 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody.
Paborsky et al., Protein Engineering, 3(6):547-553 (1990). Other tag polypeptides have been disclosed. Examples include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an α-tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using methods which are well known in the art. Such antibodies are commercially available such as from Sigma, St. Louis, USA.

According to an embodiment of this aspect of the present invention, there is provided a method of isolating a CXCR3 ligand from a biological sample or detecting the presence of CXCR3 ligands therein. It will be appreciated that some isolated soluble CXCR3 polypeptides of the present invention can selectively bind CXCL9 or CXCL10 from a biological sample.

As used herein the phrase "biological sample" refers to a biological material, such as cells, tissues and fluids such as blood, serum, plasma, lymph, bile fluid, urine, saliva, sputum, synovial fluid, semen, tears, cerebrospinal fluid, bronchioalveolar large fluid, ascites fluid, pus, conditioned medium and the like in which CXCR3 ligand is present.

Isolation of CXCR3 ligand according to this aspect of the present invention is effected by contacting the biological sample with the isolated soluble CXCR3 polypeptides of this aspect of the present invention, such that CXCR3 ligand and the molecule form a complex (using buffer, temperature conditions which allow binding of the molecule to CXCR3 ligand, see for Example Datta-Mannan and Stone 2004, supra); and isolating the complex to thereby isolate CXCR3 ligand from the biological sample.

In order to isolate the complex, the molecule is preferably immobilized on a solid support. As used herein the phrase "solid support" refers to a non-aqueous matrix to which a reagent of interest (e.g., the molecule of this aspect of the present invention) can adhere. Examples of solid supports, include, but are not limited to, solid supports formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid support can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography
column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Pat. No. 4,275,149.

Alternatively, such molecules can be used to detect the levels of CXCR3 ligand in biological samples. For diagnostic applications, molecules typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, a fluorescent or chemiluminescent compound, or a tag (such as described hereinabove and to which a labeled antibody can bind). The molecules of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. [Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc., 1987)].

The molecules of this aspect of the present invention can be included in a diagnostic kit, in which the molecule and optionally solid support and imaging reagents (e.g., antibodies, chromogenic substrate etc.) can be packaged in suitable containers with appropriate buffers and preservatives and used for diagnosis.

As used herein the term "about" refers to ± 10 %.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed.

**EXAMPLE I**

*Soluble mutant CXCR3-E3-Ig fusion protein with a single D278A mutation selectively binds CXCL9 and CXCL10, but not CXCL11*

The E3 domain of CXCR3 binds all 3 CXCR3 ligands, CXCL9, CXCL10 and CXCL11 (3). Colvin et al (3) have reported that the first aspartic acid of this domain is
critical for binding and activation of all three CXCR3 ligands, while the second aspartic acid of E3 (D282) is important for activation by CXCL10 or CXCL9, but not for CXCL11 (3). In order to further assess the binding specificity of E3 domain components, and uses thereof, a mutated variant of the CXCR3 E3 domain was generated with a single point mutation of the first Aspartic acid to Alanine (FIG. IA). As chemokines have a very short half life, a recombinant chimeric soluble molecule comprising the mutant CXCR3-E3(mut) fused to the IgGl heavy chain (Fc), CXCR3-E3(mut)-Ig, was generated.

**MATERIALS AND METHODS**

*Construction of soluble CXCR3 fragment-Ig fusion proteins:*

The nucleic acid vectors encoding soluble CXCR3 fragment-Ig fusion proteins of the present invention were constructed as follows: cDNA encoding the constant region of human (Hinge-CH2-CH3, SEQ ID NO: 58) or mouse (Hinge-CH2-CH3, SEQ ID NO: 59) IgGl heavy chain was generated by RT-PCR of RNA extracted from either human peripheral blood mononuclear cells or mouse spleen cells cultured 4 days with αCD3 and αCD28, and amplified using the following primers:

**Human IgG:**

- Sense primer: 5' CTCGAGCCCAAATCTTGTGACAAAAC 3' (SEQ ID NO: 29),
- Anti-sense primer: 5' GGGCCCTTTACCCGGGGACAGGAGA 3' (SEQ ID NO:30).

**Mouse IgG:**

- Sense primer: 5' CTCGAGGTGCCAGGGGATTGTTGTTG 3' (SEQ ID NO: 31),
- Anti-sense primer: 5' GGGCCCTTTACCCAGGAGTGGGAGA 3' (SEQ ID NO:32).

The PCR products were digested with Xhol and Apal and ligated into the mammalian expression/secretion vector pSecTag2/Hygro B (Invitrogen Life Technologies, San Diego, CA), to generate the pSecTag2-mIgG vector, having the mouse immunoglobulin coding sequences with HindIII and Xhol sites.
cDNAs encoding the different domains of mouse CXCR3 (GenBank Accession No. NM_009910, SEQ ID NO: 33) or human CXCR3 (GenBank Accession No. NM_001504, SEQ ID NO: 7) were generated by RT-PCR of RNA extracted from ConA-stimulated mouse or human splenocytes using the following primers:

**Mouse CXCR3- N-terminus:**

- Sense primer: 5' CCAAGCTTATATCACCTTGAGGTTAGTGAAC 3' (SEQ ID NO:34);
- Anti-sense primer: 5' CCGCTCGAGGAGGCTGGCAGGAAAGTT 3' (SEQ ID NO: 35).

**Mouse CXCR3-E1:**

- Sense primer: 5' CCAAGCTTGGCTGCTCCAGTGGGTTT 3' (SEQ ID NO:36),
- Anti-sense primer: 5' CCGCTCGAGGCCTGCCACTTTGCAGAGG 3' (SEQ ID NO: 37).

**Mouse CXCR3-E2:**

- Sense primer: 5' CCAAGCTTTCAGCCAACTACGATCAGCG 3' (SEQ ID NO:38),
- Anti-sense primer: 5' CCGCTCGAGGAAACCAGCCACTAGCTGC 3' (SEQ ID NO: 39).

**Mouse CXCR3-E3:**

- Sense primer: 5' CCAAGCTTGTATCCATCTCATGGATGTGGG 3' (SEQ ID NO:40),
- Anti-sense primer: 5' CCGCTCGAGGACTGACTTGGCCACATCC 3' (SEQ ID NO: 41).

**Mouse CXCR3-E3(mut):**

- Sense primer: 5' CCAAGCTTGTATCCATCTCATGGATGTGGG 3' (SEQ ID NO:42),
- Anti-sense primer: 5' CCGCTCGAGGACTGACTTGGCCACATCC 3' (SEQ ID NO:43).

**Human CXCR3- N-terminus:**

- Sense primer: 5'CCAAGCTTATATGTCCTTGAGGTTAGTGAAC 3' (SEQ ID NO:44);
Anti-sense primer: 5’ CCGCTCGAGGAGGGCTGGCAGGAAGGCCCG 3’
(SEQ ID NO: 45).

**Human CXCR3-E1:**

Sense primer: 5’ CCCAAGCTTGCTGCCGTCCAGTGGTCT 3’ (SEQ ID NO: 46),

Anti-sense primer: 5’ CCGCTCGAGCCTGCCACTTTGCGAGGCCA 3’
(SEQ ID NO: 47).

**Human CXCR3-E2:**

Sense primer: 5’ CCCAAGCTTGCCACCAGCAGCGC 3’ (SEQ ID NO: 48),

Anti-sense primer: 5’ CCGCTCGAGAAGCCTGCCACTTTGCGAGGCCA 3’
(SEQ ID NO: 49).

**Human CXCR3-E3:**

Sense primer: 5’ CCCAAGCTTGACATCCTCATGGACCTGGG 3’ (SEQ ID NO: 50),

Anti-sense primer: 5’ CCGCTCGAGAAGCCTGCCACTTTGCGAGGCCA 3’
(SEQ ID NO: 51).

**Human CXCR3-E3 (mut):**

Sense primer: 5’ CCCAAGCTTGcCATCCTCATGGACCTGGG 3’ (SEQ ID NO:52)

Anti-sense primer: 5’ CCGCTCGAGAAGCCTGCCACTTTGCGAGGCCA 3’(SEQ ID NO:53)

Each PCR product was digested with HindIII and XhoI and sub-cloned into the vector containing the mouse or human IgG1 fragment to create the fusion proteins: CXCR3-NT-Ig (SEQ ID NO:54), CXCR3-El-Ig (SEQ ID NO: 55), CXCR3-E2-Ig (SEQ ID NO: 4), CXCR3-E3-Ig (SEQ ID NO:6) and CXCR3-E3(mut)-Ig (SEQ ID NO:1). The cloned fragment sequences were verified by dideoxynucleotide sequencing (Sequenase version 2; Upstate Biotechnology, Cleveland, OH).

**Expression and purification of fusion proteins:** The pSec-CXCR-NT-Ig, pSec-CXCR-El-Ig, pSec-CXCR-E2-Ig and pSec-CXCR-E3-Ig plasmids were separately co-transfected into DG44 Chinese hamster ovary (CHO) cells that have a double deletion
for the dihydrofolate reductase (DHFR) gene (DG44 CHO DHFR<sup>−</sup> cells, provided by Dr. Lawrence Chasin from Columbia University, USA, ATCC Accession No. CRL-9096), with CHO DHFR minigene vector, which transfects DHFR-deficient CHO cells with high efficiency, using jet PEI (Polypluse transfection - Illkirch Cedex, France) according the manufacturer's protocol. Stably transfected cells were selected in a culture medium (MEM-alpha) containing hygromycin (200 µg/ml) and increasing doses of methotrexate (2.5 nM to 0.1 nM). The fusion proteins were expressed as a disulphide-linked homodimer similar to IgGl, and were purified from the culture medium by High-Trap protein G affinity column (BD Biosciences, Piscataway, NJ).

**Cytokine/chemokine binding detection by ELISA:** The specificity of binding of the mutant CXCR3-E<sub>3(mut)</sub>-Ig fusion protein to various substrates was detected by an ELISA assay as follows: Each well was coated with 10 ng of the test mouse ligand proteins -CXCL9, CXCL10, CXCL11, CCL20 (Peprotec, Rocky Hill, NJ) using coating buffer (PBSXl), incubated at 4°C overnight, and blocked with 200 µl 0.25% gelatin blocking for 2 hours at room temperature. The soluble chemokine receptor fusion proteins representing CXCR3-E2-Ig fusion protein, native CXCR3-E3-Ig or mutant CXCR3-E<sub>3(mut)</sub>-Ig fusion protein were added (10µg/ml) in 1% BSA/PBS buffer (50 µl per well), incubated overnight at 4°C and washed four times with PBS/Tween 20 (0.05%). 50µl goat anti-hlgG-HRP (Jackson ImmunoResearch, Inc., West Grove, PA) was added at 1:10000 in 1% BSA/PBS, incubated for 1 hour and washed four times with PBS/Tween 20 (0.05%). The HRP substrate solution (TMB) was then added (50 µl per well). When a blue color appeared, the reaction was terminated by adding 50 µl H<sub>2</sub>SO<sub>4</sub> (IM). O.D. was determined at 450nm with the reference filter set to 620nm.

**Statistical analysis:** The significance of differences was examined using Student's Mest. P values smaller than 0.05 were considered statistically significant.

**RESULTS**

Following verification of the mutated CXCR3-E3 sequence (FIG. IA, SEQ ID NO: 56), the nucleic acid construct encoding the recombinant soluble CXCR3-E3-Ig fusion protein was cloned into CHO cells, the resulting gene product purified, and assessed for ligand binding properties, in comparison with those of other CXCR3
Surprisingly, the mutant CXCR3-E3 fragment was found to have highly selective binding properties not previously observed in CXCR3 domains. FIG. 2A depicts the results of comparison of ligand binding properties of CXCR3-E3(mut)-Ig with that of recombinant fusion proteins constructed with different domains of CXCR3, as determined by ELISA using HRP-conjugated anti-goat antibody. The histogram shows that, surprisingly, compared to CXCR3-E2-Ig (SEQ ID NO: 4)(white); wild-type CXCR3-E3-Ig (SEQ ID NO: 6) (dark grey) and N-terminal fragment CXCR3-NT-Ig (SEQ ID NO: 5) (black), CXCR3-E3(mut)-Ig selectively binds ligands CXCL9 and CXCL10, while hardly binding CXCL11. Further experiments showed that CXCR3-E3(mut)-Ig also failed to demonstrate significant binding to several other CC or CXC ligands, including: CCL20, CXCL12, CXCL16 or CCL3 (FIG. 2B).

These results indicate that the soluble recombinant CXCR3 domain-Ig fusion proteins retain their native ligand binding properties, and that CXCR3-E3(mut)-Ig is highly selective for CXCL9 and CXCL10. Without wishing to be limited to a single hypothesis, one possible interpretation of these selective binding results is that amino acid D278 of the E3 domain of CXCR3 is critical to binding of CXCL11 ligand, but not CXCL9 or CXCL10 ligands.

**EXAMPLE II**

*Soluble mutant CXCR3-E3-Ig fusion protein with a single D278A mutation selectively neutralizes CXCL9 and CXCL10, but not CXCL11 biological activity*

In order to assess whether mutant CXCR3-E3(mut)-Ig is effective in selectively blocking the biological activity of CXCR3 ligands, mutant CXCR3-E3(mut)-Ig was assayed in a chemotaxis/migration assay, in comparison with control human isotype IgGl.

**MATERIALS AND METHODS**

*In vitro Chemotaxis assay:* Chemotaxis assays in which the chemokine-induced migration of anti-CD3/anti-CD28 activated primary CD4+ T cells (1x10^6) was determined were performed in a TransWell system (5-µm pore size, Corning Costar
Corporation, Cambridge, MA). Briefly, CD4+ cells were selected from primary spleen cells by positive selection using anti-CD4 (L3T4) MACS magnetic beads (Miltenyi Biotec, Auburn, Calif.). The enriched CD4+ cells were cultured for 48 hours with anti CD3/anti CD28. After the incubation period cells were loaded into the upper chamber of the two systems. The lower chambers were loaded with 20 ng/ml mouse CXCR3 ligands CXCL9, CXCL10 and CXCL11 (PeproTec, Rocky Hill, NJ) and 50 µg/ml of either mutant CXCR3-E3(mut)-Ig fusion protein or control isotype-matched IgG. Controls received no chemo-attractants. Cells were allowed to migrate for 3 hours under a humidified 7.5% CO₂ atmosphere at 37°C. The content of the lower chambers was collected and counted using the FACS Calibur System (BD Biosciences, Piscataway, NJ). The chemotaxis index was then calculated by dividing the number of migrating cells in the presence of chemoattractant by the number of cells migrated in its absence.

Statistical analysis: The significance of differences was examined using Student's t-test. P values smaller than 0.05 were considered statistically significant.

RESULTS

In the in-vitro chemotaxis/cell migration assay, recombinant soluble CXCR3-E3(mut)-Ig fusion protein (E3(MUT)Ig, light grey) was effective in selectively blocking CXCL9 and CXCL10-associated migration of anti CD3/anti-CD28 activated primary CD4+ T cells. FIG. 3 depicts the results of one of three independent experiments, clearly showing that the presence of 50 µg/ml CXCR3-E3(mut)-Ig fusion protein, but not control isotype IgG1 (white), effectively blocked up to 70% of CXCL9 and CXCL10-induced chemotaxis, while having no effect on chemotaxis induced by CXCL11.

EXAMPLE III

Soluble mutant CXCR3-E3-Ig fusion protein with a single D278A mutation redirects the polarization and cytokine production of mouse effector T cells

In order to assess the potential effect of selective blocking of CXCL9 and CXCL10 by the soluble mutant CXCR3-E3-Ig fusion protein on immune function, cytokine production of activated mouse pre-EAE primary T cells was assayed in the presence of CXCR3-E3-Ig fusion protein.
MATERIALS AND METHODS

Animals

Mice were used as described in detail in Example I hereinabove.

Animal models: Experimental Autoimmune Encephalomyelitis (EAE):
C57BL/6 female mice were purchased from Harlen (Israel) and maintained in IVC cages under pathogen-free conditions. At 6 weeks of age; mice were subjected to active disease induction by a single administration of MOGp33-55 (myelin oligodendrocyte glycoprotein, SEQ ID NO: 20) emulsified in Complete Freund's Adjuvant as previously described [Kassiotis and Kollias, J Exp Med (2001) 193(4):427-434]. Animals were then monitored for clinical signs daily by an observer blind to the treatment protocol. EAE was scored as follows: 0 - clinically normal; 1 - flaccid tail; 2 - hind limb paralysis; 3 - total hind limb paralysis, accompanied by an apparent front limb paralysis; 4 - total hind limb and front limb paralysis; and 5 - death.

Generation of Spleen cells

Primary spleen cells were isolated from EAE induced C57BL/6 mice (day 9). Cells (10^6 cells/ml) were cultured in a humidified 7.5 % CO_2 atmosphere at 37 °C for 72 hours and stimulated with their target autoimmune antigen, MOG_{35-53} peptide, at a concentration of 50 µg/ml. In addition, the cultures were supplemented with either recombinant CXCR3_{(mut)}-IgG fusion protein or control isotype IgG1 at a concentration of 50 ng/ml.

ELISA

Secreted levels of IL-17, IL-10, IL-12, IFN-γ, IL-2 and IL-4 were each measured by commercially available ELISA kits: IL-10, IL-2 and IFN-γ (BioLegend, San Diego, CA), IL-12 and IL-17 (Bender Medical Systems, Vienna, Austria), and IL-4 (BioLegend, San Diego, CA).

Statistical analysis: The significance of differences was examined using Student's t-test. P values smaller than 0.05 were considered statistically significant.
RESULTS

As shown in FIGs. 4A-4F, exposure of activated CD4+ T cells co-cultured with their target antigen to recombinant CXCR3(mut)-Ig fusion protein effectively reduced production of pro-inflammatory IFNγ (FIG. 4E), IL-12 (FIG. 4D), IL-17 (FIG. 4C) and IL-2 (FIG. 4F), while strongly enhancing anti-inflammatory IL-4 (FIG. 4A) and IL-10 (FIG. 4B) production. Thus, specific blocking of CXCL9 and CXCL10 by CXCR3(mut)-IgG directly re-polarizes activated CD4+ T cells from pro-inflammatory to anti-inflammatory cytokine-producing regulatory T cells.

EXAMPLE IV

Soluble mutant CXCR3-E3-Ig fusion protein with a single D278A mutation suppresses Experimental Autoimmune Encephalomyelitis (EAE)

In order to assess the therapeutic efficacy of the soluble mutant CXCR3-E3 protein in-vivo, CXCR3-E3(mut)-Ig was administered to mice during experimental autoimmune encephalomyelitis (EAE), and the severity of their symptoms monitored.

MATERIALS AND METHODS

Induction of active EAE

Experimental Adjuvant Encephalomyelitis was induced in three groups of 8 C57BL/6 mice as described in Example III above.

Soluble CXCR3-E3(mut)-Ig fusion protein administration

One day after onset of disease (day 13.), mice were treated with repeated intra-peritoneal (IP) administration (every other day) of 300 µg/mouse of either CXCR3(mut)-IgG, isotype IgG1 control, or PBS. An observer blind to the experimental procedure scored the EAE symptoms daily, as described in Example III above.

Histology:

Spinal cords of EAE induced mice, receiving CXCR3(mut)-IgG, isotype control IgG1 or PBS were removed and subjected to histology analysis. Briefly, tissue samples were fixed over night in 4% paraformaldehyde in PBS, then dehydrated, paraffin embedded and sectioned into 5 µm sections. Sections were then deparaffinized, stained with hematoxylin and eosin and analyzed using an Olympus microscope at the indicated magnification.
Statistical analysis: The significance of differences was examined using Student's t-test. P values smaller than 0.05 were considered statistically significant.

RESULTS

Administration of CXCR3\textsubscript{(mut)}-IgG to EAE induced mice completely suppressed ongoing disease, as shown in FIGs. 5A and 5B. In the two representative experiments shown, EAE symptoms in mice receiving 300 µg/mouse CXCR3\textsubscript{(mut)}-IgG (closed triangles A.) were visibly improved after 2 administrations (day 18). The CXCR3-E3\textsubscript{(mut)}-IgG treated mice were essentially symptom free after 5 treatments (day 22).

Control mice receiving isotype IgG (IgG, closed squares ■) showed progression of disease identical to that of PBS-treated controls (PBS, closed circles •).

Histological evaluation of the spinal cords of treated (CXCR3-E3\textsubscript{(mut)}-IgG and control IgG or PBS treated mice revealed significantly reduced demyelination in the spinal cords of the CXCR3-E3\textsubscript{(mut)}-IgG-treated mice (FIG. 6), consistent with the resolution of clinical symptoms observed in-vivo.

The results described herein indicate that the soluble mutant CXCR3 E3 domain fusion protein, having a single amino acid substitution of D278A, is capable of selectively binding and blocking the effects of CXCL9 and CXCL10, can modulate the cytokine profile of activated T cells from pro-inflammatory to anti-inflammatory polarity, and is effective in specifically inhibiting symptoms of CXCL9 and/or CXCL10-related disease, such as inflammatory disease, most likely via selective blocking of CXCL9 and CXCL10 signaling, while leaving CXCL11 binding and signaling unaffected.

EXAMPLE V

Soluble mutant CXCR3-E3-Ig fusion protein with a single D278A mutation redirects the polarization and cytokine production of Experimental Autoimmune Encephalomyelitis (EAE) induced SJL mouse effector T cells

In order to assess the potential effect of selective blocking of CXCL9 and CXCL10 by the soluble mutant CXCR3-E3-Ig fusion protein on immune function, cytokine production of activated mouse pre-EAE primary T cells was assayed in the presence of CXCR3-E3-Ig fusion protein.
MATERIALS AND METHODS

Animals

Mice were used as described in detail in Example I hereinabove.

Animal models: EAE: SJL female mice were maintained in IVC cages under pathogen-free conditions. At eight weeks of age, mice were subjected to active disease induction by a single administration of PLP139-151/CFA emulsified in Complete Freund's Adjuvant as previously described [Suvannavejh, G. C. et al. (2000) J Clin Invest 105:223-231]. Animals were then monitored for clinical signs daily by an observer blind to the treatment protocol. EAE was scored as follows: 0 - clinically normal; 1 - flaccid tail; 2 - hind limb paralysis; 3 - total hind limb paralysis, accompanied by an apparent front limb paralysis; 4 - total hind limb and front limb paralysis; and 5 - death.

Generation of Spleen cells

Primary spleen cells were isolated from EAE induced SJL mice (day 9).

Cells (10^6 cells/ml) were cultured in a humidified 7.5 % CO₂ atmosphere at 37°C for 72 hours and stimulated with their target autoimmune antigen, PLP139—151/CFA, at a concentration of 20 µg/ml. In addition, the cultures were supplemented with either recombinant CXCR3(mut)-IgG fusion protein or control isotype IgGl at a concentration of 50 ng/ml.

ELISA

Secreted levels of IL-17, IL-10, IFNγ and IL-4 were each measured by commercially available ELISA kits: IL-10 and IFN-γ (BioLegend, San Diego, CA), IL-17 (Bender Medical Systems, Vienna, Austria), and IL-4 (BioLegend, San Diego, CA).

Statistical analysis: The significance of differences was examined using Student's t-test. P values smaller than 0.05 were considered statistically significant.

RESULTS

As shown in FIGs. 7A-7D and similar to the results obtained in Example 3 above, exposure of activated CD4+ T cells (from EAE induced SJL mice) co-cultured with their target antigen to recombinant CXCR3(mut)-IgG fusion protein effectively reduced production of pro-inflammatory IFNγ (FIG. 7C) and IL-17 (FIG. 7D), while
strongly enhancing anti-inflammatory IL-4 (FIG. 7B) and IL-10 (FIG. 7A) production. Thus, in T cells from EAE induced SJL mice, specific blocking of CXCL9 and CXCL10 by CXCR3(mut)-IgG directly re-polarizes activated CD4+ T cells from pro-inflammatory to anti-inflammatory cytokine-producing regulatory T cells.

EXAMPLE W

Soluble mutant CXCR3-E3-Ig fusion protein with a single D278A mutation suppresses Experimental Autoimmune Encephalomyelitis (EAE) in SJL mice

In order to further assess the therapeutic efficacy of the soluble mutant CXCR3-E3 protein in-vivo, CXCR3-E3(mut)-Ig was administered to SJL mice during experimental autoimmune encephalomyelitis (EAE), and the severity of their symptoms was monitored.

MATERIALS AND METHODS

Induction of active EAE

Experimental Adjuvant Encephalomyelitis was induced in three groups of 8 SJL mice as described in Example V above.

Soluble CXCR3-E3(mut)-Ig fusion protein administration

One day after onset of disease (day 10), mice were treated with repeated intraperitoneal (IP) administration (every other day) of 300 µg/mouse of either CXCR3(mut)-IgG, isotype IgGl control, or PBS. An observer blind to the experimental procedure scored the EAE symptoms daily, as described in Example V above.

Statistical analysis: The significance of differences was examined using Student's t-test. P values smaller than 0.05 were considered statistically significant.

RESULTS

Administration of CXCR3(mut)-IgG to EAE induced SJL mice completely suppressed ongoing disease, as shown in FIG. 8. As shown, EAE symptoms in mice receiving 300 µg/mouse CXCR3(mut)-IgG (open squares) were visibly improved after 2 administrations (day 13). The CXCR3-E3(mut)-Ig treated mice were essentially symptom free after 5 treatments (day 16). Control mice receiving isotype IgG (IgG,
open triangles) showed progression of disease identical to that of PBS-treated controls (PBS, closed squares).

The results described herein indicate that the soluble mutant CXCR3 E3 domain fusion protein, having a single amino acid substitution of D278A, can modulate the cytokine profile of activated T cells from pro-inflammatory to anti-inflammatory polarity and is effective in specifically inhibiting symptoms of CXCL9 and/or CXCL10-related inflammatory disease, such as experimental autoimmune encephalomyelitis (EAE), in SJL mice similar to the results obtained in C57BL/6 mice.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications and GenBank Accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application or GenBank Accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.
List of References Cited

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Amichay et al., J. Immunol 1996;157: 4511-20;
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Wang et al., J. Biol. Chem, 1996;271:8837-42;
Youngs et al., Int J Cancer, 1997;71:257-66;
WHAT IS CLAIMED IS:

1. An isolated soluble polypeptide comprising an amino acid sequence of extracellular loop 3 (E3) domain of CXCR3 (SEQ ID NO: 9), said amino acid sequence having a substitution mutation at the amino acid corresponding to aspartic acid (D) at amino acid coordinate 278 of CXCR3 (SEQ ID NO: 11), wherein said soluble polypeptide binds at least one CXCR3 ligand.

2. The isolated soluble polypeptide of claim 1, being devoid of the N-terminal domain (SEQ ID NO: 8) of CXCR3.

3. The isolated soluble polypeptide of claim 1, wherein said aspartic acid at amino acid coordinate 278 is substituted with a non-charged amino acid.

4. The isolated polypeptide of claim 3, wherein said non-charged amino acid sequence is alanine.

5. The isolated polypeptide of claim 4, comprising the amino acid sequence as set forth in SEQ ID NO: 10.

6. The isolated polypeptide of any of claims 1-5 wherein said polypeptide has reduced binding to CXCL11 as compared to native CXCR3 E3 domain (SEQ ID NO: 9).

7. The isolated polypeptide of any of claims 1-6, wherein said polypeptide is capable of binding CXCL9 and CXCL10 with a similar affinity as native CXCR3 E3 domain (SEQ ID NO: 9).

8. An isolated soluble polypeptide comprising an amino acid sequence of CXCR3 (SEQ ID NO: 11), wherein said soluble polypeptide binds at least one CXCR3 ligand and is devoid of the N-terminal domain of CXCR3 (SEQ ID NO:8).
9. The isolated polypeptide of claim 8, wherein said amino acid sequence is a CXCR3 extracellular loop domain.

10. The isolated soluble polypeptide of claim 8, as set forth in SEQ ID NO: 11 (native E3).

11. The isolated polypeptide of claim 8, wherein said amino acid sequence is a mutated CXCR3 extracellular (E3) loop domain having a substitution mutation at the amino acid corresponding to aspartic acid (D) at amino acid coordinate 278 of CXCR3 (SEQ ID NO: 11).

12. The isolated soluble polypeptide of any of claims 1-11 further comprising a heterologous amino acid sequence contiguously attached to said amino acid sequence.

13. The isolated soluble polypeptide of claim 12, wherein said heterologous sequence is an immunoglobulin amino acid sequence.

14. The isolated soluble polypeptide of claim 12, wherein said immunoglobulin sequence is an IgG Fc amino acid sequence.

15. The isolated soluble polypeptide of claim 12 comprising the amino acid sequence as set forth in SEQ ID NO: 9 or 10 (non-mutated or mutated E3).

16. A pharmaceutical composition comprising the isolated polypeptide of any of claims 1-15 and a pharmaceutically acceptable carrier.

17. An isolated polynucleotide encoding the polypeptide of any of claims 1-15.

18. The isolated polynucleotide of claim 17, comprising the nucleic acid sequence as set forth in SEQ ID NO: 25.
19. The isolated polynucleotide of claim 13, comprising the nucleic acid sequence as set forth in SEQ ID NO: 24.

20. The isolated polynucleotide of claim 13, comprising the nucleic acid sequence as set forth in SEQ ID NO: 26.

21. The isolated polynucleotide of claim 13, comprising the nucleic acid sequence as set forth in SEQ ID NO: 23.

22. A nucleic acid construct comprising the isolated polynucleotide of any of claims 17, 18, 20 or 21.

23. A method of treating a CXCR3-associated disease in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the polypeptide of any of claims 1-15, thereby treating the CXCR3-associated disease in the subject.

24. Use of the soluble polypeptide of any of claims 1-15, for the manufacture of a medicament identified for treating a CXCR3-associated disease.

25. The use of claim 22, wherein said CXCR3-associated disease is a disease associated with increased CXCL9 or CXCL10.

26. The use of claim 22, wherein said CXCR3-associated disease is a disease associated with decreased CXCL12.

27. The method or use of any of claims 23-26, wherein the CXCR3-associated disease is selected from the group consisting of multiple sclerosis, rheumatoid arthritis, hematological diseases, cardiovascular diseases, disorders of the peripheral and central nervous system, genitourinary diseases, respiratory diseases, parasitic infection, viral infection, bacterial infection, respiratory diseases, ischemia-
reperfusion injury, allograft rejection, auto-immune disease, inflammatory disease and cancer.

28. The method or use of any of claims 23-26, wherein said CXCR3-associated disease is Multiple Sclerosis.

29. The method of claim 28, further comprising concomitantly administering to said subject an anti-Multiple Sclerosis drug selected from the group consisting of Interferon Beta Ia, Interferon Beta Ib, Glatiramer Acetate, Mitoxantrone, MethylPrednisolone, Prednisone, Prednisolone, Dexamethasone, Adreno-corticotrophic Hormone (ACTH) and Corticotropin.
The point mutation of the 3rd loop of murine CXCR3 sequence (D278A):

```
E3(mut) Primer

CCCAAGCTCTGcTATCCTCATGGAAGTGGAGTTTTTGGCCCGCAACTGTG.5,

Hind III Point mutation A to C

GTGGGAGAAAGCCACGTGGATGCTGGCGGAACTGTCAGTCCTCGAGCGG-3'

Xho I
```

**FIG. 1A**

D = aspartic acid
A = Alanine
FIG. 3
Spinal chord histology

FIG. 6
FIG. 8
### INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. C07K 14/715

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)

C07K

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

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

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
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<td>X</td>
<td>WO 00/18431 A1 (CORIXA CORP [US]) 6 April 2000 (2000-04-06)</td>
<td>1-4, 6-9, 12, 15-17, 22-28, 13, 14, 20, 21, 29</td>
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<td>Y</td>
<td>the whole document</td>
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<td>Y</td>
<td>WO 2007/094005 A2 (RAPPAPORT FAMILY INST FOR Res [IL]; KARIN NATHAN [IL]; WILDBAUM GIZI) (23 August 2007 (2007-08-23)) claim 14; examples</td>
<td>13, 14, 20, 21</td>
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### Further documents are listed in the continuation of Box C

- Special categories of cited documents
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document but published on or after the international filing date
  - "L" document which may throw doubts on prior art 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
- "A" document member of the same patent family

**Date of the actual completion of the international search**

17 June 2010

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

06/07/2010

**Name and mailing address of the ISA/**

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**Authorized officer**

Pi lat, Daniel
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<tr>
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<td>COLVIN RICHARD A ET AL: &quot;CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis&quot; MOLECULAR AND CELLULAR BIOLOGY, vol. 26, no. 15, August 2006 (2006-08) , pages 5838-5849, XP002587428 ISSN: 0270-7306 cited in the application on the whole document</td>
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Form PCT/ISA/210 (patent family annex) (April 2005)