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(71) Applicant *(for all designated States except US)*: **APOLLO LIFE SCIENCES LIMITED** [AU/AU]; Level 1, 147 Queen Street, Beaconsfield, New South Wales 2015 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **PRIEST, John, D** [AU/AU]; 12b Grafton Street, Balmain, New South Wales 2041 (AU). **WATTS, Alan, D** [AU/AU]; 31 Undercliffe Street, Dee Why, New South Wales 2099 (AU). **WHITTAKER, Jason, S.** [AU/AU]; 24 Maddison Street, Redfern East, New South Wales 2016 (AU). **PILKINGTON, Glenn, R** [AU/AU]; 6 Allara Street, Rye, Victoria 3941 (AU).

(74) Agents: **HUGHES, E., John, L.** et al.; Davies Collison Cave, 1 Nicholson Street, Melbourne, Victoria 3000 (AU).

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(54) Title: AN ISOLATED IL-12 MOLECULE OR CHIMERIC MOLECULES THEREOF

(57) Abstract: The present invention relates generally to the fields of proteins, diagnostics, therapeutics and nutrition. More particularly, the present invention provides an isolated IL-12 molecule wherein the IL-12 or chimeric molecule thereof has a profile of measurable physiochemical parameters, wherein the profile is indicative of, associated with or forms the basis of one or more pharmacological traits. The present invention further contemplates the use of the isolated IL-12 or chimeric molecule thereof in a range of diagnostic, prophylactic, therapeutic, nutritional and/or research applications.

AN ISOLATED IL-12 MOLECULE OR CHIMERIC MOLECULES THEREOF

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to the fields of proteins, diagnostics, therapeutics and nutrition. More particularly, the present invention provides an isolated IL-12 or chimeric molecules thereof comprising at least a portion of the IL-12 molecule, wherein the IL-12 or chimeric molecule thereof has a profile of measurable physiochemical parameters, wherein the profile is indicative of, associated with or forms the basis of one or more pharmacological traits. The present invention further contemplates the use of the isolated IL-12 or chimeric molecule thereof in a range of diagnostic, prophylactic, therapeutic, nutritional and/or research applications.

DESCRIPTION OF THE PRIOR ART

Reference to any prior art in this specification is not, and should not be taken as an acknowledgment or any form of suggestion that this prior art forms a part of the common general knowledge.

Human interleukin 12 (IL-12, IL12) is a heterodimer comprising two disulfide linked subunits, the alpha chain (the p35 subunit) and the beta chain (the p40 subunit). The p35 subunit is known by the following alternative names: IL12A, IL-12A, cytotoxic lymphocyte maturation factor (CLMF), cytotoxic lymphocyte maturation factor p35 (CLMF p35), cytotoxic lymphocyte maturation factor 1 (CLMF1), natural killer cell stimulatory factor 1 (NKSF1) and T-cell stimulating factor 1 (TSF1). The p40 subunit is known by the following alternative names; IL12B, IL-12B, cytotoxic lymphocyte maturation factor (CLMF), cytotoxic lymphocyte maturation factor p40 (CLMF p40),

cytotoxic lymphocyte maturation factor 2 (CLMF2), natural killer cell stimulatory factor 2 (NKSF2) and T-cell stimulating factor 2 (TSF2).

IL-12A is synthesized as a 219 amino acid peptide including a 22 amino acid signal sequence and has 2 potential N-linked glycosylation sites and a theoretical molecular mass of approximately 25kDa. IL-12B is synthesized as a 328 amino acid peptide including a 22 amino acid signal sequence, has two potential N-linked glycosylation sites and a theoretical molecular mass of 37kDa.

Expression of the alpha chain and beta chain is regulated independently as the genes for the different subunits are located on different chromosomes. The majority of cell types have the ability to express the alpha chain. However, beta chain expression is restricted to dendritic cells, phagocytic cells and cells of the monocyte/macrophage lineage and B cells, cells which predominantly express functional IL-12.

IL-12 production is induced by both innate and adaptive immune responses. Induction via the innate immune response involves pathogen products such as bacterial LPS and various cell wall components, CpG nucleic acids and double stranded RNA. Additionally, IL-12 is also induced by the process of phagocytosis of bacteria.

The induction of IL-12 expression *via* the adaptive immune response involves the interaction of antigen presenting cells (APC) with T Helper (TH) cells, through CD40-CD40L interaction. Additionally, cross linking of MHC II by TCR or CD4 induces IL-12. Importantly, at least two different signals are required for the induction of IL-12: either CD40 ligation and a co-stimulatory cytokine, a bacterial product and IFN gamma or CD40L and bacterial product.

IL-12 is a pro-inflammatory cytokine that plays an crucial role in regulating both cell mediated and innate immunity. Specifically, IL-12 is the major cytokine responsible for inducing T helper 1 (TH1) cell, cytotoxic T-cell (CTL) and natural killer (NK) cell immune responses. Furthermore, IL-12 acts on T cells and NK cells, stimulating proliferation and inducing the production of interferon gamma (IFN gamma). IL-12 also

promotes the proliferation and differentiation of naive $CD4^+$ T cells into TH1 cells that produce IFN gamma, which in turn enhances IL-12 production in dendritic cells and phagocytes resulting in a strong positive feedback mechanism leading to a powerful cell mediated immune response.

Since IL-12 is a potent activator of immune response it is beneficial in clinical situations to augment immunity, including immune responses against tumours, boosting immunity against viral infections via enhanced NK cell immunity, as well as augmenting vaccine efficiencies.

The biological effector functions exerted by IL-12 may have significant potential as a therapeutic agent to modulate physiological processes. However, minor changes to the molecule such as primary, secondary, tertiary or quaternary structure and co- or post-translational modification patterns can have a significant impact on the activity, secretion, antigenicty and clearance of the protein. It is possible, therefore, that proteins can be generated with specific primary, secondary, tertiary or quaternary structure, or co- or post-translational structure or make-up that confer unique or particularly useful properties. There is consequently a need to evaluate the physiochemical properties of proteins under different conditions of production to determine whether they have useful physiochemical characteristics or other pharmacological traits.

The problem to date is that production of commercially available proteins are carried out in cells derived from species that are evolutionary distant to humans, cells such as bacteria, yeast, fungi and insect. These cells express proteins that either lack glycosylation or exhibit glycosylation repertoires that are distinct to human cells and this impacts substantially on their clinical utility. For example, proteins expressed in yeast or fungi systems such as *Aspergillus* possess a high density of mannose which makes the protein therapeutically useless (Herscovics *et al. FASEB J* 7:540-550, 1993).

Even in non-human mammalian expression systems such as Chinese hamster ovary (CHO) cells, significant differences in the glycosylation patterns are documented compared with that of human cells. For example, most mammals, including rodents, express the enzyme

(α 1,3) galactotransferase, which generates Gal (α 1,3)-Gal (β 1,4)-GlcNAc oligosaccharides on glycoproteins. However, in humans, apes and Old World monkeys, the expression of this enzyme has become inactivated through a frameshift mutation in the gene (Larsen *et al. J Biol Chem 265*:7055-7061, 1990). Although most of the CHO cell lines used for recombinant protein synthesis, such as Dux-B11, have inactivated the gene expressing (α 1,3) Galactotransferase, they still lack a functional (α 2, 6) sialyltransferase enzyme for synthesis of (α 2, 6)-linked terminal sialic acids which are present in human cells. Furthermore, the sialic acid motifs present on CHO cell expressed glycoproteins proteins are prone to degradation by a CHO cell endogenous sialidase (Gramer *et al. Biotechnology (N.Y.) 13(7)*:692-8, 1995).

As a result, proteins produced from these non-human expression systems will exhibit physiochemical and pharmacological characteristics such as half-life, antigenicity, stability and functional potency that are distinct from human cell-derived proteins.

The recent advancement of stem cell technology has substantially increased the potential for utilizing stem cells in applications such as transplantation therapy, drug screening, toxicology studies and functional genomics. However, stem cells are routinely maintained in culture medium that contains non-human proteins and are therefore not suitable for clinical applications due to the possibility of contamination with non-human infectious material. Furthermore, culturing of stem cells in non-human derived media may result in the incorporation of non-human carbohydrate moieties thus compromising transplant application (Martin *et al. Nature Med 11(2)*:228-232, 2005). Hence, the use of specific human-derived proteins in the maintenance and/or differenttiation of stem cells will ameliorate the incorporation of xenogeneic proteins and enhance stem cell clinical utility.

Accordingly, there is a need to develop proteins and their receptors which have particularly desired physiochemical and pharmacological properties for use in diagnostic, prophylactic, therapeutic and/or nutritional research applications.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

The present invention relates generally to an isolated IL-12 or chimeric molecule thereof comprising a profile of physiochemical parameters, wherein the profile is indicative of, associated with, or forms the basis of one or more distinctive pharmacological traits. The present invention also provides an isolated IL-12 or chimeric molecule thereof comprising a physiochemical profile comprising a number of measurable physiochemical parameters, $\{[P_x]_1, [P_x]_2, \ldots [P_x]_n,\}$, wherein P_x represents a measurable physiochemical parameter and "n" is an integer ≥ 1, wherein each parameter between and including $[P_x]_1$ to $[P_x]_n$ is a different measurable physiochemical parameter, wherein the value of any one or more of the measurable physiochemical characteristics is indicative of, associated with, or forms the basis of, a distinctive pharmacological trait, T_y, or series of distinctive pharmacological traits $\{[T_y]_1, [T_y]_2, \ldots [T_y]_m\}$ wherein T_y represents a distinctive pharmacological trait and m is an integer ≥ 1 and each of $[T_y]_1$ to $[T_y]_m$ is a different pharmacological trait.

As used herein the term "distinctive" with regard to a pharmacological trait of a protein or chimeric molecule thereof refers to one or more pharmacological traits of an isolated IL-12 or chimeric molecule thereof which are distinctive for the particular physiochemical profile. In one embodiment, one or more of the pharmacological traits of an isolated IL-12 or chimeric molecule thereof is different from, or distinctive relative to a form of the same IL-12 or chimeric molecule thereof produced in a prokaryotic or lower eukaryotic cell or even a higher eukaryotic cell of a non-human species. In another embodiment, the

pharmacological traits of an isolated IL-12 or chimeric molecule thereof contribute to a desired functional outcome. As used herein, the term "measurable physiochemical parameters" or Px refers to one or more measurable characteristics of the isolated IL-12 or chimeric molecule thereof. In a particular embodiment of the present invention, the measurable physiochemical parameters of a subject isolated IL-12 or chimeric molecule thereof contribute to or are otherwise responsible for the derived pharmacological trait, Ty.

An isolated IL-12 of the present invention comprises physiochemical parameters (P_x) which define the IL-12 or chimeric molecule. The physiochemical parameters may be selected from the group consisting of apparent molecular weight (P_1), isoelectric point (pI) (P_2), number of isoforms (P_3), relative intensities of the different number of isoforms (P_4), percentage by weight carbohydrate (P_5), observed molecular weight following N-linked oligosaccharide deglycosylation (P_6), observed molecular weight following N-linked and O-linked oligosaccharide deglycosylation (P_7), percentage acidic monosaccharide content (P_8), monosaccharide content (P_9), sialic acid content (P_{10}), sulfate and phosphate content (P_{11}), Ser/Thr : GalNAc ratio (P_{12}), neutral percentage of N-linked oligosaccharide content (P_{13}), acidic percentage of N-linked oligosaccharide content (P_{14}), neutral percentage of O-linked oligosaccharide content (P_{15}), acidic percentage of O-linked oligosaccharide content (P_{16}), ratio of N-linked oligosaccharides (P_{17}), ratio of O-linked oligosaccharides (P_{18}), structure of N-linked oligosaccharide fraction (P_{19}), structure of O-linked oligosaccharide fraction (P_{20}), position and make up of N-linked oligosaccharides (P_{21}), position and make up of O-linked oligosaccharides (P_{22}), co-translational modification (P_{23}), post-translational modification (P_{24}), acylation (P_{25}), acetylation (P_{26}), amidation (P_{27}), deamidation (P_{28}), biotinylation (P_{29}), carbamylation or carbamoylation (P_{30}), carboxylation (P_{31}), decarboxylation (P_{32}), disulfide bond formation (P_{33}), fatty acid acylation (P_{34}), myristoylation (P_{35}), palmitoylation (P_{36}), stearoylation (P_{37}), formylation (P_{38}), glycation (P_{39}), glycosylation (P_{40}), glycophosphatidylinositol anchor (P_{41}), hydroxylation (P_{42}), incorporation of selenocysteine (P_{43}), lipidation (P_{44}), lipoic acid addition (P_{45}), methylation (P_{46}), N- or C-terminal blocking (P_{47}), N- or C-terminal removal (P_{48}), nitration (P_{49}), oxidation of methionine (P_{50}), phosphorylation (P_{51}), proteolytic cleavage (P_{52}), prenylation (P_{53}), farnesylation (P_{54}), geranyl geranylation (P_{55}), pyridoxal phosphate addition (P_{56}), sialylation (P_{57}), desialylation (P_{58}), sulfation (P_{59}),

ubiquitinylation or ubiquitination (P_{60}), addition of ubiquitin-like molecules (P_{61}), primary structure (P_{62}), secondary structure (P_{63}), tertiary structure (P_{64}), quaternary structure (P_{65}), chemical stability (P_{66}), thermal stability (P_{67}). A list of these parameters is summarized in Table 2.

In an embodiment, an IL-12 molecule of the present invention is characterized by a profile of one or more of the following physiochemical parameters (P_x) and pharmacological traits (T_y), comprising:

- an apparent molecular weight (P_1) of the alpha chain of about 1 to 80, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80 kDa and in one embodiment, 20 to 38 kDa;
- an apparent molecular weight (P_1) of the beta chain of about 1 to 100, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 kDa and in one embodiment, 27.5 to 55 kDa;
- an apparent molecular weight (P_1) of the disulphide linked alpha and beta chains of about 1 to 180, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180 kDa and in one embodiment, 47.5 to 93 kDa;
- a pI (P_2) range of the alpha chain of about 2 to about 14 such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and in one embodiment, 3.5 to 9.8;
- a pI (P_2) range of the beta chain of about 2 to about 14 such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and in one embodiment, 5.0 to 9.3;
- a total number of isoforms of the alpha and beta chains of about 2 to 100 isoforms (P_3), such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22,

23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 isoforms and in one embodiment 22 to 59 isoforms;

- a percentage by weight carbohydrate (P_5) of the alpha chain of about 0 to 99%, such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% and in one embodiment, 0 to 40%;
- a percentage by weight carbohydrate (P_5) of the beta chain of about 0 to 99%, such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% and in one embodiment, 0 to 37%;
- an observed molecular weight of the molecule under reduced conditions and following the removal of the N-linked oligosaccharides (P_6) of about 20 to 55 kDa;
- an observed molecular weight of the molecule under reduced conditions and following the removal of both the N-linked and O-linked oligosaccharides (P_7) of about 20 to 55 kDa;
- one or more N-glycan structures as listed in Table 9 in the N-linked fraction (P_{19});
- one or more O-glycan structures as listed in Table 10 in the O-linked fraction (P_{20});
- a site of C- mannosylation glycosylation (P_{40}) which includes Trp-319;
- an immunoreactivity profile (T_{13}) that is distinct from that of a human IL-12 molecule expressed in a non-human cell system, and in one embodiment, the protein concentration of the IL-12 of the present invention is underestimated when assayed using a quantitative immunoassay which includes a protein standard of a human IL-12 molecule expressed in *Sf*21 cells;
- a biological activity that is distinct from that of a human IL-12 expressed in a non-human cell system, and in one embodiment, the ability of IL-12 of the present

invention to induce proliferation (T_{32}) in PHA-stimulated PBMCs is significantly more potent than a human IL-12 expressed in CHO cells;

- a biological activity that is distinct from that of a human IL-12 expressed in a non-human cell system, and in one embodiment, the ability of IL-12 of the present invention to induce STAT4 activation (T_{45}) of PHA-stimulated PBMCs is significantly greater than a human IL-12 expressed in CHO cells;
- a biological activity that is distinct from that of a human IL-12 expressed in a non-human cell system, and in one embodiment, the ability of IL-12 of the present invention to induce interferon-gamma production (T_{56}) by PHA stimulated PBMCs is significantly more potent than a human IL-12 expressed in CHO cells; and
- a biological activity that is distinct from that of a human IL-12 expressed in a non-human cell system, and in one embodiment, the ability of IL-12 of the present invention to induce CD56 (T_{59}) on CD34+ human haematopoietic cells to a significantly greater extent than a human IL-12 expressed in CHO cells.

An isolated IL-12 of the present invention comprises distinctive pharmacological traits selected from the group comprising or consisting of therapeutic efficiency (T_1), effective therapeutic dose ($TCID_{50}$) (T_2), bioavailability (T_3), time between dosages to maintain therapeutic levels (T_4), rate of absorption (T_5), rate of excretion (T_6), specific activity (T_7), thermal stability (T_8), lyophilization stability (T_9), serum/plasma stability (T_{10}), serum half-life (T_{11}), solubility in blood stream (T_{12}), immunoreactivity profile (T_{13}), immunogenicity (T_{14}), inhibition by neutralizing antibodies (T_{14A}), side effects (T_{15}), receptor/ligand binding affinity (T_{16}), receptor/ligand activation (T_{17}), tissue or cell type specificity (T_{18}), ability to cross biological membranes or barriers (i.e. gut, lung, blood brain barriers, skin etc) (T_{19}), angiogenic ability (T_{19A}), tissue uptake (T_{20}), stability to degradation (T_{21}), stability to freeze-thaw (T_{22}), stability to proteases (T_{23}), stability to ubiquitination (T_{24}), ease of administration (T_{25}), mode of administration (T_{26}), compatibility with other pharmaceutical excipients or carriers (T_{27}), persistence in organism or environment (T_{28}), stability in storage (T_{29}), toxicity in an organism or environment and the like (T_{30}).

In addition, the IL-12 of the present invention may have altered biological effects on different cells types (T_{31}), including without being limited to human primary cells, such as

lymphocytes, erythrocytes, retinal cells, hepatocytes, neurons, keratinocytes, endothelial cells, endodermal cells, ectodermal cells, mesodermal cells, epithelial cells, kidney cells, liver cells, bone cells, bone marrow cells, lymph node cells, dermal cells, fibroblasts, T-cells, B-cells, plasma cells, natural killer cells, macrophages, granulocytes, neutrophils, Langerhans cells, dendritic cells, eosinophils, basophils, mammary cells, lobule cells, prostate cells, lung cells, oesophageal cells, pancreatic cells, Beta cells (insulin secreting cells), hemangioblasts, muscle cells, oval cells (hepatocytes), mesenchymal cells, brain microvessel endothelial cells, astrocytes, glial cells, various stem cells including adult and embryonic stem cells, various progenitor cells; and other human immortal, transformed or cancer cell lines.

The biological effects on the cells include effects on proliferation (T_{32}), differentiation (T_{33}), apoptosis (T_{34}), growth in cell size (T_{35}), cytokine adhesion (T_{36}), cell adhesion (T_{37}), cell spreading (T_{38}), cell motility (T_{39}), migration and invasion (T_{40}), chemotaxis (T_{41}), cell engulfment (T_{42}), signal transduction (T_{43}), recruitment of proteins to receptors/ligands (T_{44}), activation of the JAK/STAT pathway (T_{45}), activation of the Ras-erk pathway (T_{46}), activation of the AKT pathway (T_{47}), activation of the PKC pathway (T_{48}), activation of the PKA pathway (T_{49}), activation of src (T_{50}), activation of fas (T_{51}), activation of TNFR (T_{52}), activation of NFkB (T_{53}), activation of p38MAPK (T_{54}), activation of c-fos (T_{55}), secretion (T_{56}), receptor internalization (T_{57}), receptor cross-talk (T_{58}), up or down regulation of surface markers (T_{59}), alteration of FACS front/side scatter profiles (T_{60}), alteration of subgroup ratios (T_{61}), differential gene expression (T_{62}), cell necrosis (T_{63}), cell clumping (T_{64}), cell repulsion (T_{65}), binding to heparin sulfates (T_{66}), binding to glycosylated structures (T_{67}), binding to chondroitin sulfates (T_{68}), binding to extracellular matrix (such as collagen, fibronectin) (T_{69}), binding to artificial materials (such as scaffolds) (T_{70}), binding to carriers (T_{71}), binding to co-factors (T_{72}) the effect alone or in combination with other proteins on stem cell proliferation, differentiation and/or self-renewal (T_{73}) and the like. These are summarized in Table 3.

Accordingly, the present invention provides an isolated polypeptide encoded by a nucleotide sequence selected from the list consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, or a nucleotide sequence having at least about 90% identity to any one of the

above-listed sequence or a nucleotide sequence capable of hybridizing to any one of the above sequences or their complementary forms under high stringency conditions.

Yet another aspect of the present invention provides an isolated polypeptide comprising an amino acid sequence selected from the list consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 21, or an amino acid sequence having at least about 90% similarity to one or more of the above sequences.

The present invention further contemplates a pharmaceutical composition comprising at least part of the IL-12 or chimeric molecule thereof, together with a pharmaceutically acceptable carrier, co-factor and/or diluent.

With respect to the primary structure, the present invention provides an isolated IL-12 or chimeric molecule thereof, or a fragment thereof, encoded by a nucleotide sequence selected from the list consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, or a nucleotide sequence having at least about 90% identity to any one of the above-listed sequence or a nucleotide sequence capable of hybridizing to any one of the above sequences or their complementary forms under high stringency conditions.

Still, another aspect of the present invention provides an isolated nucleic acid molecule encoding IL-12 or chimeric molecule thereof or a functional part thereof comprising a sequence of nucleotides having at least 90% similarity selected from the list consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or after optimal alignment and/or being capable of hybridizing to one or more of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or their complementary forms under high stringency conditions.

In a particular embodiment, the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding a protein or chimeric molecule in or related to IL-12 having an amino acid sequence substantially as set forth in one or more of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 21 or an amino acid sequence having at least about 90% similarity to one or more of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 21 after alignment.

The present invention further extends to uses of an isolated IL-12 or chimeric molecule thereof or nucleic acid molecules encoding same in diagnostic, prophylactic, therapeutic, nutritional and/or research applications. The present invention also extends to a method of treating or preventing a condition or ameliorating the symptoms of a condition in an animal subject, said method comprising administering to said animal subject an effective amount of an isolated IL-12 or chimeric molecule thereof.

In addition, the present invention extends to uses of a protein or chimeric molecule thereof for screening small molecules, which may have a variety of diagnostic, prophylactic, therapeutic, nutritional and/or research applications.

The present invention further contemplates using an isolated IL-12 or chimeric molecule thereof as immunogens to generate antibodies for therapeutic or diagnostic applications.

The present invention further contemplates using an isolated IL-12 or chimeric molecule thereof in culture mediums for stem cells used in stem cell or related therapy.

The subject invention also provides the use of an isolated IL-12 protein or chimeric molecule thereof in the manufacture of a formulation for diagnostic, prophylactic, therapeutic, nutritional and/or research applications.

The subject invention also provides a human derived IL-12 protein or chimeric molecule thereof for use as a standard protein in an immunoassay and kits thereof. The subject invention also extends to a method for determining the level of human cell-expressed human protein or chimeric molecule thereof in a biological preparation.

TABLE 1

Sequence Identifier

Sequence Identifier	Sequence
SEQ ID NO:1	IL-12 p35 nucleotide sequence for signal peptide
SEQ ID NO:2	IL-12 p35 amino acid sequence for signal peptide
SEQ ID NO:3	IL-12 p40 nucleotide sequence for signal peptide
SEQ ID NO:4	IL-12 p40 amino acid sequence for signal peptide
SEQ ID NO:5	IL-12 p40 nucleotide sequence for signal peptide (variant)
SEQ ID NO:6	IL-12 p40 amino acid sequence for signal peptide (variant)
SEQ ID NO:7	IL-12 p35 nucleotide sequence for mature peptide
SEQ ID NO:8	IL-12 p35 amino acid sequence for mature peptide
SEQ ID NO:9	IL-12 p40 nucleotide sequence for mature peptide
SEQ ID NO:10	IL-12 p40 amino acid sequence for mature peptide
SEQ ID NO:11	IL-12 p35 nucleotide sequence for signal peptide + mature peptide
SEQ ID NO:12	IL-12 p35 amino acid sequence for signal peptide + mature peptide p35
SEQ ID NO:13	IL-12 p40 nucleotide sequence for signal peptide + mature peptide
SEQ ID NO:14	IL-12 p40 amino acid sequence for signal peptide + mature peptide
SEQ ID NO:15	IL-12 p40 nucleotide sequence for signal peptide (variant) + mature peptide
SEQ ID NO:16	IL-12 p40 amino acid sequence for signal peptide (variant) + mature peptide
SEQ ID NO:17	IL-12 nucleotide sequence for whole construct (p35 signal peptide + mature peptide + non-coding sequence + p40 signal peptide + mature peptide)
SEQ ID NO:18	IL-12 amino acid sequence for whole construct (p35 signal peptide + p35 mature peptide + p40 signal peptide + p40 mature peptide)

Sequence Identifier	Sequence
SEQ ID NO:19	IL-12 nucleotide sequence for whole construct (p35 signal peptide + p35 mature peptide + non-coding sequence + p40 signal peptide (variant) + p40 mature peptide)
SEQ ID NO:20	IL-12 amino acid sequence for whole construct (p35 signal peptide + p35 mature peptide + p40 signal peptide (variant) + p40 mature peptide)
SEQ ID NO:21	IL-12 amino acid sequence for whole construct (p35 mature peptide + p40 mature peptide)

TABLE 2

List of physiochemical parameters

P_x	Physiochemical Parameter	IL-12
P_1	Apparent molecular weight	Alpha chain: 20 to 38 kDa; Beta chain: 27.5 to 55 kDa; Disulphide linked alpha + beta chains: 47.5 to 93 kDa.
P_2	Isoelectric point (pI)	Alpha chain: 3.5 to 9.8; Beta chain: 5.0 to 9.3.
P_3	Number of isoforms	22 to 59 isoforms
P_4	Relative intensities of the different number of isoforms	
P_5	Percentage by weight carbohydrate	Alpha chain: 0 to 40%; Beta chain: 0 to 37%.
P_6	Observed molecular weight following N-linked oligosaccharide deglycosylation	20 to 55 kDa
P_7	Observed molecular weight following N-linked oligosaccharide deglycosylation and O-linked oligosaccharide deglycosylation	20 to 55 kDa

P_x	**Physiochemical Parameter**	**IL-12**
P_8	Percentage acidic monosaccharide content	
P_9	Monosaccharide content	
P_{10}	Sialic acid content	
P_{11}	Sulfate and phosphate content	
P_{12}	Ser/Thr : GalNAc ratio	
P_{13}	Neutral percentage of N-linked oligosaccharide content	
P_{14}	Acidic percentage of N-linked oligosaccharide content	
P_{15}	Neutral percentage of O-linked oligosaccharide content	
P_{16}	Acidic percentage of O-linked oligosaccharide content	
P_{17}	Ratio of N-linked oligosaccharides	
P_{18}	Ratio of O-linked oligosaccharides	
P_{19}	Structure of N-linked fraction	One or more N-glycan structures as listed in Table 9
P_{20}	Structure of O-linked fraction	One or more O-glycan structures as listed in Table 10
P_{21}	Position and make up of N-linked oligosaccharides	
P_{22}	Position and make up of O-linked oligosaccharides	
P_{23}	Co-translational modification	
P_{24}	Post-translational modification	
P_{25}	Acylation	
P_{26}	Acetylation	
P_{27}	Amidation	
P_{28}	Deamidation	

P_x	**Physiochemical Parameter**	**IL-12**
P_{29}	Biotinylation	
P_{30}	Carbamylation or carbamoylation	
P_{31}	Carboxylation	
P_{32}	Decarboxylation	
P_{33}	Disulfide bond formation	
P_{34}	Fatty acid acylation	
P_{35}	Myristoylation	
P_{36}	Palmitoylation	
P_{37}	Stearoylation	
P_{38}	Formylation	
P_{39}	Glycation	
P_{40}	Glycosylation	A site of C-mannosylation which includes Trp-319
P_{41}	Glycophosphatidylinositol anchor	
P_{42}	Hydroxylation	
P_{43}	Incorporation of selenocysteine	
P_{44}	Lipidation	
P_{45}	Lipoic acid addition	
P_{46}	Methylation	
P_{47}	N or C terminal blocking	
P_{48}	N or C terminal removal	
P_{49}	Nitration	
P_{50}	Oxidation of methionine	
P_{51}	Phosphorylation	
P_{52}	Proteolytic cleavage	
P_{53}	Prenylation	
P_{54}	Farnesylation	
P_{55}	Geranyl geranylation	
P_{56}	Pyridoxal phosphate addition	
P_{57}	Sialylation	

P_x	**Physiochemical Parameter**	**IL-12**
P_{58}	Desialylation	
P_{59}	Sulfation	
P_{60}	Ubiquitinylation or ubiquitination	
P_{61}	Addition of ubiquitin-like molecules	
P_{62}	Primary structure	
P_{63}	Secondary structure	
P_{64}	Tertiary structure	
P_{65}	Quaternary structure	
P_{66}	Chemical stability	
P_{67}	Thermal stability	

TABLE 3

List of Pharmacological traits

T_y	**Pharmacological trait**	**IL-12**
T_1	Therapeutic efficiency	
T_2	Effective therapeutic dose ($TCID_{50}$)	
T_3	Bioavailability	
T_4	Time between dosages to maintain therapeutic levels	
T_5	Rate of absorption	
T_6	Rate of excretion	
T_7	Specific activity	
T_8	Thermal stability	
T_9	Lyophilization stability	
T_{10}	Serum/plasma stability	
T_{11}	Serum half-life	
T_{12}	Solubility in blood stream	
T_{13}	Immunoreactivity Profile	Distinct from that of a recombinant human IL-12 molecule expressed in a non-human system.

T_y	**Pharmacological trait**	**IL-12**
T_{14}	Immunogenicity	
T_{14A}	Inhibitable by neutralizing antibodies	
T_{15}	Side effects	
T_{16}	Receptor/ligand binding affinity	
T_{17}	Receptor/ligand activation	
T_{18}	Tissue or cell type specificity	
T_{19}	Ability to cross biological membranes or barriers (i.e. gut, lung, blood brain barriers, skin etc)	
T_{19A}	Angiogenic ability	
T_{20}	Tissue uptake	
T_{21}	Stability to degradation	
T_{22}	Stability to freeze-thaw	
T_{23}	Stability to proteases	
T_{24}	Stability to ubiquitination	
T_{25}	Ease of administration	
T_{26}	Mode of administration	
T_{27}	Compatibility with other pharmaceutical excipients or carriers	
T_{28}	Persistence in organism or environment	
T_{29}	Stability in storage	
T_{30}	Toxicity in an organism or environment and the like	
T_{31}	Altered biological effects on different cells types	
T_{32}	Proliferation	Significantly more potent than a human IL-12 expressed in CHO cells in the proliferation (T32) of PHA-stimulated PBMCs.

T_y	**Pharmacological trait**	**IL-12**
T_{33}	Differentiation	
T_{34}	Apoptosis	
T_{35}	Growth in cell size	
T_{36}	Cytokine adhesion	
T_{37}	Cell adhesion	
T_{38}	Cell spreading	
T_{39}	Cell motility	
T_{40}	Migration and invasion	
T_{41}	Chemotaxis	
T_{42}	Cell engulfment	
T_{43}	Signal transduction	
T_{44}	Recruitment of proteins to receptors/ligands	
T_{45}	Activation of the JAK/STAT pathway	Significantly greater activation of STAT4 activation in PHA-stimulated PBMCs than that induced by a recombinant human IL-12 expressed in CHO cells.
T_{46}	Activation of the Ras-erk pathway	
T_{47}	Activation of the AKT pathway	
T_{48}	Activation of the PKC pathway and PKA pathway	
T_{49}	Activation of the PKA pathway	
T_{50}	Activation of src	
T_{51}	Activation of fas	
T_{52}	Activation of TNFR	
T_{53}	Activation of NFkB	
T_{54}	Activation of p38MAPK	
T_{55}	Activation of c-fos	
T_{56}	Secretion	Significantly more potent than a

T_y	**Pharmacological trait**	**IL-12**
		human IL-12 expressed in CHO cells in the induction of interferon-gamma production by PHA-stimulated PBMCs.
T_{57}	Receptor internalization	
T_{58}	Receptor cross-talk	
T_{59}	Up or down regulation of surface markers	Significantly greater induction of CD56 on CD34+ human haematopoietic cells than that induced by a recombinant human IL-12 expressed in CHO cells.
T_{60}	Alteration of FACS front/side scatter profiles	
T_{61}	Alteration of subgroup ratios	
T_{62}	Differential gene expression	
T_{63}	Cell necrosis	
T_{64}	Cell clumping	
T_{65}	Cell repulsion	
T_{66}	Binding to heparin sulfates	
T_{67}	Binding to glycosylated structures	
T_{68}	Binding to chondroitin sulfates	
T_{69}	Binding to extracellular matrix (such as collagen, fibronectin)	
T_{70}	Binding to artificial materials (such as scaffolds)	
T_{71}	Binding to carriers	
T_{72}	Binding to co-factors	
T_{73}	The effect alone or in combination with other proteins on stem cell proliferation, differentiation and/or	

T_y	Pharmacological trait	IL-12
	self-renewal.	

A list of abbreviations commonly used herein is provided in Tables 4 and 5.

TABLE 4

Abbreviations and alternate names

Abbreviation	Description
AAA	Amino Acid Analysis
AFC	Affinity Chromatography
bFGF	Basic Fibroblast Growth Factor, FGF2
BSA	Bovine Serum Albumin
cDLC	Combinatorial Dye Ligand Chromatography
CRD	Carbohydrate Recognition Domain
CSF	Colony Stimulating Factor
DCS	Donor Calf Serum
DeoxGlc	2-deoxyglucose
DLC	Dye Ligand pseudoaffinity Chromatography
DSC	Differential Scanning Calorimetry
ECD	Extracellular domain
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assays
EPO	Erythropoietin
EST	Expressed Sequence Tags
Fc	Fragment Crystallizable or Immunoglobulin constant region
FCS	Fetal Calf Serum
FGF2	Basic Fibroblast Growth Factor, bFGF
FTIS	Fourier Transform Infrared Spectroscopy
Fuc	Fucose
G-CSF	Granulocyte Colony Stimulating Factor
Gal	Galactose

Abbreviation	Description
GalNAc, galactosamine	2-deoxy, 2 amino galactose
GFC	Gel Filtration Chromatography
GlcA	Glucuronic acid
GlcNAc, glucosamine	2-deoxy, 2 amino glucose
Glc	Glucose
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HBS	Hepes Buffered Saline
hES	Human Embryonic Stem Cells
HIC	Hydrophobic Interaction Chromatography
HPAEC-PAD	High-pH anion-exchange chromatography with pulsed amperometric detection
HPLC	High Pressure Liquid Chromatography or High Performance Liquid Chromatography
HSA	Human Serum Albumin
HTS	High Throughput Screening
IdoA	Iduronic acid
IEC	Ion Exchange Chromatography
IEF	Isoelectric focussing
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
lacNAc	N-acetyl lactosamine
lacdiNAc	N,N'-diacetyllactosediamine
LC	Liquid Chromatography
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization - Time of Flight
Man	Mannose
MCC	Metal Chelating Chromatography
MS	Mass Spectroscopy
NacSial, NeuAc or NeuNAc	N-acetyl neuraminic acid
NGlySial, NeuGc or	N-glycolyl neuraminic acid

Abbreviation	Description
NeuGly	
PBS	Phosphate Buffered Saline
PCS	Photon Correlation Spectroscopy
PDGF-AA	Platelet Derived Growth Factor A homodimer
PNGase	Peptide-N4-(N-acetyl-β-D-glucosaminyl) Asparagine Amidase
RMLP	Receptor Mediated Ligand Chromatography
RPC	Reversed Phase Chromatography
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
Sia	Sialic acid
TCA	Trichloroacetic acid
TFF	Tangential flow filtration
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
Xyl	Xylose

TABLE 5

Abbreviations for amino acids

Amino Acid	3 Letter Code	1 Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

TABLE 6

Stem cell list

Cell type
General Stem Cell Types
Embryonic stem cells
Somatic stem cells
Germ stem cells
Human embryonic stem cells
Human epidermal stem cells
Adipose derived stem cells
Brain
Adult neural stem cells
Human neurons
Human astrocytes
Epidermis
Human keratinocyte stem cells
Human keratinocyte transient amplifying cells
Human melanocyte stem cells
Human melanocytes
Skin
Human foreskin fibroblasts
Pancreas
Human duct cells
Human pancreatic islets
Human pancreatic beta-cells
Kidney
Human adult renal stem cells
Human embryonic renal epithelial stem cells
Human kidney epithelial cells
Liver
Human hepatic oval cells
Human hepatocytes

Cell type
Human bile duct epithelial cells
Human embryonic endodermal stem cells
Human adult hepatocyte stem cells (existence controversial)
Breast
Human mammary epithelial stem cells
Lung
Bone marrow-derived stem cells
Human lung fibroblasts
Human bronchial epithelial cells
Human alveolar type II pneumocytes
Muscle
Human skeletal muscle stem cells (satellite cells)
Heart
Human cardiomyocytes
Bone marrow mesenchymal stem cells
Simple Squamous Epithelial cells
Descending Aortic Endothelial cells
Aortic Arch Endothelial cells
Aortic Smooth Muscle cells
Eye
Limbal stem cells
Corneal epithelial cells
CD34+ hematopoietic stem cells
Mesenchymal stem cells
Osteoblasts (precursor is mesenchymal stem cell)
Peripheral blood mononuclear progenitor cells (hematopoietic stem cells)
Osteoclasts (precursor is above cell type)
Stromal cells
Spleen
Human splenic precursor stem cells
Human splenocytes

Cell type
Immune cells
Human CD4+ T-cells
Human CD8+ T-cells
Human NK cells
Human monocytes
Human macrophages
Human dendritic cells
Human B-cells
Nose
Goblet cells (mucus secreting cells of the nose)
Pseudostriated ciliated columnar cells (located below olfactory region in the nose)
Pseudostratified ciliated epithelium (cells that line the nasopharangeal tubes)
Trachea
Stratified Epithelial cells (cells that line and structure the trachea)
Ciliated Columnar cells (cells that line and structure the trachea)
Goblet cells (cells that line and structure the trachea)
Basal cells (cells that line and structure the trachea)
Oesophagus
Cricopharyngeus muscle cells
Reproduction
Female primary follicles
Male spermatogonium

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphical representation comparing the proliferation of PHA-activated PBMC by IL-12 of the present invention (filled circles) and rh IL-12 expressed using CHO cells (open circles).

Figure 2 is a graphical representation comparing the activation of STAT4 in PHA-activated PBMC by IL-12 of the present invention (filled bars) and rh IL-12 expressed using CHO cells (open bars).

Figure 3 is a graphical representation comparing the levels of CD56 expression by $CD34^+$ human haematopoietic cells following treatment with IL-12 of the present invention (IL12 PI) and rh IL-12 expressed using CHO cells (CHO).

Figure 4 is a graphical representation comparing the production of interferon-gamma in PHA-activated PBMC by different concentrations of IL-12 of the present invention (filled bars) and rh IL-12 expressed using CHO cells (open bars). Error bars represent standard deviation.

Figure 5 is a graphical representation showing the *in vitro* comparison of immunoreactivity profiles between IL-12 of the present invention and a human IL-12 molecule expressed using non-human systems such as CHO and *Sf*21. OD-antibody dilution plots for the IL-12 of the present invention (filled squares), recombinant human IL-12 molecule expressed in CHO (open triangles), recombinant human IL-12 molecule expressed in *Sf*21 (filled circles) and standard curve using the supplied protein standard (recombinant human IL-12 molecule expressed in *Sf*21; open diamonds). Error bars represent standard deviation.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is to be understood that unless otherwise indicated, the subject invention is not limited to specific formulations, manufacturing methods, diagnostic methods, assay protocols, nutritional protocols, or research protocols or the like as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context already dictates otherwise. Thus, for example, reference to "a protein", "a cytokine" or "a chimeric molecule" or "a receptor" includes a single protein, cytokine or receptor or chimeric molecule as well as two or more proteins, cytokines or receptors or chimeric molecules; a "physiochemical parameter" includes a single parameter as well as two or more parameters and so forth.

The terms "compound", "active agent", "chemical agent", "pharmacologically active agent", "medicament", "active" and "drug" are used interchangeably herein to refer to a chemical compound and in particular a protein or chimeric molecule thereof that induces a desired pharmacological and/or physiological effect. The terms also encompass pharmaceutically acceptable and pharmacologically active ingredients of those active agents specifically mentioned herein including but not limited to salts, esters, amides, prodrugs, active metabolites, analogs and the like. When the terms "compound", "active agent", "chemical agent" "pharmacologically active agent", "medicament", "active" and "drug" are used, then it is to be understood that this includes the active agent *per se* as well as pharmaceutically acceptable, pharmacologically active salts, esters, amides, prodrugs, metabolites, analogs, etc.

Reference to a "compound", "active agent", "chemical agent" "pharmacologically active agent", "medicament", "active" and "drug" includes combinations of two or more actives such as two or more cytokines. A "combination" also includes multi-part such as a two-part composition where the agents are provided separately and given or dispensed separately or admixed together prior to dispensation.

For example, a multi-part pharmaceutical pack may have two or more proteins or chimeric molecules in or related to IL-12, separately maintained.

The terms "effective amount" and "therapeutically effective amount" of an agent as used herein means a sufficient amount of the protein, alone or in combination with other agents to provide the desired therapeutic or physiological effect or outcome. Undesirable effects, e.g. side effects, are sometimes manifested along with the desired therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks in determining what is an appropriate "effective amount". The exact amount required will vary from subject to subject, depending on the species, age and general condition of the subject, mode of administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using only routine experimentation.

By "pharmaceutically acceptable" carrier, excipient or diluent is meant a pharmaceutical vehicle comprised of a material that is not biologically or otherwise undesirable, i.e. the material may be administered to a subject along with the selected active agent without causing any or a substantial adverse reaction. Carriers may include excipients and other additives such as diluents, detergents, coloring agents, wetting or emulsifying agents, pH buffering agents, preservatives, and the like.

Similarly, a "pharmacologically acceptable" salt, ester, amide, prodrug or derivative of a compound as provided herein is a salt, ester, amide, prodrug or derivative that this not biologically or otherwise undesirable.

The terms "treating" and "treatment" as used herein refer to reduction in severity and/or frequency of symptoms of the condition being treated, elimination of symptoms and/or underlying cause, prevention of the occurrence of symptoms of the condition and/or their underlying cause and improvement or remediation or amelioration of damage following a condition.

"Treating" a subject may involve prevention of a condition or other adverse physiological event in a susceptible individual as well as treatment of a clinically symptomatic individual by ameliorating the symptoms of the condition.

A "subject" as used herein refers to an animal, in a particular embodiment, a mammal and in a further embodiment human who can benefit from the pharmaceutical formulations and methods of the present invention. There is no limitation on the type of animal that could benefit from the presently described pharmaceutical formulations and methods. A subject regardless of whether a human or non-human animal may be referred to as an individual, patient, animal, host or recipient. The compounds and methods of the present invention have applications in human medicine, veterinary medicine as well as in general, domestic or wild animal husbandry.

As indicated above, in a particular embodiment, the animals are humans or other primates such as orangutans, gorillas, marmosets, livestock animals, laboratory test animals, companion animals or captive wild animals, as well as avian species.

Examples of laboratory test animals include mice, rats, rabbits, guinea pigs and hamsters. Rabbits and rodent animals, such as rats and mice, provide a convenient test system or animal model. Livestock animals include sheep, cows, pigs, goats, horses and donkeys. Non-mammalian animals such as avian species, fish, and amphibians including *Xenopus* spp prokaryotes and non-mammalian eukaryotes.

The term "cytokine" is used in its most general sense and includes any of various proteins secreted by cells to regulate the immune system, modulate the functional activities of individual cells and/or tissues, and/or induce a range of physiological responses. As used herein the term "cytokine" should be understood to refer to a "complete" cytokine as well as fragments, derivatives or homologs or chimeras thereof comprising one or more amino acid additions, deletions or substitutions, but which substantially retain the biological activity of the complete cytokine.

A "cytokine receptor" is a cell membrane associated or soluble portion of the cytokine receptor involved in cytokine signalling or regulation. As used herein the term "cytokine receptor" should be understood to refer to a "complete" cytokine receptor as well as fragments, derivatives or homologs or chimeras thereof comprising one or more amino acid additions, deletions or substitutions, but which substantially retain the biological activity of the complete cytokine receptor.

The term "protein" is used in its most general sense and includes cytokines and cytokine receptors. As used herein, the term "protein" should be understood to refer to a "complete" protein as well as fragments, derivatives or homologs or chimeras thereof comprising one or more amino acid additions, deletions or substitutions, but which substantially retain the biological activity of the complete protein.

The present invention contemplates an isolated IL-12 or chimeric molecule thereof having a profile of measurable physiochemical parameters (P_x), wherein the profile is indicative of, associated with or forms the basis of one or more distinctive pharmacological traits (T_y). As used herein, the term IL-12 includes reference to the whole polypeptide as well as fragments thereof.

More particularly, the present invention provides an isolated IL-12 or chimeric molecule thereof having a physiochemical profile comprising an array of measurable physiochemical parameters, $\{[P_x]_1, [P_x]_2,\ldots[P_x]_n,\}$, wherein P_x represents a measurable physiochemical parameter and "n" is an integer ≥ 1, wherein each of $[P_x]_1$ to $[P_x]_n$ is a different measurable physiochemical parameter, wherein the value of any one or more of the measurable physiochemical characteristics is indicative of, associated with, or forms the basis of, a distinctive pharmacological trait, T_y, or a number of distinctive pharmacological traits $\{[T_y]_1, [T_y]_2, \ldots.[T_y]_m\}$ wherein T_y represents a distinctive pharmacological trait and m is an integer ≥ 1 and each of $[T_y]_1$ to $[T_y]_m$ is a different pharmacological trait.

As used herein, the term "measurable physiochemical parameters" (P_x) refers to one or more measurable characteristics of an isolated IL-12 or chimeric molecule thereof.

Exemplary "distinctive measurable physiochemical parameters" include, but are not limited to apparent molecular weight (P_1), isoelectric point (pI) (P_2), number of isoforms (P_3), relative intensities of the different number of isoforms (P_4), percentage by weight carbohydrate (P_5), observed molecular weight following N-linked oligosaccharide deglycosylation (P_6), observed molecular weight following N-linked and O-linked oligosaccharide deglycosylation (P_7), percentage acidic monosaccharide content (P_8), monosaccharide content (P_9), sialic acid content (P_{10}), sulfate and phosphate content (P_{11}), Ser/Thr:GalNAc ratio (P_{12}), neutral percentage of N-linked oligosaccharide content (P_{13}), acidic percentage of N-linked oligosaccharide content (P_{14}), neutral percentage of O-linked oligosaccharide content (P_{15}), acidic percentage of O-linked oligosaccharide content (P_{16}), ratio of N-linked oligosaccharides (P_{17}), ratio of O-linked oligosaccharides (P_{18}), structure of N-linked oligosaccharide fraction (P_{19}), structure of O-linked oligosaccharide fraction (P_{20}), position and make up of N-linked oligosaccharides (P_{21}), position and makeup of O-linked oligosaccharides (P_{22}), co-translational modification (P_{23}), post-translational modification (P_{24}), acylation (P_{25}), acetylation (P_{26}), amidation (P_{27}), deamidation (P_{28}), biotinylation (P_{29}), carbamylation or carbamoylation (P_{30}), carboxylation (P_{31}), decarboxylation (P_{32}), disulfide bond formation (P_{33}), fatty acid acylation (P_{34}), myristoylation (P_{35}), palmitoylation (P_{36}), stearoylation (P_{37}), formylation (P_{38}), glycation (P_{39}), glycosylation (P_{40}), glycophosphatidylinositol anchor (P_{41}), hydroxylation (P_{42}), incorporation of selenocysteine (P_{43}), lipidation (P_{44}), lipoic acid addition (P_{45}), methylation (P_{46}), N or C terminal blocking (P_{47}), N or C terminal removal (P_{48}), nitration (P_{49}), oxidation of methionine (P_{50}), phosphorylation (P_{51}), proteolytic cleavage (P_{52}), prenylation (P_{53}), farnesylation (P_{54}), geranyl geranylation (P_{55}), pyridoxal phosphate addition (P_{56}), sialylation (P_{57}), desialylation (P_{58}), sulfation (P_{59}), ubiquitinylation or ubiquitination (P_{60}), addition of ubiquitin-like molecules (P_{61}), primary structure (P_{62}), secondary structure (P_{63}), tertiary structure (P_{64}), quaternary structure (P_{65}), chemical stability (P_{66}), thermal stability (P_{67}). A summary of these parameters is provided in Table 2.

The term "distinctive pharmacological traits" would be readily understood by one of skill in the art to include any pharmacological or clinically relevant property of the protein or chimeric molecule of the present invention. Exemplary "pharmacological traits" which in

no way limit the invention include: therapeutic efficiency (T_1), effective therapeutic dose ($TCID_{50}$) (T_2), bioavailability (T_3), time between dosages to maintain therapeutic levels (T_4), rate of absorption (T_5), rate of excretion (T_6), specific activity (T_7), thermal stability (T_8), lyophilization stability (T_9), serum/plasma stability (T_{10}), serum half-life (T_{11}), solubility in blood stream (T_{12}), immunoreactivity profile (T_{13}), immunogenicity (T_{14}), inhibition by neutralizing antibodies (T_{14A}), side effects (T_{15}), receptor/ligand binding affinity (T_{16}), receptor/ligand activation (T_{17}), tissue or cell type specificity (T_{18}), ability to cross biological membranes or barriers (i.e. gut, lung, blood brain barriers, skin etc) (T_{19}), angiogenic ability (T_{19A}), tissue uptake (T_{20}), stability to degradation (T_{21}), stability to freeze-thaw (T_{22}), stability to proteases (T_{23}), stability to ubiquitination (T_{24}), ease of administration (T_{25}), mode of administration (T_{26}), compatibility with other pharmaceutical excipients or carriers (T_{27}), persistence in organism or environment (T_{28}), stability in storage (T_{29}), toxicity in an organism or environment and the like (T_{30}).

In addition, the protein or chimeric molecule of the present invention may have altered biological effects on different cells types (T_{31}), including but not limited to human primary cells, such as lymphocytes, erythrocytes, retinal cells, hepatocytes, neurons, keratinocytes, endothelial cells, endodermal cells, ectodermal cells, mesodermal cells, epithelial cells, kidney cells, liver cells, bone cells, bone marrow cells, lymph node cells, dermal cells, fibroblasts, T-cells, B-cells, plasma cells, natural killer cells, macrophages, neutrophils, granulocytes Langerhans cells, dendritic cells, eosinophils, basophils, mammary cells, lobule cells, prostate cells, lung cells, oesophageal cells, pancreatic cells, Beta cells (insulin secreting cells), hemangioblasts, muscle cells, oval cells (hepatocytes), mesenchymal cells, brain microvessel endothelial cells, astrocytes, glial cells, various stem cells including adult and embryonic stem cells, various progenitor cells; and other human immortal, transformed or cancer cell lines. The biological effects on the cells include effects on proliferation (T_{32}), differentiation (T_{33}), apoptosis (T_{34}), growth in cell size (T_{35}), cytokine adhesion (T_{36}), cell adhesion (T_{37}), cell spreading (T_{38}), cell motility (T_{39}), migration and invasion (T_{40}), chemotaxis (T_{41}), cell engulfment (T_{42}), signal transduction (T_{43}), recruitment of proteins to receptors/ligands (T_{44}), activation of the JAK/STAT pathway (T_{45}), activation of the Ras-erk pathway (T_{46}), activation of the AKT pathway (T_{47}), activation of the PKC pathway (T_{48}), activation of the PKA pathway (T_{49}), activation

of src (T_{50}), activation of fas (T_{51}), activation of TNFR (T_{52}), activation of NFkB (T_{53}), activation of p38MAPK (T_{54}), activation of c-fos (T_{55}), secretion (T_{56}), receptor internalization (T_{57}), receptor cross-talk (T_{58}), up or down regulation of surface markers (T_{59}), alteration of FACS front/side scatter profiles (T_{60}), alteration of subgroup ratios (T_{61}), differential gene expression (T_{62}), cell necrosis (T_{63}), cell clumping (T_{64}), cell repulsion (T_{65}), binding to heparin sulfates (T_{66}), binding to glycosylated structures (T_{67}), binding to chondroitin sulfates (T_{68}), binding to extracellular matrix (such as collagen, fibronectin) (T_{69}), binding to artificial materials (such as scaffolds) (T_{70}), binding to carriers (T_{71}), binding to co-factors (T_{72}), the effect alone or in combination with other proteins on stem cell proliferation, differentiation and/or self-renewal (T_{73}) and the like. A summary of these traits is provided in Table 3.

As used herein the term "distinctive" with regard to a pharmacological trait of a protein or a chimeric molecule of the present invention refers to one or more pharmacological traits of the protein, which are distinctive for the particular physiochemical profile. In a particular embodiment, one or more of the pharmacological traits of the isolated IL-12 or chimeric molecule thereof is different from, or distinctive relative to a form of the same protein or chimeric molecule produced in a prokaryotic or lower eukaryotic cell or even a higher non-human eukaryotic cell. In a particular embodiment, the pharmacological traits of the subject isolated IL-12 or chimeric molecule thereof are substantially similar to or functionally equivalent to a naturally occurring protein.

As used herein the term "prokaryote" refers to any prokaryotic cell, which includes any bacterial cell (including actinobacterial cells) or archaeal cell. The meaning of the term "non-mammalian eukaryote", as used herein is self-evident. However, for clarity, this term specifically includes any non-mammalian eukaryote including: yeasts such as *Saccharomyces* spp. or *Pichea* spp.; other fungi; insects, including *Drosophila* spp. and insect cell cultures; fish, including *Danio* spp.; amphibians, including *Xenopus* spp.; plants and plant cell cultures.

Reference to a "stem cell" includes embryonic or adult stem cells and includes those stem cells listed in Table 6. A protein or chimeric molecule of the present invention may be

used alone or in a cocktail of proteins to induce one or more of stem cell proliferation, differentiation or self-renewal.

Primary structure of a protein may be measured as an amino acid sequence. Secondary structure may be measured as the number and/or relative position of one or more protein secondary structures such as α-helices, parallel β-sheets, antiparallel β-sheets or turns. Tertiary structure describes the folding of the polypeptide chain to assemble the different secondary structure elements in a particular arrangement. As helices and sheets are units of secondary structure, so the domain is the unit of tertiary structure. In multi-domain proteins, tertiary structure includes the arrangement of domains relative to each other. Accordingly, tertiary structure may be measured as the presence, absence, number and/or relative position of one or more protein "domains". Exemplary domains which in no way limit the present invention include: lone helices, helix-turn-helix domains, four helix bundles, DNA binding domains, three helix bundles, Greek key helix bundles, helix-helix packing domains, β-sandwiches, aligned β-sandwiches, orthogonal β-sandwiches, β-barrels, up and down antiparallel β-sheets, Greek key topology domains, jellyroll topology domains, β-propellers, β-trefoils, β-Helices, Rossman folds, α/β horseshoes, α/β barrels, α+β topologies, disulphide rich folds, serine proteinase inhibitor domains, sea anemone toxin domains, EGF-like domains, complement C-module domain, wheat plant toxin domains, Naja (Cobra) neurotoxin domains, green mamba anticholinesterase domains, Kringle domains, mucin like region, globular domains, spacer regions. Quaternary structure is described as the arrangement of different polypeptide chains within the protein structure, with each chain possessing individual primary, secondary and tertiary structure elements. Examples include either homo- or hetro- oligomeric multimerization (e.g. dimerization or trimerization). In one embodiment, the IL-12 of the present invention exists as a homo- or hetro- dimer, trimer or oligomer.

With respect to the primary structure, the present invention provides an isolated IL-12 or chimeric molecule thereof, or a fragment thereof, encoded by a nucleotide sequence selected from the list consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, or a nucleotide sequence having at least about 90% identity to any one of the above-listed

sequence or a nucleotide sequence capable of hybridizing to any one of the above sequences or their complementary forms under high stringency conditions.

Still, another aspect of the present invention provides an isolated nucleic acid molecule encoding protein or a functional part thereof comprising a sequence of nucleotides having at least 90% similarity selected from the list consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or after optimal alignment and/or being capable of hybridizing to one or more of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or their complementary forms under high stringency conditions.

In a particular embodiment, the present invention is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding a protein, or a fragment thereof, having an amino acid sequence substantially as set forth in one or more of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 21 or an amino acid sequence having at least about 90% similarity to one or more of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 21 after optimal alignment.

Another aspect of the present invention provides an isolated IL-12 or chimeric molecule thereof, or a fragment thereof, comprising an amino acid sequence selected from the list consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 21, or an amino acid sequence having at least about 90% similarity to one or more of the above sequences.

In a particular embodiment, percentage amino acid similarity or nucleotide identity levels include at least about 90% or at least about 91% or at least about 92% or at least about 93% or at least about 94% or at least about 95% or at least about 96% or at least about 97% or at least about 98% or at least about 99% similarity or identity.

A "derivative" of a polypeptide of the present invention also encompasses a portion or a part of a full-length parent polypeptide, which retains partial transcriptional activity of the parent polypeptide and includes a variant. Such "biologically-active fragments" include deletion mutants and small peptides, for example, for at least 10, in a particular embodiment, at least 20 and in a further embodiment at least 30 contiguous amino acids,

which exhibit the requisite activity. Peptides of this type may be obtained through the application of standard recombinant nucleic acid techniques or synthesized using conventional liquid or solid phase synthesis techniques. For example, reference may be made to solution synthesis or solid phase synthesis as described, for example, in Chapter 9 entitled "*Peptide Synthesis*" by Atherton and Shephard which is included in a publication entitled "*Synthetic Vaccines*" edited by Nicholson and published by Blackwell Scientific Publications. Alternatively, peptides can be produced by digestion of an amino acid sequence of the invention with proteinases such as endoLys-C, endoArg-C, endoGlu-C and staphylococcus V8-protease. The digested fragments can be purified by, for example, high performance liquid chromatographic (HPLC) techniques. Any such fragment, irrespective of its means of generation, is to be understood as being encompassed by the term "derivative" as used herein.

The term "variant" refers, therefore, to nucleotide sequences displaying substantial sequence identity with reference nucleotide sequences or polynucleotides that hybridize with a reference sequence under stringency conditions that are defined hereinafter. The terms "nucleotide sequence", "polynucleotide" and "nucleic acid molecule" may be used herein interchangeably and encompass polynucleotides in which one or more nucleotides have been added or deleted, or replaced with different nucleotides. In this regard, it is well understood in the art that certain alterations inclusive of mutations, additions, deletions and substitutions can be made to a reference nucleotide sequence whereby the altered polynucleotide retains the biological function or activity of the reference polynucleotide or the encoded polypeptide. The term "variant" also includes naturally occurring allelic variants.

The nucleic acid molecules of the present invention may be in the form of a vector or other nucleic acid construct.

In one embodiment, the vector is DNA and may optionally comprise a selectable marker.

Examples of selectable markers include genes conferring resistance to compounds such as antibiotics, genes conferring the ability to grow on selected substrates, genes encoding

proteins that produce detectable signals such as luminescence. A wide variety of such markers are known and available, including, for example, antibiotic resistance genes such as the neomycin resistance gene (*neo*) and the hygromycin resistance gene (*hyg*). Selectable markers also include genes conferring the ability to grown on certain media substrates such as the *tk* gene (thymidine kinase) or the *hprt* gene (hypoxanthine phosphoribosyltransferase) which confer the ability to grow on HAT medium (hypoxanthine, aminopterin and thymidine); and the bacterial *gpt* gene (guanine/xanthine phosphoribosyltransferase) which allows growth on MAX medium (mycophenolic acid, adenine and xanthine). Other selectable markers for use in mammalian cells and plasmids carrying a variety of selectable markers are described in Sambrook *et al. Molecular Cloning - A Laboratory Manual*, Cold Spring Harbour, New York, USA, 1990.

The selectable marker may depend on its own promoter for expression and the marker gene may be derived from a very different organism than the organism being targeted (e.g. prokaryotic marker genes used in targeting mammalian cells). However, it is favorable to replace the original promoter with transcriptional machinery known to function in the recipient cells. A large number of transcriptional initiation regions are available for such purposes including, for example, metallothionein promoters, thymidine kinase promoters, β-actin promoters, immunoglobulin promoters, SV40 promoters and human cytomegalovirus promoters. A widely used example is the pSV2-*neo* plasmid which has the bacterial neomycin phosphotransferase gene under control of the SV40 early promoter and confers in mammalian cells resistance to G418 (an antibiotic related to neomycin). A number of other variations may be employed to enhance expression of the selectable markers in animal cells, such as the addition of a poly(A) sequence and the addition of synthetic translation initiation sequences. Both constitutive and inducible promoters may be used.

The genetic construct of the present invention may also comprise a 3' non-translated sequence. A 3' non-translated sequence refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the

mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon.

Accordingly, a genetic construct comprising a nucleic acid molecule of the present invention, operably linked to a promoter, may be cloned into a suitable vector for delivery to a cell or tissue in which regulation is faulty, malfunctioning or non-existent, in order to rectify and/or provide the appropriate regulation. Vectors comprising appropriate genetic constructs may be delivered into target eukaryotic cells by a number of different means well known to those skilled in the art of molecular biology.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particular embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity.

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence similarity", "sequence identity", "percentage of sequence similarity", "percentage of sequence identity", "substantially similar" and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 or above, such as 30 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (i.e. only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of typically 12 contiguous residues that is compared to a reference sequence. The comparison window

may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.* (*Nucl Acids Res 25:*389, 1997). A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.* (*In: Current Protocols in Molecular Biology*, John Wiley & Sons Inc. 1994-1998).

The terms "sequence similarity" and "sequence identity" as used herein refers to the extent that sequences are identical or functionally or structurally similar on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity", for example, is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, I) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "sequence identity" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software Engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software. Similar comments apply in relation to sequence similarity.

Reference herein to a low stringency includes and encompasses from at least about 0 to at least about 15% v/v formamide and from at least about 1 M to at least about 2 M salt for

hybridization, and at least about 1 M to at least about 2 M salt for washing conditions. Generally, low stringency is at from about 25-30°C to about 42°C, such as 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41 and 42°C. The temperature may be altered and higher temperatures used to replace formamide and/or to give alternative stringency conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide, such as 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30% and from at least about 0.5 M to at least about 0.9 M salt, such as 0.5, 0.6, 0.7, 0.8 or 0.9 M for hybridization, and at least about 0.5 M to at least about 0.9 M salt, such as 0.5, 0.6, 0.7, 0.8 or 0.9 M for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide, such as 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 and 50% and from at least about 0.01 M to at least about 0.15 M salt, such as 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14 and 0.15 M for hybridization, and at least about 0.01 M to at least about 0.15 M salt, such as 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14 and 0.15 M for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41$ (G+C)% (Marmur and Doty, *J Mol Biol 5:*109, 1962). However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner and Laskey, *Eur J Biochem 46:*83, 1974). Formamide is optional in these hybridization conditions. Accordingly, in a particular embodiment levels of stringency are defined as follows: low stringency is 6 x SSC buffer, 0.1% w/v SDS at 25-42°C; a moderate stringency is 2 x SSC buffer, 0.1% w/v SDS at a temperature in the range 20°C to 65°C; high stringency is 0.1 x SSC buffer, 0.1% w/v SDS at a temperature of at least 65°C.

As used herein, the terms "co- or post-translational modifications" refer to covalent modifications occurred during or after translation of the peptide chain. Exemplary co- or post-translational modifications include but are not limited to acylation (including acetylation), amidation or deamidation, biotinylation, carbamylation (or carbamoylation), carboxylation or decarboxylation, disulfide bond formation, fatty acid acylation (including myristoylation, palmitoylation and stearoylation), formylation, glycation, glycosylation, hydroxylation, incorporation of selenocysteine, lipidation, lipoic acid addition,

methylation, N- or C-terminal blocking, N- or C-terminal removal, nitration, oxidation of methionine, phosphorylation, proteolytic cleavage, prenylation (including farnesylation, geranyl geranylation), pyridoxal phosphate addition, sialylation or desialylation, sulfation, ubiquitinylation (or ubiquitination) or addition of ubiquitin-like proteins.

Acylation involves the hydrolysis of the N-terminus initiator methionine and the addition of an acetyl group to the new N-termino amino acid. Acetyl Co-A is the acetyl donor for acylation.

Amidation is the covalent linkage of an amide group to the carboxy terminus of a peptide and is frequently required for biological activity and stability of a protein. Deamidation is the hydrolytic removal of an amide group. Deamidation of amide containing amino acid residues is a rare modification that is performed by the organism to re-arrange the 3D structure and alter the charge ratio/pI.

Biotinylation is a technique whereby biotinyl groups are incorporated into molecules, either that catalyzed by holocarboxylase synthetase during enzyme biosynthesis or that undertaken *in vitro* to visualise specific substrates by incubating them with biotin-labeled probes and avidin or streptavidin that has been linked to any of a variety of substances amenable to biochemical assay.

Carbamylation (or carbamoylation) is the transfer of the carbamoyl from a carbamoyl-containing molecule (e.g., carbamoyl phosphate) to an acceptor moiety such as an amino group.

Carboxylation of glutamic acid residues is a vitamin K dependent reaction that results in the formation of a gamma carboxyglutamic acid (Gla residue). Gla residues within several proteins of the blood-clotting cascade are necessary for biological function of the proteins. Carboxylation can also occur to aspartic acid residues.

Disulfide bonds are covalent linkages that form when the thiol groups of two cysteine residues are oxidized to a disulfide. Many mammalian proteins contain disulfide bonds,

and these are crucial for the creation and maintenance of tertiary structure of the protein, and thus biological activity.

Protein synthesis in bacteria involves formylation and deformylation of N-terminal methionines. This formylation/deformylation cycle does not occur in cytoplasm of eukaryotic cells and is a unique feature of bacterial cells. In addition to the hydroxylation that occurs on glycine residues as part of the amidation process, hydroxylation can also occur in proline and lysine residues catalysed by prolyl and lysyl hydroxylase (Kivirikko *et al. FASEB Journal 3:*1609-1617, 1989).

Glycation is the uncontrolled, non-enzymatic addition of glucose or other sugars to the amino acid backbone of protein.

Glycosylation is the addition of sugar units to the polypeptide backbone and is further described hereinafter.

Hydroxylation is a reaction which is dependent on vitamin C as a co-factor. Adding to the importance of hydroxylation as a post- translation modification is that hydroxy-lysine serves as an attachment site for glycosylation.

Selenoproteins are proteins which contain selenium as a trace element by the incorporation of a unique amino acid, selenocysteine, during translation. The tRNA for selenocysteine is charged with serine and then enzymatically selenylated to produce the selenocysteinyl-tRNA. The anticodon of selenocysteinyl-tRNA interacts with a stop codon in mRNA (UGA) instead of a serine codon. An element in the 3' non-translated region (UTR) of selenoprotein mRNAs determines whether UGA is read as a stop codon or as a selenocysteine codon.

Lipidation is a generic term that encompasses the covalent attachment of lipids to proteins, this includes fatty acid acylation and prenylation.

Fatty acid acylation involves the covalent attachment of fatty acids such as the 14 carbon Myristic acid (Myristoylation), the 16 carbon Palmitic acid (Palmitoylation) and the 18 carbon Stearic acid (Stearoylation). Fatty acids are linked to proteins in the pre-Golgi compartment and may regulate the targeting of proteins to membranes (Blenis and Resh *Curr Opin Cell Biol 5(6):*984-9, 1993). Fatty acid acylation is, therefore, important in the functional activity of a protein (Bernstein *Methods Mol Biol 237*:195-204, 2004).

Prenylation involves the addition of prenyl groups, namely the 15 carbon farnesyl or the 20 carbon geranyl-geranyl group to acceptor proteins. The isoprenoid compounds, including farnesyl diphosphate or geranylgeranyl diphosphate, are derived from the cholesterol biosynthetic pathway. The isoprenoid groups are attached by a thioether link to cysteine residues within the consensus sequence CAAX, (where A is any aliphatic amino acid, except alanine) located at the carboxy terminus of proteins. Prenylation enhances proteins ability to associate with lipid membranes and all known GTP-binding and hydrolyzing proteins (G proteins) are modified in this way, making prenylation crucial for signal transduction (Rando *Biochim Biophys Acta 1300(1):*5-16, 1996; Gelb *et al. Curr Opin Chem Biol 2(1)*:40-8, 1998).

Lipoic acid is a vitamin-like antioxidant that acts as a free radical scavenger. Lipoyl-lysine is formed by attaching lipoic acid through an amide bond to lysine by lipoate protein ligase.

Protein methylation is a common modification that can regulate the activity of proteins or create new types of amino acids. Protein methyltransferases transfer a methyl group from S-adenosyl-L-methionine to nucleophilic oxygen, nitrogen, or sulfur atoms on the protein. The effects of methylation fall into two general categories. In the first, the relative levels of methyltransferases and methylesterases can control the extent of methylation at a particular carboxyl group, which in turn regulates the activity of the protein. This type of methylation is reversible. The second group of protein methylation reactions involves the irreversible modification of sulfur or nitrogen atoms in the protein. These reactions generate new amino acids with altered biochemical properties that alter the activity of the protein (Clarke *Curr Opin Cell Biol 5*:977 983, 1993).

Protein nitration is a significant post-translational modification, which operates as a transducer of nitric oxide signalling. Nitration of proteins modulates catalytic activity, cell signalling and cytoskeletal organization.

Phosphorylation refers to the addition of a phosphate group by protein kinases. Serine, threonine and tyrosine residues are the amino acids subject to phosphorylation. Phosphorylation is a critical mechanism, which regulates biological activity of a protein.

A majority of proteins are also modified by proteolytic cleavage. This may simply involve the removal of the initiation methionine. Other proteins are synthesized as inactive precursors (proproteins) that are activated by limited or specific proteolysis. Proteins destined for secretion or association with membranes (preproteins) are synthesized with a signal sequence of 12-36 predominantly hydrophobic amino acids, which is cleaved following passage through the ER membrane.

Pyridoxal phosphate is a co-enzyme derivative of vitamin B6 and participates in transaminations, decarboxylations, racemizations, and numerous modifications of amino acid side chains. All pyridoxal phosphate-requiring enzymes act via the formation of a Schiff base between the amino acid and coenzyme. Most enzymes responsible for attaching the pyridoxal-phosphate group to the lysine residue are self activating.

Sialylation refers to the attachment of sialic acid to the terminating positions of a glycoprotein via various sialyltransferase enzymes; and desialylation refers the removal of sialic acids. Sialic acids include but are not limited to, N-acetyl neuraminic acid (NeuAc) and N-glycolyl neuraminic acid (NeuGc). Sialyl structures that result from the sialylation of glycoproteins include sialyl Lewis structures, for example, sialyl Lewis a and sialyl Lewis x, and sialyl T structures, for example, Sialyl-TF and Sialyl Tn.

Sulfation occurs at tyrosine residues and is catalyzed by the enzyme tyrosylprotein sulfotransferase which occurs in the *trans*-Golgi network. It has been determined that 1 in 20 of the proteins secreted by HepG2 cells and 1 in 3 of those secreted by fibroblasts

contain at least one tyrosine sulfate residue. Sulfation has been shown to influence biological activity of proteins. Of particular interest is that the CCR5, a major HIV co-receptor, was shown to be tyrosine-sulfated and that sulfation of one or more tyrosine residues in the N-terminal extracellular domain of CCR5 are required for optimal binding of MIP-1 alpha/CCL3, MIP-1 beta/CCL4, and RANTES/CCL5 and for optimal HIV co-receptor function (Moore *J Biol Chem 278(27):*24243-24246, 2003). Sulfation can also occur on sugars. In addition, sulfation of a carbohydrate moiety of a glycoprotein can occur by the action of glycosulfotransferases such as GalNAc(β1-4)GlcNAc(β1-2)Manα4 sulfotransferase.

Post-translational modifications can encompass protein-protein linkages. Ubiquitin is a 76 amino acid protein which both self associates and covalently attaches to other proteins in mammalian cells. The attachment is via a peptide bond between the C-terminus of ubiquitin and the amino group of lysine residues in other proteins. Attachment of a chain of ubiquitin molecules to a protein targets it for proteolysis by the proteasome and is an important mechanism for regulating the steady state levels of regulatory proteins e.g. with respect to the cell cycle (Wilkinson *Annu Rev Nutr 15*:161-89, 1995). In contrast, mono-ubiquitination can play a role in direct regulation of protein function. Ubiquitin-like proteins that can also be attached covalently to proteins to influence their function and turnover include NEDD-8, SUMO-1 and Apg12.

Glycosylation is the addition of sugar residues in the polypeptide backbone. Sugar residues, such as monosaccharides, disaccharides and oligosaccharides include but are not limited to: fucose (Fuc), galactose (Gal), glucose (Glc), galactosamine (GalNAc), glucosamine (GlcNAc), mannose (Man), N-acetyl-lactosamine (lacNAc) N,N'-diacetyllactosediamine (lacdiNAc). These sugar units can attach to the polypeptide back bones in at least seven ways, namely:

(1) *via* an N-glycosidic bond to the R-group of an asparagine residue in the consensus sequence Asn-X-Ser; Asn-X-Thr; or Asn-X-Cys (N-glycosylation; N-linked glycans);

(2) *via* an O-glycosidic bond to the R-group of serine, threonine, hydroxyproline, tyrosine or hydroxylysine (O-glycosylation; O-linked glycans);

(3) *via* the R-group of tyrosine in C-linked mannose;

(4) as a glycophosphatidylinositol anchor used to secure some proteins to cell membranes;

(5) as a single monosaccharide attachment of GlcNAc to the R-group of serine or threonine. This linkage is often reversibly associated with attachment of inorganic phosphate (Yin-o-Yang);

(6) attachment of a linear polysaccharide to serine, threonine or asparagine (proteoglycans); and

(7) *via* a S-glycosidic bond to the R-group of cysteine.

N-linked glycans are beta linked to the amide nitrogen of an asparagine residue in the consensus sequence – Asn-Xaa-Ser/Thr/Cys where Xaa ≠ Pro and nearly always have an N-acetylglucosamine residue at the reducing terminus. They are biologically synthesised by the transfer of Glc3Man9GlcNAc2 from dolichol-pyrophosphate-Glc3Man9GlcNAc2 to asparagine. This is followed by enzymatic processing to form the final N-linked glycan structures. This method of synthesis means that the core pentasaccharide Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc is always present in N-linked glycans.

N-linked glycans are divided into three distinct classes: high-mannose type, complex type and hybrid type; all are formed by elongation of the core. High-mannose type N-glycans result when the core structure is predominantly substituted by mannose. Complex type N-glycans contain the core structure substituted by one or more of the sugars N-acetylglycosamine, galactose, fucose or sialic acid. Hybrid type N-glycans have structural features of both the high-mannose and complex type chains. Within each of the three classes of N-glycans there exists a wide diversity of structures which comes from the variation in the number and position of individual sugar residues, the degree of branching, and the level of phosphorylation and sulfation.

O-linked glycans are usually composed of N-acetylgalactosamine linked to serine or threonine. This type of O-linked glycan is also called mucin-type as they are often found on mucins. Unlike N-linked glycans, mucin-type O-linked glycans are synthesised one residue at a time and at least 8 core classes have been described.

Three domains can be distinguished within the larger mucin-type O-glycan chains: a core, backbone and peripheral region. The core region consists of the N-acetylgalactosamine residue attached to serine or threonine plus the sugar residue/s linked directly. The backbone region is defined as the series of galactose and N-acetylglucosamine residues linked to the core. There are two common backbone repeats: Gal(β1-3)GlcNAc (type 1) and Gal(β1-4)GlcNAc (type 2). Both backbone structures may be combined to form a long linear chain or a branched structure e.g., Gal(β1-4)GlcNAc(β1-6)[Gal(β1-4)GlcNAc(β1-3)Gal-{rest of chain}.

The peripheral region can include terminal residues such as fucose, galactose, N-acetlygalactosamine, N-acetylglucosamine, N-acetylneuraminic acid and sulfate. These terminal residues determine most of the characteristics of the O-linked glycans. For example, sialic acid residues and sulfate groups add a negative charge to the protein, increase its solubility and modify its conformation. Additionally, peripheral residues of glycosylation structures can comprise one or more of the following carbohydrate antigenic determinants in Table 7.

TABLE 7

List of carbohydrate antigenic determinants

Antigenic Name	**Antigenic Glycan Structure**
Blood group H(O), type 1	Fuc(α1-2)Gal(β1-3)GlcNAc-R
Blood group H(O), type 2	Fuc(α1-2)Gal(β1-4)GlcNAc-R
Blood group A, type 1	GalNAc(α1-3)[Fuc(α1-2)]Gal(β1-3)GlcNAc-R
Blood group A, type 2	GalNAc(α1-3)[Fuc(α1-2)]Gal(β1-4)GlcNAc-R
Blood group B, type 1	Gal(α1-3)[Fuc(α1-2)]Gal(β1-3)GlcNAc-R
Blood group B, type 2	Gal(α1-3)[Fuc(α1-2)]Gal(β1-4)GlcNAc-R
Blood group i	$[Gal(\beta 1\text{-}4)GlcNAc(\beta 1\text{-}3)]_n Gal(\beta 1\text{-}R$

Antigenic Name	Antigenic Glycan Structure
Blood group I	Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)GlcNAc(β1-3)Gal(β1-R
Lewis a (Le^a)	Gal(β1-3)[Fuc(α1-4)]GlcNAc-R
Sialyl Lewis a (sLe^a)	NeuAc(α2-3)Gal(β1-3)[Fuc(α1-4)]GlcNAc-R
Lewis b (Le^b)	Fuc(α1-2)Gal(β1-3)[Fuc(α1-4)]GlcNAc-R
Lewis x (Le^x)	Gal(β1-4)[Fuc(α1-3)]GlcNAc-R
Sialyl Lewis x (sLe^x)	NeuAc(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc-R
Lewis y (Le^y)	Fuc(α1-2)Gal(β1-4)[Fuc(α1-3)]GlcNAc-R
Forssman	GalNAc(α1-3)GalNAc(β1-3)Gal-R
Thomsen-Friedenreich (TF or T)	Gal(β1-3)GalNAc(α1-O)-Ser/Thr
Sialyl-TF (sTF) or Sialyl-T (sT)	Gal(β1-3)[NeuAc(α2-6)]GalNAc(α1-O)-Ser/Thr
Tn	GalNAc(α1-O)-Ser/Thr
Sialyl Tn (sTn)	NeuAc(α2-6)GalNAc(α1-O)-Ser/Thr

The carbohydrates will also contain several antennary structures, including mono, bi, tri and tetra outer structures.

Glycosylation may be measured by the presence, absence or pattern of N-linked glycosylation, O-linked glycosylation, C-linked mannose structure, and glycophosphatidylinositol anchor; the percentage of carbohydrate by mass; Ser/Thr – GalNAc ratio; the proportion of mono, bi, tri and tetra sugar structures or by lectin or antibody binding.

Sialylation of a protein may be measured by the immunoreactivity of the protein with an antibody directed against a particluar sialyl structure. For example, Lewis x specific antibodies react with CEACAM1 expressed from granulocytes but not with recombinant human CEACAM1 expressed in 293 cells (Lucka *et al. Glycobiology 15(1)*:87-100, 2005).

Alternatively, the presence of sialylated structures on a protein may be detected by a combination of glycosidase treatment followed by a suitable measurement procedure such as mass spectroscopy (MS), high performance liquid chromatography (HPLC) or glyco mass fingerprinting (GMF).

The apparent molecular weight of a protein includes all elements of a protein complex (cofactors and non-covalently bonded domains) and all co- or post-translational modifications (addition or removal of covalently bonded groups to and from peptide). Apparent molecular weight is often affected by co- or post-translational modifications. A protein's apparent molecular weight may be determined by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), which is also the second dimension on its two-dimensional counterpart, 2D-PAGE (two-dimensional polyacrylamide gel electrophoresis). It may be determined more accurately, however, by mass spectrometry (MS)- either by Matrix-Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) MS, which produces charged molecular ions or the more sensitive Electrospray Ionization (ESI) MS, which produces multiple-charged peaks. The apparent molecular weights of the protein may be within the range of 1 to 250 kDa. Accordingly, the isolated IL-12 or chimeric molecule of the present invention has a apparent molecular weight of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250 kDa. The molecular weight or molecular mass of a protein may be

determined by any convenient means such as electrophoresis, mass spectrometry, gradient ultracentrifugation.

The isoelectric point (or pI) of a protein is the pH at which the protein carries no net charge. This attribute may be determined by isoelectric focusing (IEF), which is also the first dimension of 2D-PAGE. Experimentally determined pI values are affected by a range of co- or post-translational modifications and therefore the difference between an experimental pI and theoretical pI may be as high as 5 units. Accordingly, an isolated IL-12 or chimeric molecule of the present invention may have a pI of 0, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10.0, 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, 10.7, 10.8, 10.9, 11.0, 11.1, 11.2, 11.3, 11.4, 11.5, 11.6, 11.7, 11.8, 11.9, 12.0, 12.1, 12.2, 12.3, 12.4, 12.5, 12.6, 12.7, 12.8, 12.9, 13.0, 13.1, 13.2, 13.3, 13.4, 13.5, 13.6, 13.7, 13.8, 13.9, or 14.0.

As used herein, the term "isoform" means multiple molecular forms of a given protein, and includes proteins differing at the level of (1) primary structure (such as due to alternate RNA splicing, or polymorphisms); (2) secondary structure (such as due to different co- or post translational modifications); and/or (3) tertiary or quaternary structure (such as due to different sub-unit interactions, homo- or hetero- oligomeric multimerization). In particular, the term "isoform" includes glycoform, which encompasses a protein having a constant primary structure but differing at the level of secondary or tertiary structure or co- or post-translational modification such as different glycosylation forms.

Chemical stability of a protein may be measured as the "half-life" of the protein in a particular solvent or environment. Typically, proteins with a molecular weight of less than 50 kDa have a half-life of approximately 5 to 20 minutes. The proteins or chimeric molecules of the present invention are contemplated to have a half-life of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56,

57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 hours. Another particularly convenient measure of chemical stability is the resistance of a protein to protease digestion, such as trypsin or chymotrypsin digestion.

The binding affinity of a protein to its ligand or receptor may be measured as the equilibrium dissociation constant (Kd) or functionally equivalent measure.

The solubility of a protein may be measured as the amount of protein that is soluble in a given solvent and/or the rate at which the protein dissolves. Furthermore, the rate and or level of solubility of a protein in solvents of differing properties such as polarity, pH, temperature and the like may also provide measurable physiochemical characteristics of the protein.

Any "measurable physiochemical parameters" may be determined, measured, quantified or qualified using any methods known to one of skill in the art. Described below is a range of methodologies which may be useful in determining, measuring, quantifying or qualifying one or more measurable physiochemical parameters of an isolated IL-12 or chimeric molecule thereof. However, it should be understood that the present invention is in no way limited to the particular methods described, or to the measurable physiochemical parameters that are measurable using these methods.

Glycoproteins can be said to have two basic components that interact with each other to create the molecule as a whole- the amino acid sequence and the carbohydrate or sugar side chains. The carbohydrate component of the molecule exists in the form of monosaccharide or oligosaccharide side chains attached to the amine side chain of Asn or the hydroxyl side chain of Ser/Thr residues of the amino acid backbone by N- or O-linkages, respectively. A monosaccharide is the term given to the smallest unit of a carbohydrate that is regarded as a sugar, having the basic formula of $(CH_2O)_n$ and most often forming a ring structure of 5 or 6 atoms (pentoses and hexoses respectively). Oligosaccharides are combinations of monosaccharides forming structures of varying complexities that may be either linear or branched but which generally do not have long

chains of tandem repeating units (such as is the case for polysaccharides). The level of branching that the oligosaccharide contains as well as the terminal and branching substitutions dramatically affect the properties of the glycoprotein as a whole, and play an important role in the biological function of the molecule. Oligosaccharides are manufactured and attached to the amino acid backbone in the endoplasmic reticulum (ER) and Golgi apparatus of the cell. Different organisms and cell types have different ratios of glycotransferases and endoglycosidases and exoglycosidases and therefore produce different oligosaccharide structures. One of the fundamental defence mechanisms of the body is the detection and destruction of aberrant isoforms and as such it is important to have correct glycosylation of a biological therapeutic not only to increase effectiveness but also to decrease detection by neutralizing antibodies.

Glycan chains are often expressed in a branched fashion, and even when they are linear, such chains are often subject to various modifications. Thus, the complete sequencing of oligosaccharides is difficult to accomplish by a single method and therefore requires iterative combinations of physical and chemical approaches that eventually yield the details of the structure under study.

Determination of the glycosylation pattern of a protein can be performed using a number of different systems, for example using SDS-PAGE. This technique relies on the fact that glycosylated proteins often migrate as diffuse bands by SDS-PAGE. Differentiation between different isoforms are performed by treating a protein with a series of agents. For example, a marked decrease in band width and change in migration position after digestion with peptide-N4-(N-acetyl-β-D-glucosaminyl) asparagine amidase (PNGase) is considered diagnostic of N-linked glycosylation.

To determine the composition of N-linked glycosylation, N-linked oligosaccharides are removed from the protein with PNGase cloned from *Flavobacterium meningosepticum* and expressed in *E. coli*. The removed N-linked oligosaccharides may be recovered from Alltech Carbograph SPE Carbon columns (Deerfield, Illinois, USA) as described by Packer *et al. Glycoconj J 5(8)*:737-47, 1998. The sample can then be taken for monosaccharide analysis, sialic acid analysis or sulfate analysis on a Dionex system with a

GP50 pump ED50 pulsed Amperometric or conductivity detector and a variety of pH anion exchange columns.

The extent of O-linked glycosylation may be determined by first removing O-linked oligosaccharides from the target protein by β-elimination. The removed O-linked oligosaccharides may be recovered from Alltech Carbograph SPE Carbon columns (Deerfield, Illinois, USA) as described by Packer *et al.* (1998, *supra*). The sample can then be taken for monosaccharide analysis, sialic acid analysis or sulfate analysis on a Dionex system with a GP50 pump ED50 pulsed Amperometric or conductivity detector and a variety of pH anion exchange columns.

Monosaccharide subunits of an oligosaccharide have variable sensitivities to acid and thus can be released from the target protein by mild trifluoro-acetic acid (TFA) conditions, moderate TFA conditions, and strong hydrochloric acid (HCl) conditions. The monosaccharide mixtures are then separated by high pH anion exchange chromatography (HPAEC) using a variety of column media, and detected using pulsed amperometric electrochemical detection (PAD).

High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been extensively used to determine monosaccharide composition. Fluorophore-based labeling methods have been introduced and many are available in kit form. A distinct advantage of fluorescent methods is an increase in sensitivity (about 50-fold). One potential disadvantage is that different monosaccharides may demonstrate different selectivity for the fluorophore during the coupling reaction, either in the hydrolyzate or in the external standard mixture. However, the increase in sensitivity and the ability to identify which monosaccharides are present from a small portion of the total amount of available glycoprotein, as well as the potential for greater sensitivity using laser-induced fluorescence, makes this approach attractive. In addition a conductivity detector may be used to determine the sulfate and phosphate composition. By using standards, the peak areas can be calculated to total amounts of each monosaccharide present. These data can indicate the level of N- and O-linked glycosylation, the extent of sialylation, and in

combination with amino acid composition, percent by weight glycosylation, percent by weight acidic glycoproteins.

Monosaccharide composition analysis of small amounts of protein is best performed with PVDF (PSQ) membranes, after electroblotting, or, if smaller aliquots are to be analyzed, on dot blots. PVDF is an ideal matrix for carbohydrate analysis because neither monosaccharides nor oligosaccharides bind to the membrane, once released by acid or enzymatic hydrolysis.

Determination of the oligosaccharide content of the target molecule is performed by a number of techniques. The sugars are first removed from the amino acid backbone by enzymatic (such as digestion with PNGase)) or chemical (such as beta elimination with hydroxide) means. The sugars may be stabilised by reduction or labeled with a fluorophore for ease of detection. The resultant free oligosaccharides are then separated either by high pH anion exchange chromatography with pulsed amperometric electrochemical detection (HPAEC-PAD), which can be used with known standards to determine the ratios of the various structures and levels of sialylation, or by fluorophore assisted carbohydrate electrophoresis (FACE) a process similar to SDS-PAGE separation of proteins. In this process the oligosaccharides are labeled with a fluorophore that imparts electrophoretic mobility. They are separated on high percentage polyacrylamide gels and the resultant band pattern provides a profile of the oligosaccharide content of the target molecule. By using standards it is possible to gain some information on the actual structures present or the bands can be cut and analysed using mass spectrometry to determine each of their structures.

Fluorophore assisted carbohydrate electrophoresis (FACE) is a polyacrylamide gel electrophoresis system designed to separate individual oligosaccharides that have been released from a glycoconjugate. Oligosaccharides are removed from the sample protein by either chemical or enzymatic means in such a way as to retain the reducing terminus. Oligosaccharides are then either digested into monosaccharides or left intact and labeled with a fluorophore (either charged or non charged). High percentage polyacrylamide gels and various buffer systems are used to migrate the oligosaccharides/monosaccharides

which migrate relative to their size/composition in much the same way as proteins. Sugars are visualised by densitometry and relative amounts of sugars can be determined by fluorophore detection. This process is compatible with MALDI-TOF MS, hence the method can be used to elucidate actual structures.

Quartz crystal microbalance and surface plasmon resonance (QCM and SPR, respectively) are two methods of obtaining biological information through the physiochemical properties of a molecule. Both measure protein-protein interactions indirectly through the change that the interaction causes in the physical characteristics of a prefabricated chip. In QCM a single crystal quartz wafer is treated with a receptor/antibody etc which interacts with the ligand of interest. This chip is oscillated by the microbalance and the frequency of the chip recorded. The protein of interest is allowed to pass over the chip and the interaction with the bound molecule causes the frequency of the wafer to change. By changing the conditions by which the ligand interacts with the chip, it is possible to determine the binding characteristics of the target molecule.

Apparent molecular weight is also a physiochemical property which can be used to determine the similarities between the protein or chimeric molecule of the present invention and those produced using alternative means.

As used herein, the term "molecular weight" is defined as the sum of atomic weights of the constituent atoms in a molecule, sometimes also referred to as "molecular mass" (Mr). Molecular weight can be determined theoretically by summing the atomic masses of the constituent atoms in a molecule. The term "apparent molecular weight" is defined as the molecular weight determined by one or more analytical techniques such as SDS page or ultra centrifugation and depends on the relationship between the molecule and the detection system. The apparent molecular weight of a protein can be determined using any one of a range of experimental methods. Analytical methods for determining the molecular weight of a protein include, without being limited to, size-exclusion chromatography (SEC), gel electrophoresis, Rayleigh light scattering, analytical ultracentrifugation, and, to some extent, time-of-flight mass spectrometry.

Gel electrophoresis is a process of determining some of the physiochemical properties (specifically apparent molecular weight and pI) of a protein and in the case of 2 dimensional electrophoresis to separate the molecule into isoforms, thereby providing information on the post-translational modifications of the protein product. Specifically, electrophoresis is the process of forcing a charged molecule (such as protein or DNA) to migrate through a gel matrix (most commonly polyacrylamide or agarose) by applying an electric potential through its body. The most common forms of electrophoresis used on proteins are isoelectric focussing, native, and SDS polyacrylamide gel electrophoresis. In isoelectric focussing a protein is placed into a polyacrylamide gel that has a pH gradient across its length. The protein will migrate to the point in the gel where it has a net charge of zero thereby giving its isoelectric point.

Glyco mass fingerprinting (GMF) is the process by which the oligosaccharide profile of a protein or one of its isoforms is identified by electrophoresis followed by specific mass spectrometric techniques. Sample protein is purified either by 1D SDS-PAGE for total profile determination or 2D gel electrophoresis for specific isoform characterization. The protein band/spot is excised from the gel and de-stained to remove contaminants. The sugars are released by chemical or enzymatic means and desalted/separated using a nanoflow LC system and a graphitised carbon column. The LC flow can be directly injected into an electrospray mass spectrometer that is used to determine the mass and subsequently the identity of the oligosaccharides present on the sample. This provides a profile or fingerprint of each isoform which can be combined with quantitative techniques such as Dionex analysis to determine the total composition of the molecule being tested.

Primary structure can be evaluated in determining the physiochemical properties of the protein or chimeric molecule of the present invention.

The primary structure of a protein can be assayed using one or more of the following systems.

Information on the primary structure of a protein can be determined using a combination of mass spectrometry (MS), DNA sequencing, amino acid composition, protein sequencing and peptide mass fingerprinting.

To determine the sequence of the amino acid backbone either N-terminal chemical sequencing, tandem mass spectrometry sequencing, or a combination of both is used. N-terminal chemical sequencing utilises Edman chemistry (Edman P "Sequence determination" *Mol Biol Biochem Biophys 8:*211-55, 1970), which states that the peptide bond between the N-terminal amino acid and the amino acid in position 2 of the protein is weaker than all other peptide bonds in the sequence. By using moderate acidic conditions the N-terminal amino acid is removed derivatised with a fluorophore (FTIC) and the retention time on a reversed-phase HPLC column determined, and compared to a standard to identify what the amino acid is. This method will determine the actual primary structure of the molecule but is not quantitative. Alternatively tandem mass spectrometry in conjunction with nanoflow liquid chromatography may be used (LC-MS/MS). In this process the protein is digested into peptides using specific endoproteases and the molecular weight of the peptides determined. High energy collision gases such as nitrogen or argon are then used to break the peptide bonds and the masses of the resultant peptides measured. By calculating the change in mass of the peptides it is possible to determine the sequence of each of the peptides (each amino acid has a unique mass). By using different proteases the peptides may then be overlapped to determine their order and thus the entire sequence of the protein.

Clearly, the combination of enzymatic digestion, chemical derivatization, liquid chromatography (LC)/MS and tandem MS provides an extremely powerful tool for AA sequence analysis. For example, the detailed structure of recombinant soluble CD4 receptor was characterized by a combination of methods, which confirmed over 95% of the primary sequence of this 369 AA glycoprotein and showed the whole nature of both N-and C-termini, the positions of attachment of the glycans, the structures of the glycans and the correct assignment of the disulfide bridges (Carr *et al. J Biol Chem 264(35)*:21286-21295, 1989).

Mass spectrometry (MS) is the process of measuring the mass of a molecule through extrapolation of its behavior in a charged environment under a vacuum. MS is very useful in stability studies and quality control. The method first requires digestion of samples by proteolytic enzymes (trypsin, V8 protease, chymotrypsin, subtilisin, and clostripain) (Franks *et al. Characterization of proteins*, Humana Press, Clifton, NJ, 1988; Hearn *et al. Methods in Enzymol 104*:190-212, 1984) and then separation of digested samples by reverse phase chromatography (RPC). With tryptic digestion in conjunction with LC-MS, the peptide map can be used to monitor the genetic stability, the homogeneity of production lots, and protein stability during fermentation, purification, dosage form manufacture and storage.

Before a mass analysis, several ways are used to interface a HPLC to a mass spectrometer: 1) direct liquid injection; 2) supercritical fluid; 3) moving belt system; 4) thermospray. The HPLC/MS interface used in Caprioli's work used a fused silica capillary column to transport the eluate from the column to the tip of the sample probe in the ionization chamber of the mass spectrometer. The probe tip is continuously bombarded with energetic Xe atoms, causing sputtering of the sample solution as it emerges from the tip of the capillary. The mass is then analyzed by the instrument (Caprioli *et al. Biochem Biophys Res Commun 146*:291-299, 1987).

MS/MS and LC/MS interfaces expand the potential applications of MS. MS/MS allows direct identification of partial to full sequence for peptides up to 25 AAs, sites of deamidation and isomerization (Carr *et al. Anal Chem 63*:2802-2824, 1991). Coupled with RPC or capillary electrophoresis (CE), MS can perform highly sensitive analysis of proteins (Figeys and Aebersold, *Electrophoresis 19*:885-892, 1998; Nguyen *et al. J Chromatogr A 705*:21-45, 1995). LC/MS allows LC methodology to separate peptides before entering the MS, such as the continuous flow FAB interfaced with microbore HPLC (Caprioli *et al.* 1987, *supra*). The latter "interface" allows the sequencing of individual peptides from complex mixtures: Fragmentation of the peptides selected by the first MS is followed by passing through a cloud of ions in a collision cell: CID (collision induced dissociation). The collision generates characteristic set of fragments, from which the sequence may be deduced, without knowing other information, such as the cDNA

sequence. In a single MS experiment, an unfractionated mixture of peptides (e.g. from an enzyme digest) is injected and the masses of the major ions are compared with those predicted from the cDNA sequence. The sequence of the recombinant human interleukin-2 was verified by fast atom bombardment (FAB)-MS analysis of CNBr and proteolytic digests (Fukuhara *et al. J Biol Chem 260*:10487-10494, 1985).

Electrospray ionization MS (ESI-MS) uses an aerosol of solution protein to introduce into a needle under a high voltage, generating a series of charged peaks of the same molecules with various charges. Because each peak generated from the differently charged species produces an estimation of the molecular weights, these estimations can be combined to increase the overall precision of the molecular weight estimation. Matrix Assisted Laser Desorption Ionization MS (MALDI-MS) uses a high concentration of a chromophore. A higher intensity laser pulse will be absorbed by the matrix and the energy absorbed evaporates part of the matrix and carries the protein sample with it into the vapor phase almost entirely. The resulting ions are then analyzed in a time of flight MS. The mild ionization may enhance the capacity of the method to provide quaternary structure information. MALDI-MS can be run rapidly in less than 15 minutes. It does not need to fragment the molecules and the result is easy to interpret as a densitometric scan of an SDS-PAGE gel, with a mass range up to over 100kDa.

Amino acid sequence can be predicted by sequencing DNA that encodes a protein. However, occasionally the actual protein sequence may be different. Traditionally, DNA sequencing reactions are just like the PCR reactions for replicating DNA (DNA denaturation, replication). By DNA cloning technology, the gene is cloned, and the nucleotide sequence determined.

The amino acid sequence of a protein can be assayed using one or more of the following systems.

Full sequence description of the protein is usually required to describe the product. Amino acid sequencing includes: in gel tryptic digestion, fractionation of the digested peptides by RPC-HPLC, screening the peptide peaks that have the most symmetrical absorbance

profile by MALDI-TOF MS, and the first peptide (N-terminal) by Edman degradation. Edman chemically derived primary sequence data is the classical method to identify proteins at the molecular level. MALDI-TOF MS can be used for N-terminal sequence analysis. However, all enzymatic digests for HPLC and peptide sequencing are recommended to first be subjected to MS or MS/MS protein identification to decrease the time and cost. The internal amino acid sequences from SDS-PAGE-separated proteins are obtained by elution of the peptides with HPLC separation after an in situ tryptic or lysyl endopeptidase digestion in the gel matrix.

Internal sequencing of the standard peptide is recommended to be run with the analyzed samples to maintain the instruments at the peak performance. More than 80% of higher eukaryotic proteins are reported to have blocked amino-termini that prevent direct amino acid sequencing. When a blocked eukaryotic protein is encountered, the presence of the sequence of the internal standard assures that the instrument is operating properly.

Edman degradation can be used for direct N-terminal sequencing with a chemical procedure, which derivatizes the N-terminal amino acids to release the amino acids and expose the amino terminal of the next AAs. The Edman sequencing includes: 1). By microbore HPLC, N-terminal sequence analysis is repeated by Edman chemistry cycles. Every cycle of the Edman chemistry can identify one amino acid. 2). After in-gel or PVDF bound protein digestions followed by HPLC separation of the resulting peptides, internal protein sequence analysis is conducted by Edman degradation chemistry.

Microbore HPLC and capillary HPLC are used for analysis and purification of peptide mixtures using RPC-HPLC. In-gel samples and PVDF samples are purified using different columns. MALDI-TOF MS analysis can be used for N-terminal analysis after HPLC fractionation. The selection criteria are: 1) The apparent purity of the HPLC fraction. 2) The mass and thus the estimated length of the peptide. The peptide mass information is useful for confirming the Edman sequencing amino acid assignments, and also in the possible detection of co- or post-translational modifications.

In-gel digests are suitable for purification on the higher sensitivity HPLC system. The internal protein sequence analysis is first enzymatically digested by SDS-PAGE. Proteins in an SDS-PAGE mini-gel can be reliably digested in-gel only with trypsin. The peptide fragments are purified by RPC-HPLC and then analyzed by MALDI-TOF MS, screening for peptides suitable for Edman sequence analysis. Proteins in a gel can only be analyzed by internal sequencing analysis, but very accurate peptides masses can be obtained, which provides additional information useful in both amino acid assignment and database searching.

PVDF-bound proteins are suitable for both N-terminal and internal Edman sequencing analysis. PVDF-bound proteins are digested with the proper enzyme (trypsin, endoproteinase Lys-C, endoproteinase Glu-C, clostripain, endoproteinase Asp-N, thermolysin) and a non-ionic detergent such as hydrogenated Triton X-100. In PVDF bound proteins, the detergents used for releasing digested peptides from the membrane can interfere with MALDI-TOF MS analysis. Before the enzyme is added, Cys is reduced with DTT and alkylated with iodoacetamide to generate carboxyamidomethyl Cys, which can be identified during N-terminal sequence analysis.

To determine the amino acid composition of a protein, the sample is hydrolyzed using phenol catalyzed strong hydrochloric acid (HCl) acidic conditions in the gaseous phase. Once the hydrolysis is performed the liberated amino acids are derivatised with a fluorophore compound that imparts a specific reversed phase characteristic on the combined molecule. The derivatized amino acids are separated using reversed phase high performance liquid chromatography (RP-HPLC) and detected with a fluorescence detector. By using external and internal standards it is possible to calculate the amount of each amino acid present in the sample from the observed peak area. This information may be used to determine sample identity and to quantify the amount of protein present in the sample. For instance, discrepancies between theoretical and actual results can be used to initially identify the possibility of a de-amidation site. In combination with monosaccharide analysis it may determine the composition % by weight glycosylation and percent by weight acidic glycoproteins. This method is limited in the information that it can provide on the actual sequence of the backbone however as there is inherent variability

due to environmental contaminants and occasional destruction of amino acids. For example, it is not possible for this method to detect point mutations in the sequence.

Peptide mass fingerprinting (PMF) is another method by which the identity of a protein may be determined. The procedure involves an initial separation of the sample by electophoretic means (either 1 or 2 dimensional), excision of the spot/band from the gel and digestion with a specific endoprotease (typically porcine trypsin). Peptides are eluted from the gel fragment and analysed by mass spectrometry to determine the peptide masses present. The resultant peptide masses are then compared to a database of theoretical mass fragments for all reported proteins (or in the case of constructs for the theoretical peptide masses of the designed sequence). The technique relies on the fact that the "fingerprint" of a protein (i.e. its combination of peptide masses) is unique. Identity can be confidently determined (greater than 90% accuracy) with as little as 4 peptides and 30% sequence coverage. Modifications such as lipid moieties and de-amidation can be identified during the PMF stage of analysis. Peaks that do not correspond to those of the identified protein are further analysed by tandem mass spectrometry (MS-MS), a technique that uses the energy created by the impact of a collision gas to break the weaker bond of the PTM. The newly freed molecule and the original peptide are then re-analysed for mass to identify the post-translational modification and the peptide fragment to which it was attached.

HPLC is classified into different modes depending on the size, charge, hydrophobicity, function or specific content of the target biomolecules. Generally, two or more chromatographic methods are used to purify a protein. It is of paramount importance to consider both the characteristics of the protein and the sample solvent when the chromatographic modes are selected.

Secondary structures of a protein can also be evaluated in characterising their properties.

The secondary structure of a protein can be assayed using one or more of the following systems.

To study the secondary structures of proteins, most commonly several spectroscopic methods should be applied and compared. Electromagnetic energy can be defined as a continuous waveform of radiation, depending on the size and shape of the wave. Different spectroscopic methods use different electromagnetic energy.

The wavelength is the extent of a single wave of radiation (the distance between two successive maxima of the waves). When the radiant energy increases, the wavelength becomes shorter. The relationship between frequency and wavenumber is:

$$\text{Wavenumber } (cm^{-1}) = \text{Frequency } (s^{-1}) / \text{ The speed of light (cm/s).}$$

The absorption of electromagnetic radiation by molecules includes vibrational and rotational transitions, and electronic transitions. Infrared (IR) and Raman spectroscopy are most commonly used to measure the vibrational energies of molecules in order to determine secondary structure. However, they are different in their approach to determine molecular absorbance.

The energy of the scattered radiation is less than the incident radiation for the Stokes line. The energy of the scattered radiation is more than the incident radiation for the anti-Stokes line. The energy increase or decrease from the excitation is related to the vibrational energy spacing in the ground electronic state of the molecule. Therefore, the wavenumber of the Stokes and anti-Stokes lines are a direct measure of the vibrational energies of the molecule.

Only the Stokes shift is observed in a Raman spectrum. The Stokes lines are at smaller wavenumbers (or higher wavelengths) than the exciting light. A high power excitation source, such as a laser, should be used to enhance the efficiency of Raman scattering. The excitation source should be monochromatic because we are interested in the energy (wavenumber) difference between the excitation and the Stokes lines.

For a vibrational motion to be IR active, the dipole moment of the molecule must change. Therefore, the symmetric stretch in carbon dioxide is not IR active because there is no

change in the dipole moment. The asymmetric stretch is IR active due to a change in dipole moment. For a vibration to be Raman active, the polarizability of the molecule must change with the vibrational motion. The symmetric stretch in carbon dioxide is Raman active because the polarizability of the molecule change. Thus, Raman spectroscopy complements IR spectroscopy (Herzberg *et al. Infrared and Raman Spectra of Polyatomic Molecules*, Van Nostrand Reinhold, New York, NY, 1945). For example, IR is not able to detect a homonuclear diatomic molecule due to the lack of dipole moments, but Raman spectroscopy can detect it because the molecular polarizability is changed by stretching and contraction of the bond, further, the interactions between electrons and nuclei are changed.

For highly symmetric polyatomic molecules with a center of inversion (such as benzene), it is more likely that bands active in the IR spectrum are not active in the Raman spectrum or vice-versa. In molecules with little or no symmetry, modes are likely to be active in both infrared and Raman spectroscopy.

IR spectroscopy measures the wavelength and intensity of the absorption of infrared light by a sample. Infrared light is so energetic that it can excite the molecular vibrations to higher energy levels. Both infrared and RAMAN spectroscopy measure the vibrations of bond lengths and angles.

IR characterizes vibrations in molecules by measuring the absorption of light of certain energies corresponding to the vibrational excitation of the molecule from $v = 0$ to $v = 1$ (or higher) states. There are selection rules that govern the ability of a molecule to be detected by infrared spectroscopy. Not all of the normal modes of vibration can be excited by infrared radiation (Herzberg *et al.* 1945, *supra*).

IR spectra can provide qualitative and quantitative information of the secondary structures of proteins, such as α-helix, β-sheet, β-turn and disordered structure. The most informative IR bands for protein analysis are amide I (1620-1700 cm^{-1}), amide II (1520-1580 cm^{-1}) and amide III (1220-1350 cm^{-1}). Amide I is the most intense absorption band in proteins. It consists of stretching vibration of the C=O (70-85% and C-N groups (10-20%). The exact

band position is dictated by the backbone conformation and the hydrogen bonding pattern. Amide II is more complex than Amide I. Amide II is governed by in-plane N-H bending (40-60%), C-N (18-40%) and C-C (10%) stretching vibrations. Amide III bands are not very useful (Krimm and Bandekar, *Adv Protein Chem 38:*181-364, 1986). Most of the β-sheet structures of FTIR amide I band usually are located at about 1629 cm^{-1} with a minimum of 1615 cm^{-1} and a maximum of 1637 cm^{-1}; the minor component may show peaks around 1696 cm^{-1} (lowest value 1685 cm^{-1}). α-helix is mainly found at 1652 cm^{-1}. An absorption near 1680 cm^{-1} is now assigned to β-turns.

The principle of Raman scattering is different from that of infrared absorption. Raman spectroscopy measures the wavelength and intensity of inelastically scattered light from molecules. The Raman scattered light occurs at wavelengths that are shifted from the incident light by the energies of molecular vibrations.

To be Raman active, for the vibration to be inelastically scattered, a change in polarizability during the vibration is essential. In the symmetric stretch, the strength of electron binding is different between the minimum and maximum internuclear distances. Therefore the polarizability changes during the vibration, and this vibrational mode scatters Raman light, the vibration is Raman active. In the asymmetric stretch the electrons are more easily polarized in the bond that expands but are less easily polarized in the bond that compresses. There is no overall change in polarizability and the asymmetric stretch is Raman inactive (Herzberg *et al.* 1945, *supra*).

Circular dichroism can be used to detect any asymmetrical structures, such as proteins. Optically active chromophores absorb different amount of right and left polarized light, this absorbance difference results in either a positive or negative absorption spectrum (Usually, the right polarized spectrum is subtracted from the left polarized spectrum). Commonly, the far UV or amide region (190-250nm) is mainly contributed from peptide bonds, providing information on the environment of the carbonyl group of the amide bond and consequently the secondary structure of the protein. α-helix usually displays two negative peaks at 208, 222 nm (Holzwarth *et al. J Am Chem Soc 178*:350, 1965), β-sheet displays one negative peak at 218 nm, random coils has a negative peak at 196 nm. Near

UV region peaks are (250-350 nm) contributed from the environment of the aromatic chromophores (Phe, Tyr, Trp). Disulfide bonds give rise to minor CD bands around 250 nm.

Intense dichroism is commonly associated with the side-chain structures being held tightly in a highly folded, three-dimensional structure. Denaturation of the protein mostly releases the steric hindrance; a weaker CD spectrum is obtained along with an increasing degree of denaturation. For example, the side chain CD spectrum of hGH is quite sensitive to the partial denaturation by adding denaturants. Some reversible chemical alterations of the molecules, such as reduction of the disulfide bonds, or alkaline titrations will change the side-chain CD spectrum. For hGH, these spectral difference can be caused by entirely the removal of a chromophores, or by affecting changes in the particular chromophore's CD response, but not by the gross denaturation or conformational changes (Aloj *et al. J Biol Chem 247*:1146-1151, 1971).

UV absorption spectroscopy is one of the most significant methods to determine protein properties. It can provide information about protein concentrations and the immediate environments of chromophoric groups. Proteins functional groups, such as amino, alcoholic (or phenolic) hydroxyl, carbonyl, carboxyl, or thiol can be transformed into strong chromophores. Visible and near UV spectroscopy are used to monitor two types of chromophores: metalloproteins (more than 400 nm) and proteins that contains Phe, Trp, Tyr residues (260-280nm). The change in UV or fluorescence signal can be negative or positive, depending on the protein sequence and solution properties.

Fluorescence measures the emission energy after the molecule has been irradiated into an excited state. Many proteins emitted fluorescence in the range of 300 to 400 nm when excited at 250 to 300 nm from their aromatic amino acids. Only proteins with Phe, Trp, Tyr residues can be measured with the order of intensity Trp>> Tyr>> Phe. Fluorescence spectra can reflect the microenvironments information that are affected by the folding of the proteins. For example, a buried Trp is usually in a hydrophobic environment and will fluoresce at maximum 325 to 330 nm range, but an exposed residue or free amino acids fluoresces at around 350 to 355 nm. An often used agent to probe protein unfolding is Bis-

ANS. The fluorescence of Bis-ANS is pH-independent. Even though its signal is weak in water, it can be increased significantly by binding to unfolding-exposed hydrophobic sites in proteins (James and Bottomley *Arch Biochem Biophy 356*:296-300, 1998).

Effective quenching of Tyr and Trp in the folded proteins causes significant signal increase upon unfolding. A simple solute may cause the change also. To maximize detection sensitivity, a signal ratio can be used. For example, In the study of rFXIII unfolding, a ratio of fluorescence intensity at 350nm to that at 330nm was used (Kurochkin *et al. J Mol Biol 248*:414-430, 1995). Conformational changes may be studied by means of excitation-energy transfer between a fluorescent donor and an absorbing acceptor, because the efficiency of transfer depends on the distance between the two chromophores (Honroe *et al. Biochem J 258*:199-204, 1989). Fluorescence was used to probe a-Antitrypsin conformation (Kwon and Yu, *Biophim Biophys Acta 1335*:265-272, 1997), to determine Tm of HSA (Farruggia *et al. Int J Biol Macromol 20:*43-51, 1997), and to detect MerP unfolding interactions (Aronsson *et al. FEBS Lett 411*:359-364, 1997).

At neutral pH, the intensity of the fluorescence emission spectrum is in the order of Trp> Tyr. At acidic pH, due to the conformational changes which disrupts the energy transfer, the fluorescence from Tyr dominates over Trp. Fluorescence studies also confirm the presence of intermediates in the guanidine-induced unfolding transition of the proteins.

Tertiary and quaternary structures of the physiochemical forms of a protein are also important in ascertaining their function.

The tertiary and quaternary structures of a protein can be assayed using one or more of the following systems.

NMR and X-ray crystallography are the most often used techniques to study the 3D structure of proteins. Other less detailed methods to probe protein tertiary structure include CD in near UV region, second-derivative of UV spectroscopy (Ackland *et al. J Chromatogr 540*:187-198, 1991) and fluorescence.

NMR is one of the main experimental methods for molecular structure and intermolecular interactions in structural biology. In addition to studying protein structures, NMR can also be utilised to study the carbohydrate structures of a protein. NMR spectroscopy is routinely used by chemists to study chemical structure using simple one-dimensional techniques. The structure of more complicated molecules can also be determined by two-dimensional techniques. Time domain NMR are used to probe molecular dynamics in solutions. Solid state NMR is used to determine the molecular structure of solids. NMR can be used to study structural and dynamic properties of proteins, nucleic acids, a variety of low molecular weight compounds of biological, pharmacological and medical interests. However, not all nuclei possess the correct property in order to be read by NMR, i.e., not all nuclei posses spin, which is required for NMR. The spin causes the nucleus to produce an NMR signal, functioning as a small magnetic field.

The crystal structure of a protein can be assayed using one or more of the following systems.

X-ray crystallography is an experimental technique that applies the fact that X-rays are diffracted by crystals. X-rays have the appropriate wavelength (in the Ångström range, ~10-8 cm) to be scattered by the electron cloud of an atom of comparable size. The electron density can be reconstructed based on the diffraction pattern obtained from X-ray scattering off the periodic assembly of molecules or atoms in the crystal. Additional phase information either from the diffraction data or from supplementing diffraction experiments should be obtained to complete the reconstruction. A model is then progressively built into the experimental electron density, refined against the data and the result is a very accurate molecular structure.

X ray diffraction has been developed to study the structure of all states of matter with any beam, e.g., ions, electrons, neutrons, and protons, with a wavelength similar to the distance between the atomic or molecular structures of interest.

Light scattering spectroscopy is based on the simple principle that larger particles scatter light more than the smaller particles. A slope base line in the 310-400nm region originates

from light scattering when large particles, such as aggregates, present in the solution (Schmid *et al. Protein structure, a practical approach*, Creighton Ed., IRI Press, Oxford, England, 1989)

Light scattering spectroscopy can be used to estimate the molecular weight of a protein and is a simple tool to monitor protein quaternary structure or protein aggregation. The degree of protein aggregation can be indicated by simple turbidity measurement. Final product pharmaceutical solutions are subjected to inspection of clarity because most aggregated proteins are present as haze and opalescence. Quasielastic light scattering spectroscopy (QELSS), sometimes called photon correlation spectroscopy (PCS), or dynamic light scattering (DLS), is a noninvasive probe of diffusion in complex fluids for macromolecules (proteins, polysaccharides, synthetic polymers, micelles, colloidal particles and aggregations). In most cases, light scattering spectroscopy yields directly the mutual diffusion coefficient of the scattering species. When applied to dilute monodisperse solutions, the diffusion coefficient obtained by QELSS can estimate the size. With polydisperse system, it estimates the width of molecular weight distribution. For accurate measurement, 200-500 mW laser power is mandatory, conventional Ar+/Kr+ gas lasers are widely used (Phillies *Anal Chem 62:*1049A-1057A, 1990). Protein aggregation was detected by human relaxin (Li *et al. Biochemistry 34*:5762-5772, 1995).

Stability of a protein is also an important determinant of function. Methods for analysing such characteristics include DSC, TGA and freeze-dry cryostage microscopy, analysis of freeze-thaw resistance, and protease resistance.

A protein may be more stable for lyophilization (freeze drying). Lyophilization is used to enhance the stability and/or shelf life of the product as it is stored in powder rather than liquid form. The process involves an initial freezing of the sample, then removal of the liquid by evaporation under vacuum. The end result is a dessicated "cake" of protein and excipients (other substances used in the formulation). The consistency of the resulting cake is critical for successful reconstitution. The lyophilization process can result in changes to the protein, especially aggregate formation though crosslinking, but also deamidation and other modifications. These can reduce efficacy by either losses, reduced activity or by

inducing immune reactions against aggregates. In order to test lyophilization stability, the protein can be formulated for lyophilization using standard stabilizers (e.g. mannitol, trehalose, Tween 80, human serum albumin and the like). After lyophilization, the amount of protein recovered can be assayed by ELISA, while its activity can be assayed by a suitable bioassay. Aggregates of the protein can be detected by HPLC or Western blot analysis.

Prior to lyophilization, the Tg or Te (define Tg or Te) of the formulation should be determined to set the maximum allowable temperature of the product during primary drying. Also, information about the crystallinity or amorphousity of the formulation helps to design the lyophilization cycle in a more rationale manner. Product information on these thermal parameters can be obtained by using differential scanning calorimetry (DSC), thermogravimetric analysis (TGA) or freeze-dry cryostage microscope.

Differential Scanning Calorimetry (DSC) is a physical thermo-analytical method to measure, characterize and analyze thermal properties of materials and determine the heat capacities, melting enthalpies and transition points accordingly. DSC scans through a temperature range at a linear rate. Individual heaters within the instrument provide heat to sample and reference pans separately, based on the "power compensated null balance" principle. During a physical transition, the absorption or evolution of the energy causes an imbalance in the amount of energy supplied to that of the sample holder. Depending on the varying thermal behavior of the sample, the energy will be taken or diffused from the sample, and the temperature difference will be sensed as an electrical signal to the computer. As a result, an automatic adjustment of the heaters makes the temperature of the sample holder identical to the reference holder. The electrical power needed for the compensation is equivalent to the calorimetric effect.

The purity of an organic substance can be estimated by DSC based on the shape and temperature of the DSC melting endotherm. The power-compensated DSC provides very high resolution compared to a heat flux DSC under the identical conditions. More well-defined and more accurate partial areas of melting can be generated from power-compensated DSC because the partial areas of melting are not "smeared" over a narrow

temperature interval, as for the lesser-resolved heat flux DSC. The power-compensated DSC produces inherently better partial melting areas and therefore better purity analysis. By the help of StepScan DSC, the power-compensated DSC can provide a direct heat capacity measurement using the traditional and time-proven means without the need for deconvolution or the extraction of sine wave amplitudes.

Thermogravimetric Analysis (TGA) measures sample mass loss and the rate of weight loss as a function of temperature or time.

As DSC, freeze-dry cryostage can reach a wide temperature range rapidly. Currently, as an preformulation and formulation study tool, simulating the lyophilization cycle in a freeze dry cryostage provides the best platform to study thermal parameters of the protein formulations on a miniature scale. Freeze dry microscope can predict the influence of formulations and process factors on freezing and drying. Only a 2-3mL sample is required for a cryostage study, which makes this technique a valuable tool to study scarce, difficult-to-obtain drugs. It is a good tool to study the effect of freezing, rate, drying rate, thawing rate on the lyophilization cycle. Annealing research may be advanced by the studies from freeze-dry cryostage microscope. Because of extensive applications of lyophilization technology, and larger demand to stabilize the extremely expensive drugs (such as proteins and gene therapy drugs), it is expected that an in-process microscopic monitor should be realized in the pharmaceutical industries soon.

The freeze-thaw resistance of a protein can be assayed using one or more of the following systems.

Co- or post translational modification such as glycosylation may protect proteins from repeated freeze/thaw cycles. To determine this, a protein or chimeric molecule of the present invention can be compared to carrier-free *E. coli*-produced counterparts. A protein are diluted into suitable medium (e.g. cell growth medium, PBS or the like) then frozen by various methods, for instance, snap frozen in liquid nitrogen, slowly frozen by being placed at –70 degrees or rapidly frozen on dry ice. The samples are then thawed either rapidly at room temperature or slowly at 4 degrees. Some samples are then refrozen and

the process are repeated for a number of cycles. The amount of protein present can be measured by ELISA, and the activity measured in a suitable bioassay chosen by a skilled artisan. The amount of activity/protein remaining is compared to the starting material to determine the resistance over many the freeze/thaw cycles.

A protein may have altered thermal stability in solution. The thermal stability of the present invention may be determined *in vitro* as follows.

A protein or chimeric molecule of the present invention can be mixed into buffer e.g. phosphate buffered saline containing carrier protein e.g. human serum albumin and incubated at a particular temperature for a particular time (e.g. 37 degrees for 7 days). The amount of protein remaining after this treatment can be determined by ELISA and compared to material stored at –70 degrees. The biological activity of the remaining protein is determined by performing a suitable bioassay chosen by a person skilled in the relevant art.

The protease resistance of a protein can be assayed using one or more of the following systems.

To compare protease resistance, solution containing a protein or chimeric molecule of the present invention and solution containing *E. coli* expressed counterparts can be incubated with a protease of choice (e.g. unpurified serum proteases, purified proteases, recombinant proteases) for different time periods. The amount of protein remaining is measured by an appropriate ELISA (e.g. one in which the epitopes recognized by the capture and detection antibodies are separated by the protease cleavage site), and the activity of the remaining protein is determined by a suitable bioassay chosen by a skilled artisan.

The bioavailability of a protein can be assayed using one or more of the following systems.

Bioavailability is the degree to which a drug or other substance becomes available to the target tissue after administration. Bioavailability may depend on half life of the drug or its ability to reach the target tissue.

Compositions comprising a protein or chimeric molecule of the present invention is injected subcutaneously or intramuscularly. The levels of the protein or its chimeric molecule can then be measured in the blood by ELISA or radioactive counts. Alternatively, the blood samples can be assayed for activity of the proteinby a suitable bioassay chosen by a skilled artisan, for instance, stimulation of proliferation of a particular target cell population. As the sample will be from plasma or serum, there may be a number of other molecules that could be responsible for the output activity. This can be controlled by using a neutralizing antibody to the protein being tested. Hence, any remaining bioactivity is due to the other serum components.

The stability or half-life of a protein can be assayed using one or more of the following systems.

A protein may have altered half-life in serum or plasma. The half-life of the protein may be determined *in vitro* as follows. Composition containing the protein can be mixed into human serum/plasma and incubated at a particular temperature for a particular time (e.g. 37 degrees for 4 hours, 12 hours etc). The amount of protein remaining after this treatment can be determined by ELISA. The biological activity of the remaining protein is determined by performing a suitable bioassay chosen by a person skilled in the relevant art. The serum chosen may be from a variety of human blood groups (e.g. A, B, AB, O etc.)

The half-life of a protein can also be determined *in vivo*. Composition containing a protein, which may be labeled by a radioactive tracer or other means, can be injected intravenously, subcutaneously, retro-orbitally, tail vein, intramuscularly or intraperitoneally) into the species of choice for the study, for instance, mouse, rat, pig, primate, human. Blood samples are taken at time points after injection and assayed for the presence of the protein (either by ELISA or by TCA-precipitable radioactive counts). A comparison composition consisting of *E. coli* or CHO-produced protein can be run as a control.

To determine the half-life of a protein, *in vivo*, male Wag/Rij rats, or other suitable animals can be injected intravenously with a protein.

Just before the administration of the substrate, a suitable volume of EDTA blood is sampled as negative control. At various time points after the injection, a suitable volume of EDTA blood can be taken from the animals using the same technique. After the last blood sampling, the animals are sacrificed. The specimen is centrifuged for 15 min at RT within 30 min of collection. The plasma samples are tested in a specific ELISA to determine the concentration of protein in each sample.

A protein may cross the blood brain barrier.

An *in vitro* assay to determine if protein of the present invention binds human brain endothelial cells can be tested using the following assays.

Radiolabeled protein can be tested for its ability to bind to human brain capillary endothelial cells. A protein can be custom conjugated with radiolabel to a specific activity using a method known in the art, for instance, with ^{125}I by the chloramine T method, or with ^{3}H.

Primary cultures of human brain endothelial cells can be grown in flat-bottom 96-well plates until five days post-confluency then lightly fixed using acetone. Cells are lysed, transferred to glass fibre membranes. Radiolabeled protein can be detected using a liquid scintillation counter.

In vivo assays for the determination of protein binding to human brain endothelial cells can be tested using the following assays.

A protein is tested for binding to human brain capillaries using sections of human brain tissue that are fresh frozen (without fixation), sectioned on a cryostat, placed on glass slides and fixed in acetone. Binding of ^{3}H-protein is examined on brain sections using quantitative autoradiography.

In vivo assay can be used to measure tissue distribution and blood clearance of human-specific protein in a primate system.

Tissue distribution and blood clearance of ^{14}C -labeled protein can be determined in, for example, male cynomolgus monkeys or other suitable primates. ^{14}C -labeled protein is administered concurrently with a ^{3}H -labeled control protein to the animals with an intravenous catheter. During the course of the study, blood samples are collected to determine the clearance of the proteins from the circulation. At 24 hours post-injection, the animals are euthanized and selected organs and representative tissues collected for the determination of isotope distribution and clearance by combustion. In addition, capillary depletion experiments are performed to samples from different regions of the brain in accordance with Triguero *et al. J of Neurochemistry 54*:1882-1888, 1990. This method removes greater than 90% of the vasculature from the brain homogenate (Triguero *et al.* cited supra).

The time-dependent redistribution of the radiolabeled protein from the capillary fraction to the parenchyma fraction is consistent with the time dependent migration of a across the blood-brain barrier.

A protein may promote or inhibit angiogenesis.

The angiogenic potential of the protein may be assessed by methods known in the art. For example, the extent of angiogenesis may be measured by microvessel sprouting in a model of angiogenesis. In this assay, rat fat microvessel fragments (RFMFs) are isolated as described in Shepherd *et al. Arterioscler Thromb Vasc Biol 24:*898-904, 2004. Epididymal fat pads are harvested from euthanized animals, minced and digested in collagenase. RFMFs and single cells are separated from lipids and adipocytes by centrifugation and suspended in 0.1% BSA in PBS. The RFMF suspension is sequentially filtered to remove tissue debris, single cells, and red blood cells from the fragments. RFMFs are suspended in cold, pH-neutralized rat-tail type 1 collagen at 15,000 RFMF/ml and plated into wells (for example, 0.25 ml/well) of 48-well plate for culture. After polymerization of the collagen, an equal volume of DMEM containing 10% FBS is added to each gel. After formation of the gels, vascular extensions characteristic of angiogenic sprouts appear by day 4 of culture. These sprouts are readily distinguished from the parent vessel fragment by the

absence of the rough, smooth-muscle associated appearance. The RFMF 3-D cultures can be treated with the protein and vessel sprout lengths can be measured at day 5 and 6 of culture.

The angiogenic potential of the protein may also be assessed by an *in vivo* angiogenesis assay described in Guedez *et al. Am J Pathol 162:*1431–1439, 2003. This assay consists of subcutaneous implantation of semiclosed silicone cylinders (angioreactors) into nude mice. Angioreactors are filled with extracellular matrix premixed with or without the protein. Vascularization within angioreactors is quantified by the intravenous injection of fluorescein isothiocyanate (FITC)-dextran before their recovery, followed by spectrofluorimetry. Angioreactors examined by immunofluorescence is able to show cells and invading angiogenic vessels at different developmental stages.

A protein may have a distinct immunoreactivity profile determined by immunoassay techniques, which involve the interaction of the molecule with one or more antibodies directed against the molecule. Examples of immunoassay techniques include enzyme-linked immunoabsorbant assays (ELISA), dot blots and immunochromatographic assays such as lateral flow tests or strip tests.

The level of the protein may be measured using an immunoassay procedure, for example, a commerically purchased ELISA kit. The protein may have a different immunoreactivity profile to non-human cell expressed protein due to the specificity of the antibodies provided in an immunoassay kit. For instance, the capture and/or detection antibodies of the immunoassay may be antibodies specifically directed against non-human cell expressed human proteins.

In addition, incorrect folding of the non-human cell expressed human protein may result in the exposure of antigenic epitopes which are not exposed on the correctly folded human cell expressed human protein. Incorrect folding may arise through, for instance, overproduction of heterologous proteins in the cytoplasm of non-human cells, for example, *E. coli* (Baneyx *Current Opinion in Biotechnology, 10*:411–421, 1999). Further, non-human cell expressed human protein may have a different pattern of post-translational

modifications to that of the protein or chimeric molecule of the present invention. For example, the non-human cell expressed human protein may exhibit abnormal quantities and/or types of carbohydrate structures, phosphate, sulfate, lipid or other residues. This may result in the exposure of antigenic epitopes which are not exposed on the protein or chimeric molecule of the present invention. Conversely, an altered pattern of post-translational modifications may result in an absence of antigenic epitopes on the protein or chimeric molecule of the present invention which are exposed on the non-human cell expressed human protein.

Any one of, or combination of, the above-mentioned factors may lead to inaccurate measurements of:

(a) naturally occurring human protein in laboratory samples or human tissues; or
(b) human cell expressed recombinant human protein in laboratory samples, human tissues or in human embryonic stem cell (hES) culture media.

The immunoreactivity profile of a human cell expressed human protein, as determined by the use of a suitable immunoassay, may provide an indication of the protein's immunogenicity in the human, as described hereinafter.

Most biologic products elicit a certain level of antibody response against them. The antibody response can, in some cases, lead to potentially serious side effects and/or loss of efficacy. For instance, some patients treated with recombinant protein expressed from non-human cells may generate neutralizing antibodies particularly during long-term therapeutic use and thereby reducing the protein's efficacy and or contribute to side effects. The protein or chimeric protein molecule expressed from human cells is unlikely to generate neutralizing antibodies therefore increasing its therapeutic efficacy compared with non-human cell expressed protein.

The immunogenicity of protein can be assayed using one or more of the following systems.

Most biologic products elicit a certain level of antibody response against them. The antibody response can, in some cases, lead to potentially serious side effects and/or loss of efficacy. For instance, some patients treated with recombinant EPO will generate neutralizing antibodies that also cross-react with the patient's own EPO. In this case, they can develop pure red cell aplasia and be resistant to EPO treatment, resulting in a need for constant dialysis.

Immunogenicity is the property of being able to evoke an immune response within an organism. Immunogenicity depends partly upon the size of the substance in question and partly upon how unlike host molecules it is. A protein may have altered immunogenicity due to its novel physiochemical characteristics. For instance, the glycosylation structure of a protein may shield or obscure the epitope(s) recognized by the antibody and therefore preventing or reducing antibody binding to the protein. Alternatively, some antibodies may recognize a glycopeptide epitope not present in the non-glycosylated version of the protein.

The ability of patient samples to recognize a protein with a distinctive physiochemical form can be determined by various immunoassays, as described herein. A properly designed immunoassay involves considerations directing to appropriate detection, quantitation and characterization of antibody responses. A number of recommendations for the design and optimization of immunoassays are outlined in Mire-Sluis *et al. J Immunol Methods 289(1-2)*:1-16, 2004, which is incorporated by reference.

The use of protein on therapeutic implants can be assayed using one or more of the following systems.

The present invention extends to the use of a protein to manipulate stem cells. A major therapeutic use of stem cells is in regeneration of tissue, cartilage or bone. In one embodiment, the cells are likely to be introduced to the body in a biocompatible three-dimensional matrix. The implant will consist of a mixture of cells, the scaffold, growth factors and accessory components such as biodegradable polymers, proteoglycans and the like. Incorporation of a protein into these matrices during their construction is proposed to regulate the behavior of the cells. Such implants may be used for the formation of bone,

the growth of neurons from progenitor cells, chondrocyte implantation for cartilage replacement and other applications. Human cell-derived proteins may reduce the quantity and/or variety of xenogeneic proteins from stem cell culture conditions and thereby reduce the risks of infection by non-human pathogens.

An IL-12 or chimeric molecule of the present invention may interact differently with the matrix used for the formation of the implant, as well as regulating the cells incorporated within the implant. It is anticipated that the combination of a protein or chimeric molecule of the present invention with the implant components will result in one or more of the following pharmacological traits, such as higher proliferation, enhanced differentiation, maintenance in a desired state of differentiation, greater lineage specificity of differentiation, enhanced secretion of matrix components, better 3-dimensional structure formation, enhanced signaling, better structural performance, reduced toxicity, reduced side effects, reduced inflammation, reduced immune cell infiltrate, reduced rejection, longer duration of the implant, longer function of the implant, better stimulation of the cells surrounding the implant, better tissue regeneration, better organ function, or better tissue remodeling.

The effects of proteins on differential gene expression can be assayed using one or more of the following systems.

The differences in gene expression can be analyzed in cells exposed to a protein.

Microarray technology enables the simultaneous determination of the mRNA expression of almost all genes in an organism's genome. This method uses gene "chips" in which oligonucleotides corresponding to the sequences of different genes are attached to a solid support. Labeled cDNA derived from mRNA isolated from the cell or tissue of interest is incubated with the chips to allow hybridisation between cDNA and the attached complementary sequence. A control is also used, and following hybridisation and washing the signal from both is compared. Specialised software is used to determine which genes are up or down regulated or which have unchanged expression. Many thousands of genes can be analysed on each chip. For example using Affymetrix technology, the Human

Genome U133 (HG-U133) Set, consisting of two GeneChip (registered trade mark) arrays, contains almost 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 well-substantiated human genes. The GeneChip (registered trade mark) Mouse Genome 430 2.0 contains over 39,000 transcripts on a single array.

This type of analysis reveals changes in the global mRNA expression pattern and therefore differences in the expression of genes not known to be controlled by a particular stimulus may be uncovered. This technology is hence suitable to analyze the induced gene expression associated with protein or chimeric molecule of the present invention.

The definition of known and novel genes regulated by the particular stimulus will assist in the identification of the biochemical pathways that are important in the biological activity of the particular protein or chimeric molecule of the present invention. This information will be useful in the identification of novel therapeutic targets.

The system could also be used to look at differences in gene expression induced by a protein or chimeric molecule of the present invention as compared to commercially available products.

The effects of protein on binding ability can be assayed using one or more of the following systems.

The binding ability of an IL-12 or chimeric molecule of the present invention to various substances, including extracellular matrix, artificial materials, heparin sulfates, carriers or co-factors can be investigated.

The effects of a protein on the ability of a particular protein to bind an extracellular matrix can be determined using the following assays.

A surface is coated with extracellular matrix proteins, including but not limited to collagen, vitronectin, fibronectin, laminin, in an appropriate buffer. The unbound sites can be blocked by methods known in the art, for instance, by incubation with BSA solution. The surface is washed, for instance, with PBS solutions, then a solution containing the protein

to be tested, for instance an IL-12 or chimeric molecule of the present invention, is added to the surface. After coating, the surface is washed and incubated with an antibody that recognizes a protein. Bound antibody is then detected, for instance, by an enzyme-linked secondary antibody that recognizes the primary antibody. The bound antibodies are visualized by incubating with the appropriate substrate and observing a colour change reaction. Glycosylated proteins may adhere more strongly to the extracellular matrix proteins than unglycosylated proteins.

Alternatively, an equivalent amount (specified by ELISA concentration or bioassay activity units) of an IL-12 or chimeric molecule of the present invention, or a counterpart protein expressed by non-human cells, are incubated with matrix coated wells, then following washing of the wells the amount bound is determined by ELISA. The amount of bound protein can be indirectly measured by a drop in ELISA reactivity following incubation of the sample with the coated surface.

The ability of protein to bind artificial materials can be assayed using one or more of the following systems.

In order to determine the binding ability of a protein to artificial materials, a surface is coated with artificial material, including but not limited to metals, scaffolds, in an appropriate buffer. The surface is washed, for instance, with PBS solutions, then a solution containing the protein to be tested, for instance an IL-12 or chimeric molecule of the present invention, is added to the surface. After coating, the surface is washed and incubated with an antibody that recognizes a protein. Bound antibody is then detected, for instance, by a enzyme-linked secondary antibody that recognizes the primary antibody. The bound antibodies are visualized by incubating with the appropriate substrate and observing a color change reaction.

Alternatively, an equivalent amount (specified by ELISA concentration or bioassay activity units) of an IL-12 or chimeric molecule of the present invention, and a counterpart protein expressed by non-human cells, are incubated with wells coated by artificial materials, the wells are then washed and the amount bound is determined by ELISA. The

amount bound can be indirectly measured by a drop in ELISA reactivity following incubation of the sample with the coated surface.

Ability to bind to artificial surfaces may have biological consequences, for instance, in stent coating. Alternatively, a scaffold coated with an IL-12 or chimeric molecule of the present invention is used to seed cells on. The cell growth and differentiation is then monitored and compared to uncoated or differentially coated scaffolds.

The ability of protein to bind to heparin sulfates can be assayed using one or more of the following systems.

An IL-12 or chimeric molecule of the present invention is expected to interact differentially with heparin sulfates due to their physiochemical form. These differences are expected to be evident in experimental models of cell proliferation, differentiation, migration and the like. The combination of a protein with heparin sulfates is expected to have distinctive pharmacological traits for a given treatment. This may be an increase in serum half-life, bioavailability, reduced immune-related clearance, greater efficacy, reduced dosage fewer side effects and related advantages.

The ability of protein to bind to carriers or co-factors can be assayed using one or more of the following systems.

Proteins are typically bound to other molecules when they are present in plasma. These molecules may be termed "carriers" or "co-factors" and will influence such factors as bioavailability or serum half life.

Incubating purified versions of the proteins in plasma and analyzing the resulting solution by size exclusion chromatography can determine the interaction of a protein or chimeric molecule of the present invention with their binding partners. If the protein binds a co-factor, the resulting complex will have a larger molecular weight, resulting in an altered elution time. The complex can be compared for biological activity, *in vitro* or *in vivo* half-life and bioavailability.

The effects of protein on bioassays can be assayed using one or more of the following systems.

Various bioassays can be performed to test the activity of an IL-12 or chimeric molecule of the present invention, including assays on cell proliferation, cell differentiation, cell apoptosis, cell size, cytokine/cytokine receptor adhesion, cell adhesion, cell spreading, cell motility, migration and invasion, chemotaxis, ligand-receptor binding, receptor activation, signal transduction, and alteration of subgroup ratios.

The effects of protein on cell proliferation can be assayed using one or more of the following systems.

Cells, in a particular embodiment, exponentially growing cells, are incubated in a growth medium in the presence of an IL-12 or chimeric molecule of the present invention. This can be performed in flasks or 96 well plates. The cells are grown for a period of time and then the number of cells is determined by either a direct (e.g. cell counting) or an indirect (MTT, MTS, tritiated thymidine) method. The increase or decrease in proliferation is determined by comparison with a medium only control assay. Different concentrations of protein can be used in parallel series of experiments to get a dose response profile. This can be used to determine the ED50 and ED100 (the dose required to generate the half maximal and maximal response effectively).

The effects of protein on cell differentiation or maintenance of cells in an undifferentiated state can be assayed using one or more of the following systems.

Cells are incubated in a growth medium in the presence of an IL-12 or chimeric molecule of the present invention. After a suitable period of time, the cells are assayed for indicators of differentiation. This may be the expression of particular markers on the cell surface, cytoplasmic markers, an alteration in the cell dimensions, shape or cytoplasmic characteristics. The markers may include proteins, sugar structures (e.g. glycosaminocglycans such as heparin sulfates, chondroitin sulfates etc.) lipids

(glycosphingolipids or lipid bilayer components). These changes can be assayed by a number of techniques including microscopy, western blot, FACS staining or forward/side scatter profiles.

The effects of protein on cell apoptosis can be assayed using one or more of the following systems.

Apoptosis is defined as programmed cell death, and is distinct from other methods of cell death such as necrosis. It is characterized by defined changes in the cells, such as activation of signaling pathways (e.g. Fas, TNFR) resulting in the activation of a subset of proteases know as caspases. Initiator caspase activation leads to the activation of the executioner caspases which cleave a variety of cellular proteins resulting in nuclear fragmentation, cleavage of nuclear lamins, blebbing of the cytoplasm and destruction of the cell. Apoptosis can be induced by protein ligands such as FasL, TNFa and lymphotoxin or by signals such as UV light and substances causing DNA damage.

Cells are incubated in a growth medium in the presence of protein and or other agents as suitable for the assay. For instance, the presence of agents able to block transcription (actinomycin D) or translation (cycloheximide) may be required. Following incubation for an appropriate period, the number of cells is determined by a suitable method. A decrease in cell number may indicate apoptosis. Other indications of apoptosis may be obtained by staining of the cells, for instance, for annexins or observing characteristic laddering patterns of DNA. Further evidence for the confirmation of apoptosis may be achieved by preventing the expression of apoptotic markers by incubating with cell permeable caspases inhibitors (e.g. z-VAD FMK), then assaying for apoptotic markers.

An IL-12 or chimeric molecule of the present invention may prevent apoptosis by providing a survival signal through cellular survival pathways such as the Bcl2 or Akt pathways. Activation of these pathways can be confirmed by western blotting for an increase in cellular Bcl2 expression, or for an increase in the activated (phosphorylated) form of Akt using a phospho-specific antibody directed against Akt.

For this assay, cells are incubated in the presence or absence of the survival factor (e.g. IL-3 and certain immune cells). A proportion of cells incubated in the absence of the survival factor will die by apoptosis upon extended culture, whereas cells incubated in sufficient quantities of survival factor will survive or proliferate. Activation of the cellular pathways responsible for these effects can be determined by western blotting, immunocytochemistry and FACS analysis.

The effects of a protein on the inhibition of apoptosis can be assayed using one or more of the following systems.

A protein is tested for *in vitro* activity to protect rat-, mouse-and human cortical neural cells from cell death under hypoxic conditions and with glucose deprivation. For this, neural cell cultures are prepared from rat embryos. To evaluate the effects of the protein, the cells are maintained in modular incubator chambers in a water-jacketed incubator for up to 48 hours at 37° C, in serum-free medium with 30 mM glucose and humidified 95% air/5%CO_2 (normoxia) or in serum-free medium without glucose and humidified 95% N_2/5% CO_2 (hypoxia and glucose deprivation), in the absence or presence of the protein. The cell cultures are exposed to hypoxia and glucose deprivation for less than 24 hour and thereafter returned to normoxic conditions for the remainder of 24 hour. The cytotoxicity is analyzed by the fluorescence of Alamar blue, which reports cell viability as a function of metabolic activity.

In another method, the neural cell cultures are exposed for 24 hours to 1 mM L-glutamate or a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) under normoxic conditions, in the absence or presence of various concentrations of the protein. The cytotoxicity is analyzed by the fluorescence of Alamar blue, which reports cell-viability as a function of metabolic activity.

A protein may affect the growth, apoptosis, development, or differentiation of a variety of cells. These changes can be reflected by, among other measurable parameters, changes in the cell size and changes in cytoplasmic complexity, which are due to intracellular organelle development. For instance, keratinocytes induced to differentiate by suspension

culture exhibit downregulation of surface markers such as integrins, an increase in cell size and cytoplasmic complexity. The effects of a protein on cell size, or cytoplasmic complexity can be assayed using one or more of the following systems.

FACS measures the amount of light scattered off by a cell when a beam of laser is incident on it. An argon laser providing light with a wavelength of 488nm is frequently used. The larger the size of the cell, the greater the disruption of the beam of light in the forward direction, hence the level of forward scatter corresponds to the size of the cell. In order to measure changes in cell size, cells treated with a protein are diluted in sheath fluid and injected into the flow cytometer (FACSVantage SE, Becton Dickinson). Untreated cells act as a control. The cells pass through a beam of light and the amount of forward scattering of the light corresponds to the size of the cells.

Changes in intracellular organelle growth and development (cytoplasmic complexity) can also be measured by FACS. The intracellular organelles of the cell scatter light sideways. Hence, change in cytoplasmic complexity can be measured by the amount of side scattering of light by the cells by the above method, and the level of complexity of intracellular organelles and the level of granularity of the cell can be estimated by measuring the level of side scatter of light given off by the cells.

The effect of a protein on cell size or cytoplasmic complexity can be assessed by using FACS to compare the profiles given off by, for instance, 20,000 treated cells with the signals emitted by identical number of untreated cells. By comparing the signals from the different treated populations of cells, the relative changes in cell size and cytoplasmic complexity can be determined.

The effects of a protein on cell growth, apoptosis, development, or differentiation can be assayed using one or more of the following systems.

Protein-induced apoptosis and changes in cell growth or cycles can be assessed by labeling the DNA of treated cells with dyes such as propidium iodine which has an excitation wavelength in the range of 488 nm and emission at 620 nm. Cells undergoing apoptosis

has condensed DNA as well as different size and granularity. These factors give specific forward and size scatter profiles as well as fluorescence signal, and hence the population of cells undergoing apoptosis can be differentiated from normal cells. The amount of DNA in a cell also reflects which state of the cell cycle the cell is in. For instance, a cell in G_2 stage will have twice the amount of DNA as a cell in G_0 state. This will be reflected by a doubling of the fluorescence signal given off by a cell in G_2 phase. The effect of a protein can be assessed by using FACS to compare the fluorescence signals given off by for instance, 20,000 treated cells with the signals emitted by identical number of untreated cells.

The protein may also alter the expression of various proteins. The effects of the protein on protein expression by cells can be assayed using one or more of the following systems.

To assess the increase and decrease in expression of a protein in an entire cell, the cells can be fixed and permeabilised, then incubated with fluorescence conjugated antibody targeting the epitope of the protein of interest. A large variety of fluorescent labels can be used with an Argon laser system. Fluorescent molecules such as FITC, Alexa Fluor 488, Cyanine 2, Cyanine 3 are commonly used for this experiment. This method can also be used to estimate the changes in expression of surface markers and proteins by labeling non-permeabilised cells where only the epitope exposed on the cell surface can be labeled with antibodies. The effect of a protein can be assessed by using FACS to compare the fluorescence signals given off by, for instance, 20,000 treated cells with the signals emitted by identical number of untreated cells.

The effects of a protein on ligand/receptor adhesion can be assayed using one or more of the following systems.

A protein may be more or less adhesive to substrates compared to those of a previously known physiochemical form. The interaction may be with protein receptors for sugar structures (e.g. selectins, such as L-selectin and P-selectin), with extracellular matrix components such as fibronectin, collagens, vitronectins, and laminins, or with non-protein components such as sugar molecules (heparin sulfates, other glycosaminoglycans).

A protein may also interact differently with non-biological origin materials such as tissue culture plastics, medical device components (e.g. stents or other implants) or dental materials. In the case of medical devices this may alter the engraftment rates, the interaction of the implant with particular classes of cell type or the type of linkage formed with the body.

Any suitable assays for protein adhesion can be employed. For instance, a solution containing an IL-12 or chimeric molecule of the present invention is incubated with a binding partner, in a particular embodiment, on an immobilised surface. Following incubation, the amount of the IL-12 or the chimeric molecule present in the solution is assayed by ELISA and the difference between the amount remaining and the starting material is what has bound to the binding partner. For instance, the interaction between the IL-12 or the chimeric molecule and an extracellular matrix protein could be determined by first coating wells of a 96 well plate with the ECM protein (e.g. fibronectin). Non-specific binding is then blocked by incubation with a BSA solution. Following washing, a known concentration of an IL-12 or its chimeric molecule solution is added for a defined period. The solution is then removed and assayed for the amount of IL-12 or its chimeric molecule remaining in solution. The amount bound to the ECM protein can be determined by incubating the wells with an antibody to an IL-12 or its chimeric molecule, then detecting with an appropriate system (either a labeled secondary antibody or by biotin-avidin enzyme complexes such as those used for ELISA).

Methods for determining the amount bound to other surfaces may involve hydrolyzing a protein from the inert implant surface, then measuring the amino acids present in the solution.

The effects of a protein on cell adhesion can be assayed using one or more of the following systems.

Cell adhesion to matrix (e.g. extracellular matrix components such as fibronectin, vitronectin, collagen, laminin etc.) is mediated at least in part by the integrin molecules. Integrin molecules consist of alpha and beta subunits, and the particular combinations of

alpha and beta subunit give rise to the binding specificity to a particular ligand (e.g. a2b1 integrin binds collagen, a5b1 binds fibronectin etc). The integrins subunits have large extracellular domains responsible for binding ligand, and shorter cytoplasmic domains responsible for interaction with the cytoskeleton. In the presence of ligand, the cytoplasmic domains are responsible for the induction of signal transduction events ("outside in signaling"). The affinity of integrins for their ligands can be modulated by extracellular signaling events that in turn lead to changes in the cytoplasmic tails of the integrins ("inside out signaling").

Incubation with a protein can potentially alter cell adhesion in a number of ways. First, it can alter qualitatively the expression of particular integrin subsets, leading to changes in binding ability. Secondly, the amount of a particular integrin expressed may alter, leading to altered cell binding to its target matrix. Thirdly, the affinity of a particular integrin may be altered without changing its surface expression (inside-out signaling). All these changes may alter the binding of cells to either a spectrum of ligands, or alter the binding to a particular ligand.

A protein can be tested in Cell-ECM adhesion assays which are generally performed in 96 well plate. Wells are coated with matrix, then unbound sites within the wells are blocked with BSA. A defined number of cells are incubated with the coated wells, then unbound cells are washed away and the bound cells incubated in the presence or absence of the protein. The number of cells is determined by an indirect method such as MTT/MTS. Alternatively, the cells are labeled with a radioactive label (e.g. ^{51}Cr) and a known amount of radioactivity (i.e. cells) is added to each well. The amount of bound radioactivity is determined and calculated as a percentage of the amount loaded.

Cells also adhere to other cells, for instance, adhesion of one population of cells to a monolayer of another type of cells. To assay for this, the suspension cells added to the monolayer cells would be labeled with radioactivity. The cells are then incubated in the presence or absence of a protein. The unbound cells would be washed away and the remaining mixed population of cells can be lysed and assayed for the amount of radioactivity present.

The effects of a protein on cell spreading can be assayed using one or more of the following systems.

A protein may have altered effects on cell spreading. Initiation of cell spreading is a key step in cell motility and invasive behavior. Cells spreading can be initiated *in vitro* in a number of ways. Plating a suspension of cells onto ECM components will result in attachment and ligand binding by integrin receptors. This initiates signal transduction events resulting in the activation of a family of the Cdc42, Rac and Rho small GTPases. Activation of these proteins results in actin polymerization and an extension of a lamellipodium, resulting in gradual flattening of the cells and contact of more integrins with their receptors. Eventually the cells have flattened totally and formed focal adhesions (large structures containing integrins and signaling proteins). Cell spreading can also be initiated by stimulation of adherent cells with growth factors, again resulting in activation of the Cdc42/Rac/Rho proteins and lamellipodium formation.

Cell spreading can be quantitated by examining a large number of cells at different time points following stimulation with a protein. The area of each cell can be determined using image analysis programs and the percentage of cells spread as well as the degree of cell spreading can be compared with time. More rapid spreading may be initiated by a higher activation of the Cdc42/Rac/Rho pathways, alternatively, temporal, qualitative and quantitative differences in their activation may be observed with a protein. This in turn may reflect differences in the signaling events induced by the protein.

The effects of a protein on cell motility, migration and invasion can be assayed using one or more of the following systems.

Cells adherent to a tissue culture dish do not remain statically anchored to one spot, but rather constantly extend and retract portions of their cell body. When viewed under time-lapse photography, the cells can be observed to move around the dish, either as isolated single cells or as a cell colony. This motion may be either "random walk" (i.e. not directed in a particular direction), or directional. Both types of motion can be increased by the

addition of growth factors. Time-lapse photography can be used to quantitate the overall distance covered by the cells in a given time period, as well as the overall directionality.

In the case of directional migration, cells will move towards a source of chemoattractant by sensing the chemical gradient and orienting their migration machinery towards it. In many instances, the chemoattractant is a growth factor. Directional migration can be quantitated by providing a source of chemoattractant (e.g. *via* a thin pipette) then imaging the cells migrating towards it with time-lapse photography.

An alternative system for determining directed migration is the Boyden chamber assay. In this assay, cells are placed in an upper chamber that is connected to a lower chamber *via* small holes in the partitioning membrane. Growth medium is put in both chambers, but chemoattractant is added only to the lower chamber, resulting in a diffusion gradient between the two chambers. The cells are attracted to the growth factor source and migrate through the holes in the separation membrane and on to the lower side of the membrane. After a number of hours, the membrane is removed and the number of cells that has migrated onto the bottom of the membrane is determined.

The process of cellular invasion utilises many of the same components as migration. Cell invasion can be modeled using layers of extracellular matrix through which the cells invade. For instance, Matrigel is a mixture of basement membrane components (ECM components, growth factors etc.) that is liquid at 4 degrees but rapidly sets at 37 degrees to form a gel. This can be used to coat the upper surface of a Boyden chamber, and the chemoattractant added to the lower layer. For cells to pass onto the lower surface of the membrane, they must degrade the matrigel using enzymes such as collagenases and matrix metalloproteinases (MMPs) as well as migrating directionally towards the chemoattractant. This assay mimics the various processes required for cellular invasion.

The effects of a protein on chemotaxis can be assayed using one or more of the following systems.

The migration of cells toward the chemoattractant can be measured *in vitro* in a Boyden chamber. A protein is placed in the lower chamber and an appropriate target cell population is placed in the upper chamber. To mimic the *in vitro* process of immune cells migrating from the blood to sites of inflammation, migration through a layer of cells may be measured. Coating the upper surface of the well of the Boyden chamber with a confluent sheet of cells, for instance, epithelial, endothelial or fibroblastic cells, will prevent direct migration of immune cells through the holes in the well. Instead, the cells will need to adhere to the monolayer and migrate through it towards the protein to be tested. The presence of cells on the under surface of the Boyden chamber or in the medium in the lower well in only those wells treated with the protein is indicative of the chemotactic ability of the protein. To show that the effect is specific to a protein, a neutralising antibody can be incubated with the protein in the lower chamber.

Alternatively, to test the ability of a substance (chemical, protein, sugar) to prevent chemotaxis, the substance is included in the lower chamber of the Boyden chamber along with a solution containing known chemotactic ability (this may be a specific chemokine, conditioned medium from a cell source or cells secreting a range of chemokines). A susceptible target cell population is then added to the upper chamber and the assay performed as described above.

The effects of a protein on ligand-receptor binding can be assayed using one or more of the following systems.

A protein may have different ligand-receptor binding abilities. Ligand-receptor binding can be measured by various parameters, for instance, the dissociation constant (Kd), dissociation rate constant (off rate) (k^-), association rate constant (on rate) (k^+). Differences in ligand-receptor binding may correlate with different timing and activation of signaling, leading to different biological outcomes.

Ligand-receptor binding can be measured and analysed by either Scatchard plot or by other means such as Biacore.

For Scatchard analysis, a protein labeled with, for instance, radioactively labeled (eg, ^{125}I), is incubated in the presence of differing amounts of cold competitor of a protein with cells, or extracts thereof, expressing the corresponding ligand or receptor. The amount of specifically bound labeled protein is determined and the binding parameters calculated.

For the Biacore, the corresponding recombinant ligand or receptor of the protein is coupled to the detection unit. Solutions containing a protein of choice are then passed over the detection cell and binding is determined by a change in the properties of the detection unit. On rates can be determined by passing solutions containing the protein over the detection cell until a fixed reading is recorded (when the available sites are all occupied). A solution not containing the protein is then passed over the cell and the protein dissociates from the corresponding ligand or receptor, giving the off rate.

The effects of a protein on receptor activation can be assayed using one or more of the following systems.

Interaction with a protein and its corresponding ligand or receptor may be paralleled by differences in the signaling events induced from the cell's endogenous protein. The timing of interaction may be characteristic of a protein as definitely on/off rates or dissociation constants.

Activated receptors are often internalized by the cells. The receptor/ligand complex can then be dissociated (e.g., be lowering the pH within cellular vesicles, resulting in detachment of the ligand) and the receptor recycled to the cell surface. Alternatively, the complex may be targeted for destruction. In this case the receptors are effectively down-regulated and unable to generate more signal, whereas when they are recycled they are able to repeat the signaling process. Differential receptor binding or activation may result in the receptor being switched from a destruction to a recycling pathway, resulting in a stronger biological response.

The effects of a protein on signal transduction can be assayed using one or more of the following systems.

Binding of ligands or receptors to the protein may initiate signaling, which may include reverse signaling, through a variety of cytoplasmic proteins. Reverse signaling occurs when a membrane-bound form of a ligand transduces a signal following binding by a soluble or membrane bound version of its receptor. Reverse signaling can also occur after binding of the membrane bound ligand by an antibody. These signaling events (including reverse signaling events) lead to changes in gene and protein expression. Hence, a protein can induce or inhibit different signal transductions in various pathways or other signal transduction events, such as the activation of JAK/STAT pathway, Ras-erk pathway, AKT pathway, the activation of PKC, PKA, Src, Fas, TNFR, NFkB, p38MAPK, c-Fos, recruitment of proteins to receptors, receptor phosphorylation, receptor internalization, receptor cross-talk or secretion.

The ligands or receptors recruited to the protein may be unique to the protein, due to different conformations of the ligand or receptors being induced. One way of assaying for these differences is to immunoprecipitate the ligand or receptor using an antibody crosslinked to sepahrose beads. Following immunoprecipitation and washing, the proteins are loaded on a 2D gel and the comparative spot patterns are analysed. Different spots can be cut out and identified by mass spectrometry.

The effects of a protein on up regulation and down regulation of surface markers can be assayed using one or more of the following systems.

Cells may have a variety of responses to a protein. There are a range of proteins on cell surfaces responsible for communication between the cells and the extracellular environment. Through regulated processes of endocytosis and exocytosis, various proteins are transported to and from the cell surface. Typical proteins found on the cells surface includes receptors, binding proteins, regulatory proteins and signaling molecules. Changes in expression and degradation rate of the proteins also changes the level of the proteins on

the cell surface. Some proteins are also stored in intracellular reservoirs where specific signals can induce trafficking of proteins between this storage and the cellular membrane.

Cells are incubated for an appropriate amount of time in medium containing a protein and their responses can be compared with cells exposed to the same medium without the protein. The proteins on the cell membrane can be solubilised and separated from the cells by centrifugation. The level of expression of a specific protein can be measured by Western blotting. Cells can also be labeled with fluorescence conjugated antibodies, and visualized under confocal microscopy system or counted by fluorescence activated cell sorting (FACS). This will detect any changes in expression and distribution of proteins on the cells. By using multiple antibodies, changes in protein interaction can also be studied by confocal microscopy and immuno-precipitation. Similarly, these experiments can be extended to *in vivo* animal models. Cells from specific part of animals treated with the protein may be extracted and examined with identical methodologies.

Cells induced to differentiate *in vitro* or *in vivo* by the addition of the protein will express differentiation markers that distinguish them from the untreated cells. Some cells, for instance, progenitor or stem cells, can differentiate into many subpopulations, distinguishable by their surface markers. A protein may stimulate the progenitor cells to differentiate into subgroups in a particular ratio.

A protein may have effects upon cell repulsion.

The effects of the protein on the modulation of the growth and guidance of cells and neurons is a convenient assay for cell repulsion.

Disrupting the interactions between subunits and other components of a protein leads to a way to inhibit the biological effects of the protein. Compounds inhibiting such biological effects are identified by a number of ways.

High throughput screening programs use a library of small chemical entities (chemicals or peptides) to generate lead compounds for clinical development. A number of assays can be

used to screen a library compounds for their ability to affect a biologically relevant endpoint. Each potential compound in a library is tested with a particular assay in a single well, and the ability of the compound to affect the assay determined. Some examples of the assays are provided below:

For this assay, cells are plated into a microtitre plate (96 plate, 384 plate or the like). The cells will have a readout mechanism for activation of a protein. This may involve assaying for cell growth, assaying for stimulation of a particular pathway (e.g., FRET based techniques), assaying for induction of a reporter gene (e.g., CAT, beta-galactosidase, fluorescent proteins), assaying for apoptosis and assaying for differentiation. Cells are then exposed to the protein in the presence or absence of a particular small molecule. The drug can be added before, after or during the addition of the protein. After an appropriate period of time, the individual wells are read using an appropriate method (eg, Fluorescence for FRET or induction of fluorescent proteins, cell number by MTT, beta-galactosidase activity etc). Control wells without addition of any drug or cytokine serve as comparisons. Any molecule able to inhibit the receptor/cytokine complex will give a different readout to the control wells. Further experiments will be required to show specificity of the inhibition. Alternatively, the drug could affect the detection method by a non-cytokine, non-receptor mechanism (a false positive).

A ligand or receptor of a protein is immobilised on a solid surface. The protein and the compound to be tested are then added. This can be performed by adding a protein first, then the compound; the compound first, then a protein; or the compound and the protein can be added together. Bound protein is then detected by an appropriate detection antibody. The detection antibody can be labeled with an enzyme (e.g., alkaline phosphatase or Horse-radish peroxidase for colorimetric detection) or a fluorescent tag for fluorescence detection. Alternatively, a protein can be labeled (e.g., Biotin, radioactive labeling) and be detected with an appropriate technique (e.g., for Biotin labeling, streptavidin linked to a colorimetric detection system, for radiolabeling the complex is solubilised and counted). Inhibition of protein binding is measured by a drop in the reading compared to the control wells.

Soluble ligands or receptors of a protein are bound to beads. This binding reaction can be either an adsorption process or involve chemically linking them to the plate. The beads are incubated with the protein and a candidate compound in an appropriate well. This can be performed as the protein first, then compound; compound first then the protein; or compound and the protein together. A fluorescently labeled detection antibody that recognizes the protein is then added. The unbound antibody is removed and the beads are passed through a FACS. The amount of fluorescence detected will decrease if a compound inhibits the interaction of the protein with its receptor.

To enable screening of multiple interactions between protein and its corresponding ligand/receptor against one inhibitory compound, the ability of the FACS machine to analyse scatter profiles is used. A bead with a larger diameter will have a different scatter profile to that of a smaller bead, and this can be separated out for analysis ("gating").

A number of different proteins, one of which is the protein of interest, are each linked to beads of a particular diameter. A mixture of ligands/receptors to the above-mentioned proteins are then added to the bead mixture in the presence of one candidate compound. The bound ligands/receptors are then detected using a specific secondary antibodies that is fluorescently labeled. The antibodies can be all labeled with the same detection fluorophore. The ability of the compound to prevent binding of a protein to its ligand/receptor is then determined by running the sample though a FACS machine and gating for each known bead size. The individual binding results are then analysed separately. The major benefit of this method of analysis is that the screening each compound can be tested in parallel with a number of proteins to decrease the time taken for screening proportionally.

A protein may also be characterised by its crystal structure. The physiochemical form of a protein may provide a unique 3D crystal structure. In addition, the crystal structure of the protein-ligand/receptor complex may also be generated using a protein. Since the IL-12 of the present invention provides a protein which is substantially similar to a human naturally occurring form, the complex is likely to be a more reflective representation of the *in vivo* structure of the naturally occurring protein-ligand/receptor complex. Once a crystal

structure has been obtained, interactions between an IL-12 of the present invention and potential compounds inhibiting such interactions can be identified.

Once potential compounds are identified by high throughput screening or from the crystal structure of the protein-ligand/receptor complex, a process of rational drug design can begin.

There are several steps commonly taken in the design of a mimetic from a compound having a given desired property. First, the particular parts of the compound that are critical and/or important in determining the desired property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. Alanine scans of peptides are commonly used to refine such peptide motifs. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modeled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, x-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modeling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modeled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic. Modeling can be used to generate inhibitors which interact with the linear sequence or a three-dimensional configuration.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted onto it can conveniently be selected so that the mimetic is easy to synthesize, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological

activity of the lead compound. Alternatively, where the mimetic is peptide-based, further stability can be achieved by cyclizing the peptide, increasing its rigidity. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimization or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

The goal of rational drug design is to produce structural analogs of biologically active polypeptides of interest or of small molecules with which they interact (e.g. agonists, antagonists, inhibitors or enhancers) in order to fashion drugs which are, for example, more active or stable forms of the polypeptide, or which, e.g. enhance or interfere with the function of a polypeptide *in vivo*. See, e.g. Hodgson (*Bio/Technology 9:*19-21, 1991). In one approach, one first determines the three-dimensional structure of a protein of interest by x-ray crystallography, by computer modeling or most typically, by a combination of approaches. Useful information regarding the structure of a polypeptide may also be gained by modeling based on the structure of homologous proteins. An example of rational drug design is the development of HIV protease inhibitors (Erickson *et al. Science 249:*527-533, 1990). In addition, target molecules may be analyzed by an alanine scan (Wells, *Methods Enzymol 202:*2699-2705, 1991). In this technique, an amino acid residue is replaced by Ala and its effect on the peptide's activity is determined. Each of the amino acid residues of the peptide is analyzed in this manner to determine the important regions of the peptide.

It is also possible to isolate a target-specific antibody, selected by a functional assay and then to solve its crystal structure. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analog of the original receptor. The anti-id could then be used to identify and isolate peptides from banks of chemically or biologically produced banks of peptides. Selected peptides would then act as the pharmacore.

In one aspect, a protein is used as an immunogen to generate antibodies. The physiochemical form of a protein may raise antibodies to the protein; glycopeptides specific to the protein; or antibodies directed to another co- or post-translationally modified peptide within the protein.

The protein may present epitopes not normally accessible (but possibly present) *in vivo*. For instance, there may be regions within a receptor domain that are normally in contact with another component of a heteromeric receptor. These epitopes may be used to generate monoclonal antibodies that cross react with the endogenous receptor. Such antibodies may block interaction of one receptor component with another and therefore prevent signal transduction. This may be therapeutically useful in the case of overexpression of a cytokine or receptor. The antibodies may also be therapeutically useful in diseases where the receptor is overexpressed and signals without needing the ligand.

The antibodies are also useful to detect the levels of the protein during the treatment of the disease (e.g., serum levels for half-life determination).

In addition, the antibodies are useful as diagnostic for determining the presence of a protein in a particular sample.

Reference to an "antibody" or "antibodies" includes reference to all the various forms of antibodies, including but not limited to: full antibodies (e.g. having an intact Fc region), including, for example, monoclonal antibodies; antigen-binding antibody fragments, including, for example, Fv, Fab, Fab' and $F(ab')_2$ fragments; humanized antibodies; human antibodies (e.g., produced in transgenic animals or through phage display); and immunoglobulin-derived polypeptides produced through genetic engineering techniques. Unless otherwise specified, the terms "antibody" or "antibodies" and as used herein encompasses both full antibodies and antigen-binding fragments thereof.

Unless stated otherwise, specificity in respect of an antibody of the present invention is intended to mean that the antibody binds substantially only to its target antigen with no appreciable binding to unrelated proteins. However, it is possible that an antibody will be

designed or selected to bind to two or more related proteins. A related protein includes different splice variants or fragments of the same protein or homologous proteins from different species. Such antibodies are still considered to have specificity for those proteins and are encompassed by the present invention. The term "substantially" means in this context that there is no detectable binding to a non-target antigen above basal, i.e. non-specific, levels.

The antibodies of the present invention may be prepared by well-known procedures. See, for example, Monoclonal Antibodies, *Hybridomas: A New Dimension in Biological Analyses*, Kennet *et al.* (eds.), Plenum Press, New York (1980); and *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1988).

One method for producing an antibody comprises immunizing a non-human animal, such as a mouse or a transgenic mouse, with a protein of the present invention, or immunogenic parts thereof, such as, for example, a peptide containing the receptor binding domain, whereby antibodies directed against the polypeptide of a protein, or immunogenic parts thereof, are generated in the animal. Various means of increasing the antigenicity of a particular protein, such as administering adjuvants or conjugated antigens, comprising the antigen against which an antibody response is desired and another component, are well known to those in the art and may be utilized. Immunizations typically involve an initial immunization followed by a series of booster immunizations. Animals may be bled and the serum assayed for antibody titer. Animals may be boosted until the titer plateaus. Conjugates may be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Both polyclonal and monoclonal antibodies can be produced by this method. The methods for obtaining both types of antibodies are well known in the art. Polyclonal antibodies are less favored but are relatively easily prepared by injection of a suitable animal with an effective amount of a protein, or immunogenic parts thereof, collecting serum from the animal and isolating specific antibodies to a protein by any of the known immunoadsorbent

techniques. Antibodies produced by this technique are generally less favoured, because of the potential for heterogeneity of the product.

The use of monoclonal antibodies is particularly favored because of the ability to produce them in large quantities and the homogeneity of the product. Monoclonal antibodies may be produced by conventional procedures.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al. Nature 256*:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using for example, the techniques described in Clackson *et al. Nature 352*:624-628, 1991 and Marks *et al. J Mol Biol 222*:581-597, 1991.

The present invention contemplates a method for producing a hybridoma cell line which comprises immunizing a non-human animal, such as a mouse or a transgenic mouse, with a protein of the present invention; harvesting spleen cells from the immunized animal; fusing the harvested spleen cells to a myeloma cell line to generate hybridoma cells; and identifying a hybridoma cell line that produces a monoclonal antibody that binds a protein.

Such hybridoma cell lines and the monoclonal antibodies produced by them are encompassed by the present invention. Monoclonal antibodies secreted by the hybridoma

cell lines are purified by conventional techniques. Hybridomas or the monoclonal antibodies produced by them may be screened further to identify monoclonal antibodies with particularly desirable properties, such as the ability to inhibit cytokine-signaling through its receptor.

A protein or immunogenic part thereof that may be used to immunize animals in the initial stages of the production of the antibodies of the present invention should be from a human-expressed source.

Antigen-binding fragments of antibodies of the present invention may be produced by conventional techniques. Examples of such fragments include, but are not limited to, Fab, Fab', $F(ab')_2$ and Fv fragments, including single chain Fv fragments (termed sFv or scFv). Antibody fragments and derivatives produced by genetic engineering techniques, such as disulfide stabilized Fv fragments (dsFv), single chain variable region domain (Abs) molecules, minibodies and diabodies are also contemplated for use in accordance with the present invention.

Such fragments and derivatives of monoclonal antibodies directed against a protein may be prepared and screened for desired properties, by known techniques, including the assays herein described. The assays provide the means to identify fragments and derivatives of the antibodies of the present invention that bind to a protein, as well as identify those fragments and derivatives that also retain the activity of inhibiting signaling by a protein. Certain of the techniques involve isolating DNA encoding a polypeptide chain (or a portion thereof) of a mAb of interest, and manipulating the DNA through recombinant DNA technology. The DNA may be fused to another DNA of interest, or altered (e.g. by mutagenesis or other conventional techniques) to add, delete, or substitute one or more amino acid residues.

DNA encoding antibody polypeptides (e.g. heavy or light chain, variable region only or full length) may be isolated from B-cells of mice that have been immunized with a protein of the present invention. The DNA may be isolated using conventional procedures. Phage display is another example of a known technique whereby derivatives of antibodies may be

prepared. In one approach, polypeptides that are components of an antibody of interest are expressed in any suitable recombinant expression system, and the expressed polypeptides are allowed to assemble to form antibody molecules.

Single chain antibodies may be formed by linking heavy and light chain variable region (Fv region) fragments *via* an amino acid bridge (short peptide linker), resulting in a single polypeptide chain. Such single-chain Fvs (scFvs) have been prepared by fusing DNA encoding a peptide linker between DNAs encoding the two variable region polypeptides (VL and VH). The resulting antibody fragments can form dimers or trimers, depending on the length of a flexible linker between the two variable domains (Kortt *et al. Protein Engineering 10:*423, 1997). Techniques developed for the production of single chain antibodies include those described in U.S. Patent No. 4,946,778; Bird (*Science 242:*423, 1988), Huston *et al.* (*Proc Natl Acad Sci USA 85:*5879, 1988) and Ward *et al.* (*Nature 334:*544, 1989). Single chain antibodies derived from antibodies provided herein are encompassed by the present invention.

In one embodiment, the present invention provides antibody fragments or chimeric, recombinant or synthetic forms of the antibodies that bind to the protein of the present invention and inhibit signaling by the protein.

Techniques are known for deriving an antibody of a different subclass or isotype from an antibody of interest, i.e., subclass switching. Thus, IgG1 or IgG4 monoclonal antibodies may be derived from an IgM monoclonal antibody, for example, and vice versa. Such techniques allow the preparation of new antibodies that possess the antigen-binding properties of a given antibody (the parent antibody), but also exhibit biological properties associated with an antibody isotype or subclass different from that of the parent antibody. Recombinant DNA techniques may be employed. Cloned DNA encoding particular antibody polypeptides may be employed in such procedures, e.g. DNA encoding the constant region of an antibody of the desired isotype.

The monoclonal production process described above may be used in animals, for example mice, to produce monoclonal antibodies. Conventional antibodies derived from such

animals, for example murine antibodies, are known to be generally unsuitable for administration to humans as they may cause an immune response. Therefore, such antibodies may need to be modified in order to provide antibodies suitable for administration to humans. Processes for preparing chimeric and/or humanized antibodies are well known in the art and are described in further detail below.

The monoclonal antibodies herein specifically include "chimeric" antibodies in which the variable domain of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a non-human species (e.g., murine), while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from humans, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison *et al. Proc Natl Acad Sci USA 81*:6851-6855, 1984).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from the non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which the complementarity determining regions (CDRs) of the recipient are replaced by the corresponding CDRs from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired properties, for example specificity, and affinity. In some instances, framework region residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the complementarity determining regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework region residues are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al. Nature 321*:522-525, 1986; Reichmann *et al. Nature 332*:323-329, 1988; Presta, *Curr Op Struct Biol 2*:593-596, 1992; Liu *et al. Proc Natl Acad Sci USA 84*:3439,

1987; Larrick *et al. Bio/Technology 7:*934, 1989; and Winter and Harris, *TIPS 14:*139, 1993.

In a further embodiment, the present invention provides an immunoassay kit with the ability to assay the level of human protein expressed from human cells present in a biological preparation, including a biological preparation comprising the naturally occurring human protein.

A biological preparation which can be assayed using the immunoassay kit of the present invention includes but is not limited to laboratory samples, cells, tissues, blood, serum, plasma, urine, stool, saliva and sputum.

The immunoassay kit of the present invention comprises a solid phase support matrix, not limited to but including a membrane, dipstick, bead, gel, tube or a multi-well, flat-bottomed, round-bottomed or v-bottomed microplate, for example, a 96-well microplate; a preparation of antibody directed against the human protein of interest (the capture antibody); a preparation of blocking solution (for example, BSA or casein); a preparation of secondary antibody (the detection antibody), also directed against the human protein of interest and conjugated to a suitable detection molecule (for example, alkaline phosphatase); a solution of chromagenic substrate (for example, nitro blue tetrazolium); a solution of additional substrate (for example, 5-bromo-4-chloro-3-indolyl phosphate); a stock solution of substrate buffer (for example, 0.1M Tris-HCL (pH 7.5) and 0.1M NaCl, 50mM $MgCl_2$); a preparation of the protein of the present invention with known concentration (the standard); and instructions for use.

A suitable detection molecule may be chosen from the list consisting an enzyme, a dye, a fluorescent molecule, a chemiluminescent, an isotope or such agents as colloidal gold conjugated to molecules including, but not limited to, such molecules as staphylococcal protein A or streptococcal protein G.

In a particular embodiment, the capture and detection antibodies are monoclonal antibodies, the production of which comprises immunizing a non-human animal, such as a

mouse or a transgenic mouse, with a protein of the present invention, followed by standard methods, as hereinbefore described. Monoclonal antibodies may alternatively be produced by recombinant methods, as hereinbefore described and may comprise human or chimeric antibody portions or domains.

In another embodiment, the capture and detection antibodies are polyclonal antibodies, the production of which comprises immunizing a non-human animal, such as a mouse, rabbit, goat or horse, with a protein of the present invention, followed by standard methods, as hereinbefore described.

The components of the immunoassay kit are provided in predetermined ratios, with the relative amounts of the various reagents suitably varied to provide for concentrations in solution of the reagents that substantially maximize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients, which on dissolution provide for each reagent solution having the appropriate concentration for combining with the biological preparation to be tested.

The instructions for use may detail the method for using the immunoassay kit of the present invention. For example, the instructions for use may describe the method for coating the solid phase support matrix with a prepared solution of capture antibody under suitable conditions, for example, overnight at 4°C. The instructions for use may further detail blocking non-specific protein binding sites with the prepared blocking solution; adding and incubating serially diluted sample containing the of the present invention under suitable conditions, for example, 1 hour at 37°C or 2 hours at room temperature, followed by a series of washes using a suitable buffer known in the art, for example, a solution of 0.05% Tween 20 in 0.1M PBS (pH 7.2). In addition, the instructions may provide that a preparation of detection antibody is applied followed by incubation under suitable conditions, for example, 1 hour at 37°C or 2 hours at room temperature, followed by a further series of washes. A working solution of detection buffer is prepared from the supplied detection substrate(s) and substrate buffer, then added to each well under a suitable conditions ranging from 5 minutes at room temperature to 1 hour at 37°C. The chromatogenic reaction may be halted with the addition of 1N NaOH or 2N H_2SO_4.

In an alternative embodiment, the instructions for use may provide the simultaneous addition of any combination of any or all of the above components to be added in predetermined ratios, with the relative amounts of the various reagents suitably varied to provide for concentrations in solution of the reagents that substantially maximize the formation of a measurable signal from formation of a complex.

The level of colored product, or fluorescent or chemiluminescent or radioactive or other signal generated by the bound, conjugated detection reagents can be measured using an ELISA-plate reader or spectrophotometer, at an appropriate optical density (OD), or as emitted light, using a spectrophotometer, fluorometer or flow cytometer, at an appropriate wavelength, or using a radioactivity counter, at an appropriate energy spectrum, or by a densitometer, or visually by comparison to a chart or guide. A serially diluted solution of the standard preparation is assayed in parallel with the above sample. A standard curve or chart is generated and the level of the protein present within the sample can be interpolated from the standard curve or chart.

The subject invention also provides a human derived protein for use as a standard protein in an immunoassay. The present invention further extends to a method for determining the level of human cell-expressed human protein in a biological preparation comprising a suitable assay for measuring the human protein wherein the assay comprises (a) combining the biological preparation with one or more antibodies directed against the human protein; (b) determining the level of binding of the or each antibody to the human protein or the chimeric molecule in the biological preparation; (c) combining a standard human protein sample with one or more antibodies directed against the human protein; (d) determining the level of binding of the or each antibody to the standard human protein sample; (e) comparing the level of the or each antibody bound to the human protein in the biological preparation to the level of the or each antibody bound to the standard human protein sample.

In particular, the standard human protein sample is a preparation comprising the protein of the present invention.

The biological preparation includes but is not limited to laboratory samples, cells, tissues, blood, serum, plasma, urine, stool, saliva and sputum. The biological preparation is bound to one or more capture antibody as described hereinbefore or by methods known in the art. For instance, the solid phase support matrix is first coated with a prepared solution of capture antibody under suitable conditions (for example, overnight at 4°C); followed by blocking non-specific protein binding sites with the prepared blocking solution; then adding and incubating serially diluted sample containing a protein of the present invention under suitable conditions (for example, 1 hour at 37°C or 2 hours at room temperature), followed by a series of washes using a suitable buffer known in the art (for example, a solution of 0.05% Tween 20 in 0.1M PBS (pH 7.2)).

The biological preparation is then combined with one or more detection antibodies conjugated to a suitable detection molecule as described herein. For instance, applying a preparation of detection antibody followed by incubation under suitable conditions (for example, 1 hour at 37°C or 2 hours at room temperature), followed by a further series of washes.

Determination of the level of binding may be carried out as described hereinbefore or by methods known in the art. For instance, a working solution of detection buffer is prepared from the detection substrate(s) and substrate buffer, then adding to each well under a suitable conditions ranging from 5 minutes at room temperature to 1 hour at 37°C. The chromatogenic reaction may be halted with the addition of 1N NaOH or 2N H_2SO_4.

In a particular embodiment, the present invention contemplates an isolated IL-12 as hereinbefore described.

In an embodiment, an IL-12 of the present invention is characterized by a profile of one or more of the following physiochemical parameters (P_x) and pharmacological traits (T_y), comprising:

- an apparent molecular weight (P_1) of the alpha chain of about 1 to 80, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26,

27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80 kDa and in one embodiment, 20 to 38 kDa;

- an apparent molecular weight (P_1) of the beta chain of about 1 to 100, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 kDa and in one embodiment, 27.5 to 55 kDa;

- an apparent molecular weight (P_1) of the disulphide linked alpha and beta chains of about 1 to 180, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180 kDa and in one embodiment, 47.5 to 93 kDa;

- a pI (P_2) range of the alpha chain of about 2 to about 14 such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and in one embodiment, 3.5 to 9.8;

- a pI (P_2) range of the beta chain of about 2 to about 14 such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and in one embodiment, 5.0 to 9.3;

- a total number of isoforms of the alpha and beta chains of about 2 to 100 isoforms (P_3), such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 isoforms and in one embodiment 22 to 59 isoforms;

- a percentage by weight carbohydrate (P_5) of the alpha chain of about 0 to 99%, such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66,

67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% and in one embodiment, 0 to 40%;

- a percentage by weight carbohydrate (P_5) of the beta chain of about 0 to 99%, such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% and in one embodiment, 0 to 37%;
- an observed molecular weight of the molecule under reduced conditions and following the removal of the N-linked oligosaccharides (P_6) of about 20 to 55 kDa;
- an observed molecular weight of the molecule under reduced conditions and following the removal of both the N-linked and O-linked oligosaccharides (P_7) of about 20 to 55 kDa;
- one or more N-glycan structures as listed in Table 9 in the N-linked fraction (P_{19});
- one or more O-glycan structures as listed in Table 10 in the O-linked fraction (P_{20});
- a site of C- mannosylation glycosylation (P_{40}) which includes Trp-319;
- an immunoreactivity profile (T_{13}) that is distinct from that of a human IL-12 molecule expressed in a non-human cell system, and in one embodiment, the protein concentration of the IL-12 of the present invention is underestimated when assayed using a quantitative immunoassay which includes a protein standard of a human IL-12 molecule expressed in *Sf*21 cells;
- a biological activity that is distinct from that of a human IL-12 expressed in a non-human cell system, and in one embodiment, the ability of IL-12 of the present invention to induce proliferation (T_{32}) in PHA-stimulated PBMCs is significantly more potent than a human IL-12 expressed in CHO cells;
- a biological activity that is distinct from that of a human IL-12 expressed in a non-human cell system, and in one embodiment, the ability of IL-12 of the present invention to induce STAT4 activation (T_{45}) of PHA-stimulated PBMCs is significantly greater than a human IL-12 expressed in CHO cells;
- a biological activity that is distinct from that of a human IL-12 expressed in a non-human cell system, and in one embodiment, the ability of IL-12 of the present

invention to induce interferon-gamma production (T_{56}) by PHA stimulated PBMCs is significantly more potent than a human IL-12 expressed in CHO cells; and

- a biological activity that is distinct from that of a human IL-12 expressed in a non-human cell system, and in one embodiment, the ability of IL-12 of the present invention to induce CD56 (T_{59}) on CD34+ human haematopoietic cells to a significantly greater extent than a human IL-12 expressed in CHO cells.

The physiochemical form of the protein of the present invention may be achieved by modifying the host cell by a variety of ways known in the art, including but not limited to the introduction of one or more transgene into the host cell that encodes an enzyme or enzymes that will produce the desired physiochemical form. Such transgenes include various types of sialyltransferases, such as ST3Gal1, ST3Gal2, ST3Gal3, ST3Gal4, ST3Gal5, ST3Gal6, ST6Gal1, ST6Gal2, ST6GalNAc1, ST6GalNAc2, ST6GalNAc3, ST6GalNAc4, ST6GalNAc5, ST8Sia1, ST8Sia2, ST8Sia3, ST8Sia4, ST8Sia5, ST8Sia6; galactosyltransferases, such as GalT1, GalT2; fucosyltransferases such as FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7, FUT8, FUT9, FUT10, FUT11; sulfotransferases; GlcNAc transferases such as GNT1, GNT2, GNT3, GNT4, GNT5; antenna-cleaving enzymes and endoglycosidases.

For instance, inefficient terminal sialylation of N-glycan structures that results in reduced serum half-life of an expressed protein such as recombinant human AchE can be ameliorated by the addition of a rat beta-galactoside alpha-2,6-sialyltransferase transgene to HEK 293 cells (Chitlaru *et al. J Biochem 336*:647-658, 1998; Chitlaru *et al. J Biochem 363*:619-631, 2002).

Similarly, inefficient formation of particular Lewis x groups such as sialyl Lewis x structures on N-glycan structures that results in reduced ligand binding of an expressed protein such as recombinant human PSGL-1 can be ameliorated by the addition of a fucosyltransferase transgene to HEK 293 cells (Fritz *et al. PNAS 95*:12283-12288, 1998).

In one embodiment, a protein is produced using a human cell line transformed with either α-2,3 or α-2,6 sialytransferase, or both α-2,3 sialytransferase and α-2,6 sialytransferase

("sialylated-protein"). Examples of sialylated-protein include sialylated-IL-12, sialylated-sialylated-IL-12 p35 and sialylated-IL-12 p40.

In particular, the sialylated-protein is characterized by a profile of physiochemical parameters (P_x) comprising one or more physiochemical parameters. Monosaccharide (P_9) and sialic acid contents (P_{10}) of the sialylated-protein are, when normalized to GalNAc, 1 to 0.1-100 NeuNAc; and when normalized to 3 times of mannose 3 to 0.1-100 NeuNAc. Neutral percentage of N-linked oligosaccharides (P_{13}) of the sialylated-protein is 0 to 99% such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99%. Acidic percentage of N-linked oligosaccharides (P_{14}) of the sialylated-protein is 1 to 100% such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%. Neutral percentage of O-linked oligosaccharides (P_{15}) of the sialylated-protein is 0 to 99% such as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99%. Acidic percentage of O-linked oligosaccharides (P_{16}) of the sialylated-protein is 1 to 100% such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%. The *in vivo* half-life (T_{11}) of the sialylated-protein is increased in comparison to the half-life of the protein of the invention expressed without the transgene.

In one embodiment, the sialylated-protein contains at least one of the structural formulae described herein or at least one of the structural formulae described herein where one or more NeuNAc linkage is a α 2,6 linkage in the N-linked fraction.

In one embodiment, the sialylated-protein contains at least one of the structural formulae described herein or at least one of the structural formulae described herein where one or more NeuNAc linkage is a α 2,6 linkage in the O-linked fraction.

In one embodiment, the protein of the invention is produced using a human cell line transformed with FUT3 ("fucosylated-protein"). Examples of fucosylated-protein include fucosylated-IL-12, fucosylated-IL-12 p35 and fucosylated-IL-12 p40.

In particular, the fucosylated-protein is characterized by a profile of physiochemical parameters (P_x) comprising one or more of physiochemical parameters. Monosaccharide (P_9) and sialic acid contents (P_{10}) of the fucosylated-protein are, when normalized to GalNAc, 1 to 0.1-100 NeuNAc; and when normalized to 3 times of mannose 3 to 0.1-100 NeuNAc.

In one embodiment, the fucosylated-protein has a higher proportion of structure containing Lewis structures (such as Lewis a, Lewis b, Lewis x or Lewis y) or sialyl Lewis structures (such as sialyl Lewis a or sialyl Lewis x).

In one embodiment, the fucosylated-protein has altered binding affinity to ligands in comparison to the binding affinity of the protein of the invention expressed without the transgene.

Human host cells suitable for the introduction of the cloned DNA sequence comprising a the IL-12 of the present invention include but are not limited to HEK 293 and any derivatives thereof, HEK 293 c18, HEK 293-T, HEK 293 CEN4, HEK 293F, HEK 293FT, HEK 293E, AD- 293 (Stratagene), 293A (Invitrogen), Hela cells and any derivatives thereof, HepG2, PA-1 Jurkat, THP-1, HL-60, H9, HuT 78, Hep-2, Hep G2, MRC-5, PER.C6, SKO-007, U266, Y2 (Apollo), WI-38, WI-L2.

The physiochemical form of IL-12 of the present invention may be achieved by modifying the host cell by a variety of ways known in the art, including but not limited to the introduction of a transgene into the host cell that encodes an enzyme or enzymes that will produce the desired physiochemical form. The introduction of specific DNA sequences can be used to optimize the integration of the cloned DNA sequence into the host cell genome, the various types of integration including but not limited to site-specific, targeted, direct or enzyme-mediated integration.

The DNA of IL-12 can be introduced into suitable host cells by various transfection methods known in the art, for instance, using chemical reagents such as DEAE-dextran, calcium phosphate, artificial liposomes, or by direct microinjection, electroporation, biolistic particle delivery or infection or transfection with viral constructs as described below.

DEAE-dextran is a cationic polymer that associates with negatively charged nucleic acids. An excess of positive charge, contributed by the polymer in the DNA/polymer complex allows the complex to come into closer association with the negatively charged cell membrane. Uptake of the complex is presumably by endocytosis. Other synthetic cationic polymers including polybrene, polyethyleneimine and dendrimers have also been used for transfection.

Calcium phosphate co-precipitation can be used for transient and stable transfection of a variety of cell types. The DNA is mixed with calcium chloride in a controlled manner and added to a buffered saline/phosphate solution and the mixture is incubated at room temperature. A precipitate is generated and is taken up by the cells via endocytosis or phagocytosis.

The most commonly used synthetic lipid component of liposomes for liposome-mediated gene delivery is one which has overall net positive charge at physiological pH. Often the cationic lipid is mixed with a neutral lipid such as L-dioleoyl phosphatidylethanolamine (DOPE). The cationic portion of the lipid molecule associates with the negatively charged

nucleic acids, resulting in compaction of the nucleic acid in a liposome/nucleic acid complex. Uptake of the complex is by endocytosis.

Direct microinjection of DNA into cultured cells or nuclei is an effective, although laborious technique, which is not appropriate if a large number of transfected cells are required.

Electroporation utilizes an electric pulse, which generates pores that allow the passage of nucleic acids into the cells. This technique requires fine-tuning and optimization for duration and strength of the pulse for each type of cell used. Commercially available electroporation device includes Amaxa Biosystems' Nucleofector Kits (Amaxa Biosystems, Germany).

This method relies upon high velocity delivery of nucleic acids on microprojectiles to recipient cells.

Infection or transfection with viral or retroviral constructs include the use of retrovirus, such as lentivirus, or DNA viruses, such as adenovirus. The process involves using a viral or retroviral vector to transfer a foreign gene to the host's cells.

In some embodiments, the protein is produced by either transient methods or from stably transfected cell lines. Transient transfection is performed using either adherent or suspension cell lines. For adherent cell lines, the cells are grown in serum containing medium (between 2-10% serum) and in medium such as DMEM, DMEM/F12 (JRH). Serum used can be fetal calf serum (FCS), donor calf serum (DCS), new born calf serum (NBCS) or the like. Plasmid vectors are introduced into the cells by standard methods known in the art. In a particular embodiment, the DNA of the protein is transfected using DEAE dextran or calcium phosphate precipitation. Following transfection, the cells are switched to an appropriate collection medium (e.g. serum free DMEM/F12) for collection of the expressed protein.

Transient expression of the protein from suspension cells can be performed by introducing the plasmid vector using the methods outlined above. The suspension cells can be grown in either serum containing medium, or in serum free medium (e.g. Freestyle medium (Invitrogen), CD293 medium (Invitrogen), Excell medium (JRH) or the like). The transfection can be performed in the absence of serum by transfecting in an appropriate media using a suitable transfection method, for instance, lipofectamine in OptiMEM medium.

Transient expression usually results in a peak of expression 2-3 days after transfection. Episomal vectors are replicated within the cell and give sustained expression. Therefore, to obtain large amounts of product, episomal expression vectors are transfected into cells and the cells are expanded. A protein is expressed into the medium, which is collected as the cells are expanded over a period of weeks. The expression medium can be serum containing or serum free and the cells can be either adherent or suspension adapted.

Stable clones are obtained by transfection of the expression vector into the cells, then selecting with an appropriate agent, for instance, phleomycin, hygromycin, puromycin, neomycin G418, methotrexate or the like. Stable clones will survive selection as the plasmid contains a resistance gene in addition to the gene encoding the protein. One to two days after introduction of the gene, selection is begun on either the whole population of cells (stable pools) or on cells plated at clonal density. A non-transfected population of cells is also selected to determine the efficacy of cell killing by the selective agent. For adherent cells, the cells are allowed to grow on a tissue culture plate until visible separate clones are obtained. They are then removed from the plate by trypsinization, or physical removal and placed into tissue culture wells (eg, one clone per well of a 96 well plate). For suspension cells, limiting dilution cloning is performed subsequent to selection. The clones are then expanded, then either characterized and/or subjected to a further round of limiting dilution analysis.

Stable clones growing in serum containing medium can be adapted by gradual reduction of serum levels followed by detachment and growth under low serum in suspension. The serum levels are then reduced further until serum free status is achieved. Some growth

media allow more rapid adaptation (e.g. a straight swap from serum containing adherent conditions to serum free suspension growth), an example of which is Invitrogen's CD293 media.

Following growth in serum free media, the clones can begin media optimization. The clones are tested for production characteristics, for example, integral viable cell number, in many different growth media until an optimum formulation or formulations are obtained. This may depend on the method of production of the product. For instance, the cells may be expanded in one medium, then additives that enhance expression added prior to product collection.

The over-expressed protein may accumulate within host cells. Recovery of intracellular protein involves treatment of the host cells with lysis buffers including but not limited to buffers containing: NP40, Triton X-100, Triton X-114, sodium dodecyl sulfate (SDS), sodium cholate, sodium deoxycholate, CHAPS, CHAPSO, Brij-35, Brij-58, Tween-20, Tween-80, Octylglucoside and Octylthioglucoside. Alternative methods of host cell lysis may include sonication, homogenization, french press treatment and repeated cycles of freeze thawing and treatment of the cells with hypotonic solutions.

The final product can be produced in many different sorts of bioreactors, by way of non-limiting examples, including stirred tank, airlift, packed bed perfusion, microcarriers, hollow fibre, bag technologies, cell factories. The methods may be continuous culture, batch, fed batch or induction. Peptones may be added to low serum cultures to achieve increases in volumetric protein production.

The IL-12 of the present invention is purified using a purification strategy. Purification methods include but are not limited to: tangential flow filtration (TFF); ammonium sulfate precipitation; size exclusion chromatography (SEC); gel filtration chromatography (GFC); affinity chromatography (AFC); Protein A Affinity Purification; Receptor mediated Ligand Chromatography (RMLC); dye ligand chromatography (DLC); ion exchange chromotogaphy (IEC), including anion or cation exchange chromatography (AEC or

CEC); reversed-phase chromatography (RPC); hydrophobic interaction chromatography (HIC); metal chelating chromatography (MCC).

TFF is a rapid and efficient method for biomolecule separation and is used for concentrating, desalting, or fractionating samples. TFF can concentrate samples as large as hundreds of litres down to as little as 10 ml. In conjunction with a suitable molecular weight cut off membrane, TFF can separate and isolate biomolecules of differing size and molecular weight (nominal molecular weight cutoff (NMWC) 5 KDa, 10 KDa, 30 KDa, 100 KDa). The process of diafiltration involving dilution of the sample followed by re-concentration can be used to desalt or exchange the sample buffer.

Salting out or ammonium sulfate precipitation is useful for concentrating dilute solutions of proteins. It is also useful for fractionating a mixture of proteins. Increases in the ionic strength of a solution containing protein causes a reduction in the repulsive effect of like charges between protein molecules. It also reduces the forces holding the solvation shell around the protein molecules. When these forces are sufficiently reduced, the protein will precipitate; hydrophobic proteins precipitating at lower salt concentrations than hydrophilic proteins. Fractionation of protein mixtures by the stepwise increase in the ionic strength followed by centrifugation can be a very effective way of partly purifying proteins.

SEC separates proteins by size, based on the flow of the sample through a porous matrix. SEC has the same principle as GFC when it is used to separate molecules in aqueous systems. In SEC, molecules larger than pores of the packing elute with the solvent front first and are completely excluded. Intermediate sizes of molecules, between the completely excluded and the retained, pass through the pores of the matrix according to their sizes. Small molecules which freely pass in and out of the pores are retained. Therefore, different sizes of proteins have different elution volume and retention times. For structurally similar molecules, the larger the molecular sizes, the earlier they elute out. Before running any samples, a standard curve should be established to determine the working limits and reference retention time.

When the protein shapes are the same, molecular weight can be screened in the elutes from the column rapidly by UV absorption, fluorescence or light scattering, according to the packing materials of various pore sizes on the column. Photon correlation spectroscopy (PCS) has been usually performed on static samples and for liquid chromatographic detection. Low angle laser light scattering has also been coupled to chromatographic detection to detect the molecular weights directly, independent of the shapes of the proteins (Carr *et al. Anal Biochem 175:*492-499, 1988). SEC-HPLC was used to detect hGH degradation and aggregation (Pikal *et al. Pharm Res 8:*427-436, 1991). It was also used for estimation of contamination in studying β-galactosidase (Yoshioka *et al. Pharm Res 10:*103-108, 1993).

AFC purifies biological molecules according to specific interactions between their chemical structures and the suitable affinity ligands. The target molecule is adsorbed by a complementary immobilized ligand specifically and reversibly. The ligand can be an inhibitor, substrate, analog or cofactor, or an antibody which can recognize the target molecules specifically. Subsequently, the adsorbed molecules are either eluted by competitive displacement, or by the conformation change through a pH or ionic strength shift.

Protein A Affinity Purification is an example of affinity purification utilising the affinity of certain bacterial proteins that bind generally to antibodies, regardless of the antibody's specificity to antigen. Protein A, Protein G and Protein L are three that have well characterised antibody-binding properties. These proteins have been produced recombinantly and used routinely for affinity purification of key antibody types from a variety of species. A genetically engineered recombinant form of Protein A and G, called Protein A/G, is also available. These antibody-binding proteins can be immobilized to support matrixes. This method has been modified to purify recombinant proteins that have had the Protein A binding region of an antibody (Fc region) linked to the target protein. Binding to the immobilised Protein A molecule is performed under physiological conditions and eluted by change in pH or ionic strength.

RMLC is a special kind of AFC utilising the inherent affinity of a receptor for its cognate target molecule. The receptor molecule is immobilised on a suitable chromatography support matrix via reactive amines, reactive hydrogens, carbonyl, carboxyl or sulfhydryl groups. In one example of RMLC, the receptor-Fc chimera molecule is immobilised on Protein A sepharose beads via affinity of the Fc portion of the receptor to the Protein A. This method has the advantage of immobilising the receptor in an orientation that exposes its ligand-binding site to its cognate cytokine. Adsorption of the target molecule to the receptor is performed under physiological conditions and elution is achieved by change in pH or ionic strength.

DLC is a kind of ALC utilizing the ability of reactive dyes to bind proteins in a selective and reversible manner. The dyes are generally monochlorotriazine compounds. The reactive chloro group allows easy immobilization of the triazine dye to a support matrix, such as Sepharose or agarose, and, more recently, to nylon membranes.

The initial discovery of the ability of these dyes to bind proteins came from the observation that blue dextran (a conjugate of cibacron blue FG-3A), used as a void volume marker on gel filtration columns, could retard the elution of certain proteins. A number of studies have been carried out on the specificity of the dyes for particular proteins, mostly using the prototype cibacron blue dye. The dyes appear to be most effective at binding proteins and enzymes that utilize nucleotide cofactors, such as kinases and dehydrogenases, although other proteins such as serum albumin also bind tightly. It has been proposed that the aromatic triazine dye structure resembles the nucleotide structure of nicotinamide adenine dinucleotide (NAD) and that the dye interacts with the dinucleotide fold in these proteins. In many cases, bound proteins can be eluted from the columns by a substrate or nucleotide cofactor in a competitive fashion, and dyes have been shown to compete for substrate-binding sites in free solution. It seems likely that these dyes can bind proteins by electrostatic and hydrophobic interactions and by more specific "pseudoaffinity" interactions with ligand-binding sites. Enhancing the specificity of dye ligands by modification to further resemble ligands (biomimetic dyes) has been successful in the purification of a number of dehydrogenases and proteases (McGettrick *et al. Methods Mol Biol 244*:151-7, 2004).

Ion Exchange Chromatography (IEC) purifies proteins using protein retention on columns resulting from the electrostatic interactions between the ion exchange column matrix and the proteins. When the pH of the mobile phase is above the pI of the target protein will be negatively charged and will interact with an anion exchange column (AEC). When the pH of the mobile phase is below the pI of the target protein the protein will be positively charged and a cation exchange column (CEC) should be used. The target proteins are eluted by increasing the concentrations of a counter ion with the same charge as the target molecule.

RPC separates biological molecules according to the hydrophobic interactions between the molecule and a chromatographic support matrix. Ionizable compounds are best analyzed in their neutral form by controlling the pH of the separation. Mobile phase additives, such as trifluoroacetic acid, increase protein hydrophobicity by forming ion pairs which strongly adsorb to the stationary phase. By changing the polarity of the mobile phase, the biological molecules are eluted from the chromatographic support.

HIC is similar to RPC, but with a larger nominal pore size. In HIC, the elution solvent uses an aqueous salt solution, instead of the aqueous or organic mobile phases used in RPC. Also, the order of sample elution is reversed from that obtained from RPC. The surfaces of proteins consist of hydrophilic residues and hydrophobic "patches", which are usually located in the interior of the folded proteins to stabilize the proteins. When the hydrophobic patches become exposed to the aqueous environment, they will disrupt the normal solvation properties of the protein, which is thermodynamically unfavorable. In the aqueous mobile phase, the higher the concentrations of inorganic salts (e.g. ammonium sulfate), the higher surface tension, thereby increasing the strength of hydrophobic interactions between the hydrophobic groups of the HIC resin and the proteins, which are adsorbed. However, while descending the salt concentration gradient, the surface tension of the aqueous mobile phase is decreased, thus reducing the hydrophobic interaction, resulting in the proteins desorbing from the hydrophobic groups of the column.

MCC is a technique in which proteins are separated on the basis of their affinity for chelated metal ions. Various metal ions including but not limited to Cu^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+} or Ni^{2+} are immobilized on the stationary phase of a chromatographic support via a covalently bound chelating ligand (e.g. iminodiacetic acid). Free coordination sites of the metal ions are used to bind different proteins and peptides. Elution can occur by displacement of the protein with a competitive molecule or by changing the pH. For instance, a lowering of the pH in the buffer results in a reduced binding affinity of the protein-metal ion complex and desorption of the protein. Alternatively, bound proteins can be eluted from the column using a descending pH gradient, in the form of a step gradient or as linear gradient.

The physiochemical form of the protein of the present invention may be achieved by chemical and/or enzymatic modification to the expressed molecule in a variety of ways known in the art.

The present invention contemplates chemical or enzymatic coupling of carbohydrates to the peptide chain of a protein at a time after the protein or chimeric molecule is expressed and purified. Chemical and/or enzymatic coupling procedures may be used to modify, increase or decrease the number or profile of carbohydrate substituents. Depending on the coupling mode used, the sugar(s) may be attached to (a) amide group of arginine, (b) free carboxyl groups, (c) sulfhydroxyl groups such as those of cysteine, (d) hydroxyl groups such as those of serine, threonine, hydroxylysine or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, (f) the amide group of glutamine, or (g) the amino groups such as those of histidine, arginine or lysine. Additions can be carried out chemically or enzymatically. For example serial addition of sugar units to the protein can be performed using appropriate recombinant glycosyltransferases. Glycosyltransferases can also be used to add sugars that have covalently attached substituents. For example, sialic acid with covalently attached polyethylene glycol (PEG) can be transferred by a sialyltransferase to a terminal galactosyl residue to increase molecular size and serum half-life.

The carbohydrate side chain of a protein can also be modified chemically or enzymatically to incorporate a variety of functionalities, including phosphate, sulfate, hydroxyl, carboxylate, O-sulfate and N-acetyl groups.

Carbohydrates present on a protein may also be removed chemically or enzymatically. Trifluoromethanesulfonic acid or an equivalent compound can be used for chemical deglycosylation. This treatment can result in the cleavage of most or all sugars, except the linking sugar, while leaving the polypeptide intact. Individual sugars or the entire chain can also be removed from a protein by a variety of endoglycosidases and exoglycosidases.

The glycan component of a protein may be modified synthetically by treatment with sialidases, or mild acid treatment to remove any residual sialic acids; treatment with exo- or endo- glycosidases to trim down the antennae of N-linked oligosaccharides or shorten O-linked oligosaccharides. It may also be treated with fucosidases or sulfatases to remove side groups such as fucose and sulfate. Pseudo glycan structures such as polyethylene glycol or dextrans may be chemically added to the amino acid backbone, or a glycotransferase cocktail can be used with sugar-dUDP precursors to synthetically add sugar subunits to the glycan.

The present invention contemplates a protein such as IL-12 chemically or enzymatically coupled to radionuclides.

Iodination procedures may be used to attach iodine isotopes (e.g. ^{123}I) to the peptide chain of the protein. In particular, the isotope(s) may be attached to a (a) phenolic ring of a tyrosine, or (b) the imidazole ring of a histidine on the peptide chain of the protein or the chimeric molecule thereof. Iodination may be performed using the Chloramine-T, iodine monochloride, triiodide, electrolytic, enzymatic, conjugation, demetallation, iodogen or iodo-bead methods.

Technetium labeling procedures may be used to attach ^{99m}Tc to an IL-12 of the present invention using a method known in the art, for instance, by the reduction of $^{99m}TcO_4^-$ with

a reducing agent (e.g. stannous chloride) followed by ^{99m}Tc labelling of an IL-12 via a bifunctional chelating agent, for instance, diethylenetriamine pentaacetic acid (DTPA).

The present invention contemplates an IL-12 chemically or enzymatically coupled to chemotherapeutic agents. Suitable agents (e.g. zoledronic acid) may be conjugated to the the IL-12 using methods known in the art, for instance, by a N-hydroxysulfosuccinimide enhanced carbodiimide-mediated coupling reaction.

The present invention contemplates an IL-12 chemically or enzymatically coupled to toxins. Suitable toxins, including melittin, vanous toxin, truncated pseudomonas exotoxin, ricin, gelonin and diptheria toxin may be conjugated to the IL-12 using a method known in the art, for instance, by maleimide or carbodiimide coupling chemistry.

An isolated IL-12 described herein may be delivered to the subject by any means that produces contact of the isolated IL-12 with the target receptor or ligand in the subject. In a particular embodiment, an IL-12 of the present invention is delivered to the subject as a "pharmaceutical composition".

In another aspect, the present invention contemplates a pharmaceutical composition comprising one or more isolated IL-12 molecules as hereinbefore described together with a pharmaceutically acceptable carrier or diluent.

Composition forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dilution medium comprising, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of surfactants. The preventions of the action of microorganisms can be brought about by various anti-bacterial and anti-fungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like. In many cases, it

will be favorable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with the active ingredient and optionally other active ingredients as required, followed by filtered sterilization or other appropriate means of sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, suitable methods of preparation include vacuum drying and the freeze-drying technique which yield a powder of active ingredient plus any additionally desired ingredient.

When the active agent is suitably protected, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet or administered *via* breast milk. For oral therapeutic administration, the active ingredient may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers and the like. Such compositions and preparations should contain at least 1% by weight of active agent. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active agent in such therapeutically useful compositions is such that a suitable dosage will be obtained. In a particular embodiment, compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 200 mg of modulator. Alternative dosage amounts include from about 1 μg to about 1000 mg and from about 10 μg to about 500 mg. These dosages may be per individual or per kg body weight. Administration may be per hour, day, week, month or year.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter. A binder such as gum, acacia, corn starch or gelatin; excipients such as

dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The present invention also contemplates topical formulations. In a topical composition, the active agent may be suspended within a cream or lotion or wax or other liquid solution such that topical application of the cream or lotion or wax or liquid solution results in the introduction of the active agent to a biological surface in the subject. The term "biological surface" as used herein, contemplates any surface on or within the organism. Examples of "biological surfaces" to which the topical compositions of the present invention may be applied include any epithelial surface such as the skin, respiratory tract, gastrointestinal tract and genitourinary tract.

In addition to traditional cream, emulsion, patch or spray formulations, the agents of the present invention may also be delivered topically and/or transdermally using a range of iontophoric or poration based methodologies.

"Iontophoresis" is predicated on the ability of an electric current to cause charged particles to move. A pair of adjacent electrodes placed on the skin set up an electrical potential between the skin and the capillaries below. At the positive electrode, positively charged drug molecules are driven away from the skin's surface toward the capillaries. Conversely, negatively charged drug molecules would be forced through the skin at the negative electrode. Because the current can be literally switched on and off and modified,

iontophoretic delivery enables rapid onset and offset, and drug delivery is highly controllable and programmable.

Poration technologies, use high-frequency pulses of energy, in a variety of forms (such as radio frequency radiation, laser, heat or sound) to temporarily disrupt the stratum corneum, the layer of skin that stops many drug molecules crossing into the bloodstream. It is important to note that unlike iontophoresis, the energy used in poration technologies is not used to transport the drug across the skin, but facilitates its movement. Poration provides a "window" through which drug substances can pass much more readily and rapidly than they would normally.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, anti-bacterial and anti-fungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art and except insofar as any conventional media or agent is incompatible with the modulator; their use in the pharmaceutical compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

In an additional embodiment, the pharmaceutical composition of the present invention can be used alone or in conjunction with other biologics, drugs or therapies for treatment of HIV infections, herpes virus infections, cryptosporidiosis, pulmonary francisella tularensis subsp. novicida infection, atypical mycobacterium infection, mycosis fungoides, Sezary syndrome, Crohn's disease, inflammatory bowel disease, cutaneous leishmaniasis, multiple sclerosis, relapsing remitting multiple sclerosis, psoriasis, plaque psoriasis, asthma, hypereosinophilic syndrome, uveitis, graft vs host disease (GVHD), myelodysplastic syndromes, Wegener's granulomatosis, IL-2 induced hypotension and a variety of cancers including: cutaneous T-Cell lymphoma; cervical squamous cell carcinoma; cervical adenocarcinoma; lymphoma, advanced solid tumours, renal cancer, melanoma, ovarian epithelial cancer, peritoneal cavity cancer, breast cancer, non-small cell lung cancer, liver cancer, bladder cancer, adenocarcinoma of the prostate, Waldenstrom's macroglobulinemia, grade 1 follicular lymphoma, grade 2 follicular lymphoma, diffuse

small, lymphocytic/marginal zone lymphoma, mantle cell lymphoma, Kaposi's sarcoma, neuroblastoma, lymphocytic lymphoma, myeloid leukemia, Hodgkin's disease, plasma cell tumor; skin cancer, pancreatic cancer, gastric cancer, colorectal cancer, leukemia, testicular cancer, multiple myeloma, kidney tumor, endometrial cancer, AIDS-related small noncleaved cell lymphoma, AIDS-related immunoblastic large cell lymphoma, AIDS-related peripheral/systemic lymphoma, skin metastases, lip and oral cavity cancer, head and neck cancer, oropharyngeal cancer, adult acute lymphoblastic leukemia, adult acute myeloid leukemia, childhood acute lymphoblastic leukemia, childhood acute myeloid leukemia, neuroblastoma, rectal carcinoma and non-Hodgkin's lymphoma.

In a further embodiment, the pharmaceutical composition of the present invention can be used alone or in conjunction with other biologics, drugs or therapies for treatment of metastatic cancers that exhibit an immunogenic phenotype.

However, the pharmaceutical composition of the present invention has higher pharmaceutical efficacy, increased thermal stability, increased serum half-life or higher solubility in the bloodstream when compared with human IL-12 expressed in non-human cell lines. The present invention also shows reduced risks for immune-related clearance or related side effects. Because of these improved properties, the composition of the present invention can be administered at a lower frequency than a similar protein expressed in non-human cell lines. Decreased frequency of administration is anticipated to enhance patient compliance resulting in improved treatment outcomes. The quality of life of the patient is also elevated.

Accordingly, in one embodiment, the pharmaceutical composition of the present invention can be administered in a therapeutically effective amount to patients in the same way a protein expressed in non-human cell lines is administered. The therapeutic amount is that amount of the composition necessary for the desired *in vivo* activity. The exact amount of composition administered is a matter of preference subject to such factors as the exact type of condition being treated, the condition of the patient being treated and the other ingredients in the composition. The pharmaceutical compositions containing the isoforms of the IL-12 of the present invention may be formulated at a strength effective for

administration by various means to a human patient experiencing one or more of the above disease conditions. Average therapeutically effective amounts of the composition may vary. Effective doses are anticipated to range from 0.1ng/kg body weight to 20μg/kg body weight; or based upon the recommendations and prescription of a qualified physician.

The present invention further extends to uses of the isolated IL-12 comprising at least part of the IL-12 and a composition comprising same in a variety of therapeutic and/or diagnostic applications.

More particularly, the present invention extends to a method of treating or preventing a condition in a mammalian subject, wherein the condition can be ameliorated by increasing the amount or activity of the IL-12 of the present invention, the method comprising administering to said mammalian subject an effective amount of an isolated protein, a fragment thereof or a composition comprising the isolated protein.

The subject invention also provides a pharmaceutical composition comprising an isolated IL-12 of the present invention, for use, alone or in combination with other proteins expressed from human cell lines, for example, bFGF, EPO, G-CSF, SCF, in the culturing hES and other human progenitor cells, in particular, human haematopoietic progenitor cells, thus reducing the potential risks of transferring animal-derived infectious agents and thus allowing hES and other human progenitor cells to be applicable for therapeutic applications.

The present invention is further described by the following non-limiting examples.

EXAMPLE 1

(a) Production of a DNA construct expressing human IL-12

pUMVC3-IL12 was purchased from Aldevron and contained the sequences of p35 and p40 in tandem.

(b) Preparation of Megaprep of pUMVC3-IL-12

750ml of sterile LB broth containing ampicillin (100µg/ml) was inoculated with 750µl of overnight culture of *E. coli* transformed with pUMVC3-IL-12. The culture was incubated at 37°C with shaking for 16 hours. Plasmid was prepared in accordance with a Qiagen Endofree Plasmid Mega Kit (Qiagen Mega Prep Kit #12381).

EXAMPLE 2

(a) Production of IL-12 of the Present Invention

At day 0, five 500 cm2 tissue culture dishes (Corning) were seeded with 3 ´ 107 cells of transformed embryonal human kidney cell line, for example HEK 293 or derivatives thereof, HEK 293 c18, HEK 293T, 293 CEN4, HEK 293F, HEK 293FT, HEK 293E, AD-293 (Stratagene), or 293A (Invitrogen). Cells were seeded in 90 ml per plate of Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12) (JRH Biosciences), the medium being supplemented with 10% (v/v) donor calf serum (DCS, JRH Biosciences), 4 mM L-glutamine (Amresco), 50 U/ml Penicillin G and 50 mg/ml Streptomycin Sulphate (JRH Biosciences). The plates were incubated at 37 °C and 5% CO2 overnight.

At day 1, transfection was performed using calcium phosphate. 1 hour before transfection, the medium in each plate was replaced with 120 ml of fresh DMEM/F12 supplemented with 10% (v/v) DCS, 4 mM L-glutamine, 50 U/ml Penicillin G and 50 mg/ml Streptomycin Sulphate. Calcium phosphate / DNA precipitate was prepared by adding

1200 mg of plasmid DNA harbouring the gene for human IL-12 and 3000 ml of 2.5 M CaCl2 in sterile 1xTE (10mM Tris and 1 mM EDTA pH 7.3 (Sigma)) to a final volume of 30 ml (solution A). Solution A was added drop-wise to 30 ml of 2 x HEPES Buffered Saline (273.8 mM NaCl, 10 mM KCl, 11.1 mM (+)-D-Glucose, 41.96 mM HEPES free acid and 1.5 mM Na2HPO4.7H2O pH 7.06-7.12 (Sigma)) (solution B) with a 10 ml pipette. During the course of addition, bubbles were gently blown through solution B. The mixture was incubated at 25°C for 20 minutes. 12 ml of the mixture was added drop-wise to each plate. After 4 hours the medium containing the transfection mixture was removed and 100 ml of DMEM/F12 supplemented with 10% (v/v) DCS, 4 mM L-glutamine, 50 U/ml Penicillin G and 50 mg/ml Streptomycin Sulphate, and a final concentration of 3.5 mM HCl, with the medium having a final pH of 7, was added to each plate. The plates were incubated at 37°C and 5% CO2 overnight.

At day 2, the cell culture supernatant was discarded. The contents in the plates were washed twice with 50 ml of DMEM/F12 medium per plate and 100 ml of fresh serum-free DMEM/F12 medium supplemented with 40 mM N-acetyl-D-mannosamine (New Zealand Pharmaceuticals), 10 mM L-Glutamine (Amresco), 0.5 g/L Mannose (Sigma) and 1% (v/v) Penicillin-Streptomycin was added to each plate. The plates were incubated at 37 °C and 5% CO2 overnight.

At day 3, the cell culture supernatant was collected and 100 ml fresh serum-free DMEM/F12 medium supplemented with 40 mM N-acetyl-D-mannosamine, 10 mM L-Glutamine, 4.1 g/L Mannose, and 50 U/ml Penicillin G and 50 mg/ml Streptomycin Sulphate was added to each plate. The plates were incubated at 37 °C and 5% CO2 overnight. 1 mM PMSF and 5 mM EDTA were added to the collected cell culture supernatant and the mixture was stored at 4 °C.

At day 4, the cell culture supernatant was collected. 1 mM PMSF and 5 mM EDTA was added to the collected cell culture supernatant and combined with the day 3 collection before particulate removal using a 0.45 micron low-protein binding filter (Durapore, Millipore). The mixture was either stored at 4°C or used immediately. For long-term storage, the supernatant was kept at -70°C.

(b) Isolation and Purification of IL-12

Two different methods were used as the initial step in the purification of IL-12

1. dye-ligand chromatography, and
2. tangential flow filtration (TFF).

In the process of dye-ligand chromatography (DLC), a library of immobilised reactive dye was used to screen IL-12 for efficient binding and release in a batch purification microtitre format. Suitable dye-protein combinations were then tested in a small-scale column format.

In small-scale purification, 5 ml samples of thawed cell culture supernatant were passed through 0.5 ml dye-ligand columns at pH 6. In this optimisation step optimal reactive dye-cytokine combinations were selected for maximal recovery in fractions for up scaling in bulk DLC.

For bulk scale DLC reactive dye number 63 High (Zymatrix) was selected as the reactive dye with the best binding and elution properties for IL-12. The filtered cell culture supernatant was pH adjusted to 6 with a final concentration of 20 mM MES (Sigma) and 5 mM MgCl2 (Sigma) pH6 and passed under gravity flow over 4.0 ml or 8.0 ml column bodies (Alltech, Extract Clean Filter columns) with 3 ml or 6 ml respectively of DLC resin pre-equilibrated to pH 6 with 50 mM MES/5 mM MgCl2. The column was washed with Buffer A (20 mM MES/5 mM MgCl2 pH 6) until fractions were free of protein as monitored by colourmetric protein assay (Biorad protein assay). IL-12 was eluted using three elution buffers in the following order:

Elute 1:	Buffer C (50 mM Tris-Cl/10 mM EDTA pH 8)
Elute 2:	EN1.0 (50 mM Tris-Cl/10 mM EDTA/1.0 M NaCl pH 8)
Elute 3:	EN2.0 (50 mM Tris-Cl/10 mM EDTA/2.0 M NaCl pH 8)

The eluted fractions were assayed by silver-stained 1D SDS PAGE using 4 – 20 % Tris-Glycine gels (Invitrogen). IL-12 was found to bind to reactive dye 63 High and was found to elute in Buffer EN1.0. DLC Fractions containing IL-12 were pooled for desalting prior to cation exchange chromatography. The concentrated sample was then desalted using a fast desalting column (HiPrep 26/10 Desalting, (Pharmacia)) at a flow rate of 4 ml/min into 50 mM MES pH 5.6 (Sigma).

The alternative first step involved the use of tangential flow filtration (TFF). In this process, one litre of filtered cell culture supernatant was concentrated 10 fold using a TFF device (Pelicon XL, Ultracell, Millipore). The sample was pumped at 150 ml/min across 150 cm2 of regenerated cellulose membrane, with a nominal molecular weight cut-off of 5 KDa until the sample had concentrated down to a volume of 100 ml. The concentrated sample was diafiltered by the addition of an equal volume of buffer 50 mM MES pH 5.6 followed by another concentration down to 100 ml. This diafiltration step was repeated twice with a final concentration to 100 ml. The concentrated diafiltered sample was then filtered through a 0.45 micron low-protein binding filter (Durapore, Millipore).

The IL-12-containing fractions from either DLC or TFF methods were then passed over a cation exchange column (Bio-Rad Laboratories, Uno S6) pre-equilibrated to pH 5.6 with 50 mM MES pH 5.6 (Sigma). The bound IL-12 was then eluted from the column with a optimised gradient from 50 mM MES pH 5.6 to 50 mM MES pH 6.5 containing 1 M NaCl at a flow rate of 2ml/min. The resulting fractions were analysed for apparent molecular weight and level of purity by silver-stained 1D SDS PAGE using 4 – 20 % Tris-Glycine gels (Invitrogen). Fractions containing IL-12 were pooled and concentrated to less than 1 ml for size exclusion chromatography using a centrifugal filter device (Amicon Ultra, Viva Spin).

Size exclusion chromatography was performed on the concentrated fractions using Superdex 200 preparative grade 16/70 (Pharmacia, Uppsala, Sweden) column. An isocratic flow of 1 %(w/v) Ammonium Bicarbonate was used at a flow rate of 1 ml/min. Total run time was 130 min with peaks eluting between 40 and 100 minutes. The eluted fractions

were assayed by silver-stained 1D SDS PAGE using 4 – 20 % Tris-Glycine gels (Invitrogen). The peak eluting at approximately 70 minutes was found to contain IL-12.

The purified IL-12 was found to have two subunits with an apparent MW of around 38 kDa and 46 kDa and to be at least 95 % pure as assessed by silver stained SDS PAGE using 4 – 20 % Tris-Glycine gels (Invitrogen) . The final concentration of the IL-12 was found to be 1660 μg/ml as estimated by absorption at 280 nm using a molar extinction co-efficient of 81330 M-1 cm-1.

EXAMPLE 3

(a) Characterization of IL-12 by Two-Dimensional Polyacrylamide Electrophoresis

The sample collected from Example 2 was buffer exchanged by dialysis or desalting column (Pharmacia HR 10/10 Fast Desalting Column) into repurified (18 MOhm) water and dried using a SpeedVac concentrator. Alternatively, the sample underwent precipitation, for example, TCA precipitation, using methods known in the art. The dried sample was then re-dissolved into 240μl MSD buffer (5M urea, 2M thiourea, 65mM DTT, 2% (w/v) CHAPS, 2% (w/v) sulfobetaine 3-10, 0.2% (v/v) carrier ampholytes, 40mM Tris, 0.002% (w/v) bromophenol blue, water). The samples were then reduced and alkylated by incubation in 5mM tributylphosphine, 1M acrylamide at room temperature for 1 hour then centrifuged at 15000g for 8 minutes.

Isoelectric focusing (IEF) was performed using either precast 11 cm or precast 17 cm gel pH 3-10 immobolised pH gradient IEF strips (BioRad or Amersham). The IEF strips were re-hydrated in the sample in a rehydration tray (Amersham) at room temperature until all the solution was taken up by the strips. The IEF strips were placed into the focusing chamber and covered with paraffin oil. IEF was performed (100 V for 1 hour, 200V for 1 hour, 600V for 2 hours, 1000 V for 2 hours, 2000 V for 2 hours, 3500 V for 12 hours and 100 V for up to 12 hours until the strips had reached approxiamtely 35 kV hours in the case of 11cm strips; 85kV hours in the case of 17cm strips (using the same V ramp up procedure)).

Following isoelectric focusing the strips were reduced and alkylated before being applied to a second dimension gel. The strips were incubated in 1 ´ Tris/HCl pH 8.8, 6M urea, 2% (w/v) SDS, 2% (v/v) glycerol, 5mM tributylphosphine (TBP), 2.5% (v/v) acrylamide solution for at least 20 minutes.

The 11cm strips were separated on the second dimension by Criterion pre poured (11 ´ 8cm 1mm thick) 10-20% Tris HCl gradient gels (BioRad). 17cm strips were separated on 17 x 17 cm, 1.5mm thick, self poured 10-20% Tris glycine gradient gels. Precision or Kaleidoscope molecular weight markers (BioRad) were also applied to the gel.

The SDS-PAGE was run using either a Criterion or Protean II electrophoresis system (BioRad) (40mA per gel for approximately 1 hour (until the buffer front was about to run off the end of the gel) for 11 cm gels and 15mA constant current per gel for 21 hours for 17cm gels). The buffer used was 192 mM glycine, 0.1% (w/v) SDS, 24.8 mM Tris base at pH 8.3.

The completed second dimension gels were stained using Deep Purple fluorescent stain. The gels were fixed in a 7.5% acetic acid (v/v) in 10% methanol for a minimum of 1 hour before being incubated in 300mM Na2CO3, 35mM NaHCO3 for 2 x 30 minutes, then incubated in 1:200 dilution Deep Purple stain for at least 1 hour in the dark with agitation. The gels were then destained by 2 x 15 minute incubations in 10% MeOH, 7% HAc. The gel was imaged using a Typhoon imager (Amersham) or a LAS3000 imager (Fuji).

The software ImageJ (http://rsb.info.nih.gov/ij/) was used to analyse the relative intensities of the protein spots on the gel. Densitometry was performed on the spots within a selected area of the gel and a background subtraction was conducted using the appropriate region of the gel lacking protein spots. A volume integration was performed on each protein spot of interest from which the centre of mass for the spot was calculated. Relative percentage intensities were calculated for each protein spot and by normalising the combined value of the intensities of all spots to 100%, the intensity of each protein spot relative to the other spots in the gel was determined.

The charge of the isoforms (pKa values) were determined by measuring the respective distance of the spots from the left side of the gel using ImageJ. Since the relationship between the pI values of the strip and the physical distance of the gel was linear, the pI values corresponding to the different pKa values of the isoform spots were readily determined.

The major protein spots in the resulting gel corresponds to isoforms of the alpha and beta chains of IL-12. The low intensity spots may be represent either alpha or beta chains of IL-12 or low level contaminants, however, these cannot be confirmed by PMF due to the low intensity. Examination of the gel revealed that IL-12 of the present invention contains 22 to 59 isoforms, inclusive of both alpha and beta chains. Table 8 shows key properties of these isoforms: the pI values (± 1.0), the apparent molecular weights (± 20%), and the relative intensities (± 20% of the actual value or ± 2% of the total, whichever is larger). The values listed correspond to the intensity weighted center within the selected area of gel containing the spot and hence, are only reflective of the pI and molecular weight of the protein at one particular reading within the selected area of the gel. Taking into consideration the inherent variability of size and position of protein spots within 2D gels, the pI values for the alpha chain of the IL-12 of the present invention were determined to range from about 3.5 to 9.8 and the pI values of the beta chain of the IL-12 of the present invention were determined to range from about 5.0 to 9.3, based on the values listed in Table 8; and the apparent molecular weights of the alpha chain of the IL-12 of the present invention were determined to range from 20 to 38 kDa and the apparent molecular weights of the beta chain of the IL-12 of the present invention were determined to range from about 27.5 to 55 kDa, based on the values listed in Table 8.

TABLE 8

Molecular weights and pI values of isoforms of IL-12

Spot No	Isoelectric Point (pI)		Molecular Weight (kDa)		Relative Intensity (%) (Normalized Value)	
		Range		Range		Range
2	6.01	±1.00	46.11	±9.22	0.17	±2.00
3	6.01	±1.00	42.75	±8.55	0.53	±2.00
4	6.02	±1.00	38.59	±7.72	0.80	±2.00
5	6.06	±1.00	34.83	±6.97	0.66	±2.00
6	6.26	±1.00	45.14	±9.03	0.30	±2.00
7	6.27	±1.00	42.10	±8.42	0.92	±2.00
8	6.29	±1.00	38.37	±7.67	2.64	±2.00
9	6.32	±1.00	34.80	±6.96	1.64	±2.00
10	6.54	±1.00	45.06	±9.01	0.43	±2.00
11	6.54	±1.00	41.88	±8.38	1.83	±2.00
12	6.55	±1.00	38.15	±7.63	4.74	±2.00
13	6.57	±1.00	34.72	±6.94	3.87	±2.00
14	6.84	±1.00	44.57	±8.91	1.02	±2.00
15	6.84	±1.00	41.41	±8.28	2.48	±2.00
16	6.85	±1.00	38.15	±7.63	5.02	±2.00
17	6.86	±1.00	34.87	±6.97	5.63	±2.00
18	7.16	±1.00	44.01	±8.80	1.67	±2.00
19	7.16	±1.00	41.44	±8.29	2.95	±2.00
20	7.16	±1.00	38.16	±7.63	5.73	±2.00
21	7.17	±1.00	34.76	±6.95	5.64	±2.00
22	7.50	±1.00	43.73	±8.75	0.25	±2.00
23	7.50	±1.00	40.98	±8.20	0.65	±2.00
24	7.51	±1.00	38.03	±7.61	1.33	±2.00
25	7.50	±1.00	35.40	±7.08	1.38	±2.00
26	8.31	±1.00	40.61	±8.12	0.21	±2.00
27	8.30	±1.00	36.89	±7.38	0.33	±2.00

Spot No	Isoelectric Point (pI)		Molecular Weight (kDa)		Relative Intensity (%) (Normalized Value)	
		Range		Range		Range
28	4.32	±1.00	31.88	±6.38	0.09	±2.00
29	4.46	±1.00	32.02	±6.40	0.20	±2.00
30	4.46	±1.00	30.93	±6.19	0.28	±2.00
31	4.61	±1.00	31.79	±6.36	0.72	±2.00
32	4.63	±1.00	30.57	±6.11	1.06	±2.00
33	4.81	±1.00	31.17	±6.23	2.18	±2.00
34	4.82	±1.00	29.72	±5.94	1.73	±2.00
35	5.02	±1.00	30.94	±6.19	2.67	±2.00
36	5.04	±1.00	29.43	±5.89	3.44	±2.00
37	5.04	±1.00	28.34	±5.67	0.65	±2.00
38	5.28	±1.00	30.42	±6.08	3.29	±2.00
39	5.30	±1.00	28.99	±5.80	3.41	±2.00
40	5.29	±1.00	27.90	±5.58	0.90	±2.00
41	5.59	±1.00	30.12	±6.02	3.36	±2.00
42	5.61	±1.00	28.59	±5.72	3.69	±2.00
43	5.61	±1.00	27.16	±5.43	1.14	±2.00
44	6.00	±1.00	29.64	±5.93	2.27	±2.00
45	6.00	±1.00	28.27	±5.65	3.32	±2.00
46	6.01	±1.00	26.78	±5.36	0.87	±2.00
47	6.42	±1.00	29.11	±5.82	1.49	±2.00
48	6.43	±1.00	27.96	±5.59	2.09	±2.00
49	6.44	±1.00	26.49	±5.30	0.85	±2.00
50	6.91	±1.00	28.98	±5.80	0.55	±2.00
51	6.91	±1.00	27.94	±5.59	1.55	±2.00
52	6.92	±1.00	26.42	±5.28	0.49	±2.00
53	7.49	±1.00	28.73	±5.75	0.19	±2.00
54	7.48	±1.00	27.85	±5.57	0.93	±2.00
55	7.50	±1.00	26.37	±5.27	0.31	±2.00

Spot No	Isoelectric Point (pI)		Molecular Weight (kDa)		Relative Intensity (%) (Normalized Value)	
		Range		Range		Range
56	8.15	±1.00	27.54	±5.51	0.61	±2.00
57	8.15	±1.00	26.52	±5.30	0.17	±2.00
58	8.90	±1.00	27.74	±5.55	0.34	±2.00
59	8.89	±1.00	26.65	±5.33	0.17	±2.00
60	9.76	±1.00	37.09	±7.42	2.22	±2.00

(b) One-Dimensional Polyacrylamide Electrophoresis

The collected sample from Example 2 was dried and then re-solubilised into 60ml of 1D sample buffer (10% glycerol, 0.1% SDS, 10mM DTT, 63mM trisTris-HCl; i.e. under reduced conditions) and heated at 100°C for 5 minutes. For PNGaseF treatment, a 30 µL aliquot of the sample was taken and NP40 added to a final concentration of 0.5 %. 5 µL of PNGaseF was added and the sample was incubated at 37 °C for 3 hours. For glycosidase cocktail treatment of the sample, an aliquot was taken and NP40 was added to a final concentration of 0.5%. 1µL of PNGase F, and 1 µL each of Sialidase A (neuramidase), O-Glycanase, β (1-4)-Galactosidase and β-N-Acetylglucosaminidase was added. Treated and untreated samples were incubated at 37 °C for 3 hours. Treated and untreated samples were run on a pre-cast Tris gel, for example, a Tris 4-20% gradient gel (BioRad) or Tris HCl gradient gel (Invitrogen). Precision molecular weight markers (BioRad catalogue number 161-0363) were also applied to the gel. Criterion 4-20% or 18% gels were used for 1D SDS-PAGE (BioRad catalogue numbers: 345-0033 or 345-0024). The SDS-PAGE was run using either a Mini Protean II or a Criterion electrophoresis system (BioRad) at 200 V for approximately 1 h or until the buffer front was about to run off the end of the gel. The buffer used was 192 mM glycine, 0.1% (w/v) SDS, 24.8 mM Tris base at pH 8.3. The completed gels were fixed for at least 30 minutes in 10% MeOH and 7% HAc. The gel was then stained using Sypro Ruby gel stain (BioRad) for at least 3 h and destained with 10% MeOH and 7% HAc for at least 30 minutes. Alternatively the gels were stained using

Deep Purple (Amersham) as per the manufacturers instructions. The gel was imaged using a FX laser densitometer (BioRad) and the appropriate filter.

The apparent molecular weight of the IL-12 of the present invention (as observed by SDS-PAGE) under reduced conditions and following the release of N-linked oligosaccharides (by PNGase treatment) was 20 to 55 kDa. The apparent molecular weight of the IL-12 of the present invention (as observed by SDS-PAGE) under reduced conditions and following the release of N-linked oligosaccharides (by PNGase treatment) and O-linked oligosaccharides (by glycosidase cocktail) was 20 to 55 kDa.

(c) N-Terminal Sequencing

Protein bands are cut from the gel prepared above (two-dimensional gel) and are placed into a 0.5ml tube and 100ml extraction buffer is added (100mM Sodium acetate, 0.1%SDS, 50mM DTT pH 5.5). The gel slices are incubated at 37°C for 16 hours with shaking. The supernatant is applied to a ProSorb membrane (ABI) as per the manufacturers instruction and sequenced using an automated 494 Protein Sequencer (Applied Biosystems) as per the manufacturers instructions. The sequence generated is used to confirm the identity of the proteins to be human IL-12.

(d) Peptide Mass Fingerprinting

Protein bands were cut from the gel prepared above (either from a two-dimensional gel or a one-dimensional gel) and washed with 25ml of wash buffer (50% acetonitrile in 50mM NH4HCO3). The gel pieces were left at room temperature for at least 1 hour and dried by vacuum centrifugation for 30 minutes. The gel pieces and 12ml of trypsin solution (20mg trypsin, 1200ml NH4HCO3) was placed in each sample well and incubated at 4°C for 1 hour. The remaining trypsin solution was removed and 20ml 50mM NH4HCO3 was added. The mixture was incubated overnight at 37°C with gentle shaking. The peptide samples were concentrated and desalted using C18 Zip-Tips (Millipore, Bedford, MA) or pre-fabricated micro-columns containing Poros R2 (Perseptive Biosystems, Framingham, MA) chromatography resin. Bound peptides were eluted in 0.8 µl of matrix solution (α-

cyano-4-hydroxy cinnamic acid (Sigma), 8 mg/ml in 70% acetonitrile / 1% formic acid) directly onto a target plate. Peptide mass fingerprints of tryptic peptides were generated by matrix-assisted laser desorption / ionisation time-of-flight mass spectrometry (MALDI-TOF MS) using a Perseptive Biosystems Voyager DE-STR. Spectra were obtained in reflectron mode using an accelerating voltage of 20 kV. Mass calibration was performed using trypsin autolysis peaks, 2211.11 Da and 842.51 Da as internal standards. Data generated from peptide mass fingerprinting (PMF) was used to confirm the identity of the protein. Searches (primarily of Homo sapien (Human) and mammalian entries) were performed in databases such the SWISS-PROT and TrEMBL, via the program PeptIdent (www.expasy.ch/tools/peptident.html). Identification parametres included peptide mass tolerance of 0.1Da, a maximum of one missed tryptic cleavage per peptide, and the methionine sulfoxide and cysteine-acrylamide modifications. Identifications were based on the number of matching peptide masses and the total percentage of the amino acid sequence that those peptides covered, in comparison to other database entries. Generally, a peptide match with at least 30% total sequence coverage was required for confidence in identification, but very low and high mass proteins, and those resulting from protein fragmentation, may not always meet this criterion, therefore requiring further identification.

Where inconclusive or no protein identification could be obtained from MALDI-TOF PMF analysis, the remaining peptide mixture or the identical spot cut from a replicate gel was subjected to tryptic digest and analysed by electrospray ionization tandem MS (ESI-MS/MS). For ESI-MS/MS, peptides were eluted from Poros R2 micro-columns in 1-2 *l of 70% acetonitrile, 1% formic acid directly into borosilicate nanoelectrospray needles (Micromass, Manchester, UK). Tandem MS was performed using a Q-Tof hybrid quadrupole / orthogonal-acceleration TOF mass spectrometer (Micromass). Nanoelectrospray needles containing the sample were mounted in the source and stable flow obtained using capillary voltages of 900-1200V. Precursor ion scans were performed to detect mass to charge ratio (m/z) values for peptides within the mixture. The m/z of each individual precursor ion was selected for fragmentation and collided with argon gas using collision energies of 18-30eV. Fragment ions (corresponding to the loss of amino acids from the precursor peptide) were recorded and processed using MassLynx Version 3.4

(Micromass). Amino acid sequences were deduced by the mass differences between y- or b-ion 'ladder' series using the program MassSeq (Micromass) and confirmed by manual interpretation. Peptide sequences were then used to search the NCBI and TrEMBL databases using the program BLASTP "short nearly exact matches". A minimum of two matching peptides were required to provide confidence in a given identification.

The identity of the gel spots were confirmed to be IL-12.

Further, an observed 1Da shift in the masses of tryptic peptides would indicate the asparagine residues (N) of NX(S/T/C) motifs found in the theoretical amino acid sequence of human IL-12 are modified to aspartic acid (D), consistent with the known ability of PNGase F to induce an N to D residue modification upon removal of associated N-linked oligosaccharides. This observation would allow for the identification of confirmed sites of N-glycosylation of the IL-12 of the present invention.

EXAMPLE 4

Analysis of Amino Acid, Monosaccharide, Oligosaccharide, Phosphate, Sulfate and Isoform Composition of IL-12

(a) Preparation of Samples for Amino Acid, Monosaccharide, Oligosaccharide, Phosphate, Sulfate and Isoform Analysis

For characterisation of monosaccharide and oligosaccharide glycosylation and phosphate and sulfate post-translational modifications, the saccharides are first removed from the polypeptide backbone by hydrolytic or enzymatic means. The sample buffer components are also removed and exchanged with water to avoid inhibition of the hydrolysis and enzymatic reactions before analysis began. A solution of purified IL-12 in PBS is dialysed extensively against 4 litres of deionised ultrafiltered water (18 MOhm) for four days with two changes per day using a regenerated cellulose dialysis membrane (Spectrapore) with a nominal molecular weight cut-off (NMWC) of 5 KDa. After dialysis the solution is dried using a Savant Speed Vac (New York, USA). The dried down sample is then resuspended

in 2 ml of deionised ultrafiltered water (18 MOhm) and divided into aliquots for the various analyses.

(b) Analysis of Amino Acid Composition by the Gas Phase Hydrolysis Method

Amino acids in the samples are analysed using precolumn derivatisation with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC). The stable fluorescent amino acid derivatives are separated and quantified by reversed phise (C18) HPLC. The procedure employed is based on the Waters AccQTag amino acid analysis methodology.

Three samples of the IL-12 preparation are taken and dried in a Speed Vac . The dried samples are then hydrolysed for 24 hours at 110°C. After hydrolysis the samples are dried again before derivatisation as follows. The dried samples are re-dissolved in 10 μL of an internal amino acid standard solution (α-aminobutyric acid, AABA), 35 μL of borate buffer is added followed by 15 μL of AQC derivatising reagent. The reaction mix is heated at 50°C for 12 minutes in a heating block. The derivatised amino acid sample is transferred to the autosampler of a HPLC system consisting of a Waters Alliance 2695 Separation Module, a Waters 474 Fluorescence Detector and a Waters 2487 Dual λ Absorbance Detector in series. The control and analysis software is Waters Empower Pro Module (Waters Corporation, Milford. MA, USA). The samples are passed over a Waters AccQTag column (15cm x 3.9mm ID) using chromatographic parameters (i.e. suitable eluents and gradient flows) known in the art.

(c) Analysis of Neutral and Amino Monosaccharide composition

Two samples of the IL-12 preparation are taken and treated in two different ways to liberate monosaccharides. Each treatment, as described below, is performed in triplicate.

1. Hydrolysed with 2 M trifluroacetic acid (TFA) heated to 100° C for four hours to release neutral sugars (galactose, glucose, fucose and mannose).
2. Hydrolysed with 4 M HCl heated to 100° C for four hours to release amino sugars (N-acetyl-galactosamine, N-acetyl-glucosamine).

All of the hydrolysates are lyophilised using a Speed Vac system, redissolved in 200 µl water containing 0.8 nmols of internal standard. For neutral and amino sugars the internal standard is 2-deoxy-glucose. The samples are then centrifuged at 10,000 g for 30 minutes to remove protein debris. The supernatant is transferred to a fresh tube and analysed by high pH anion exchange chromatography using a Dionex LC 50 system with a GP50 pump and an ED50 pulsed amperometric detector (Dionex Ltd). Analysis of neutral and amino sugars is performed using a Dionex CarboPac PA-20 column. Elution is performed with an isocratic hydroxide concentration of 10 mM over 20 minutes. This is achieved with the Dionex EG50 eluent generation system.

(d) Analysis of Acidic Monosaccharide Composition

A sample of the IL-12 preparation is taken and treated in the following way to liberate sialic acid monosaccharides. The treatment is performed in triplicate. The sample is hydrolysed with 0.1 M TFA at 80° C for 40 minutes to release N-Acetyl and N-Glycolyl neuraminic acid. The hydrolysates are lyophilised using a Speed Vac, redissolved in 200 µl water containing 0.8 nmols of internal standard. For sialic acid analysis the internal standard is lactobionic acid. Samples are then centrifuged at 10,000 g for 30 minutes. The supernatant is transferred to a fresh tube and analysed by high pH anion exchange chromatography using a Dionex LC 50 system with a GP50 pump and an ED50 pulsed amperometric detector. Analysis of sialic acids is performed using a Dionex CarboPac PA1 using using chromatographic parameters (i.e. suitable eluents and gradient flows) known in the art.

(e) Analysis of Oligosaccharide Composition

For analysis of oligosaccharide composition two samples of the IL-12 preparation were taken in triplicate and treated in one of the following ways:

1. Release of N-linked oligosaccharides was achieved with the enzyme Peptide-N4-(N-acetyl-β-D-glucosaminyl) Asparagine Amidise (PNGase). First, a

1/5th volume of denaturation solution (2 % SDS (Sigma)/1 M β-mercaptoethanol (Sigma)) was added to the sample. The sample was heated to 100 °C for 5 minutes. A 1/10th volume of 15 % Triton-X100 (Sigma) was added to the sample. The sample was mixed gently and allowed to cool to room temperature. 25 Units of PNGase (Sigma) was added and incubated overnight at 37°C.

2. Release of O-linked oligosaccharides was achieved by the process of β-elimination. First, a 1/2 volume of 4M sodium borohydride (freshly made) (Sigma) solution was added to the sample. A ½ volume of 0.4 M NaOH (BDH, HPLC grade) was added to the sample. The sample was incubated at 50° C for 16 hours. The sample was cooled on ice and a ½ volume of 0.4 M acetic acid (Sigma) was added to the sample.

Both the N-linked and O-linked samples were further processed to remove buffer components using a Carbo Pac graphitised carbon SPE column. The column equilibration and elution conditions were was follows:

First, the column was pre-equilibrated with 1 column volume of 80 % acetonitrile (Sigma) followed by two column volumes of H_2O. The sample was loaded under gravity flow and the column washed with two column volumes of H_2O. To elute neutral oligosaccharides 2 ml of 50 % acetonitrile was applied to the column. To elute acidic oligosaccharides 2 ml of 50 % acetonitrile/0.1% formic acid was applied to the column. Any remaining oligosaccharides were eluted by the addition of 2 ml of 80 % acetonitrile/0.1 % formic acid. Individual fractions from the SPE columns containing the neutral or acidic N-linked oligosaccharides and the neutral or acidic O-linked oligosaccharides were dried down to completion using a Speed Vac. The samples were redissolved in 200 µl water and analysed by high pH anion exchange chromatography using a Dionex LC 20 system with a GP50 pump and an ED50 pulsed amperometric detector. Analysis of neutral and acidic oligosaccharides was performed using a CarboPac PA100 column and chromatographic parameters (i.e. suitable eluents and gradient flows) known in the art.

(f) Analysis of Sulfate and Phosphate Composition

Sulfate/phosphate analysis is performed essentially by the method described by Harrison and Packer (Harrison and Packer *Methods Mol Biol 125*:211-216, 2000). A sample of the IL-12 preparation is taken for sulfate/phosphate analysis and hydrolysed in 4 M HCl at 100 °C for four hours. The HCl is removed by drying the samples in a Speed Vac system. Samples are then redissolved into 200 µl H_2O. A suitable volume of sample is injected onto a Dionex LC 50 system with a GP50 pump and a ED50 conductivity detector. Separation is performed by a Dionex IonPac IS11 Anion exchange column using chromatographic parameters (i.e. suitable eluents and gradient flows) known in the art.

(g) Further Separation of Protein Isoforms

Further separation of IL-12 isoforms is performed using a pellicular anion exchange column. A suitable volume of sample, for example, 24 µl, is separated through a ProPac SAX-10 column (Dionex Ltd) using a Dionex SUMMIT system with UV-Vis detector (Dionex Ltd). Separation is performed using suitable eluents and gradients known in the art. IL-12 isoforms are found to elute in a pattern of distinct peaks.

EXAMPLE 5

Glyco Mass Fingerprinting

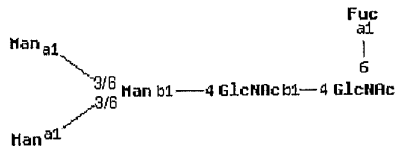
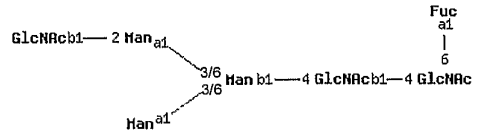
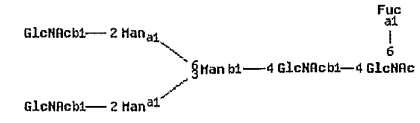
The IL-12 of the present invention was separated using 2D gel electrophoretic techniques as in Example 3 and blotted onto polyvinyl difluorethane (PVDF) membrane. The spots were stained using one of a standard array of protein stains (Colloidal Coomassie Blue, Sypro Ruby or Deep Purple), and the isoform relative amounts quantified using densitometry algorithms. The individual spots were excised and treated with an array of deglycosylating enzymes and/or chemical means, as appropriate, to remove the oligosaccharides present according to methods described in this document. Once removed, the N-linked, O-linked and C-linked oligosaccharides were separated and analysed on a liquid chromatography-electrospray mass spectrometry system (LC-MS) using a graphitised carbon column and organic solvent (MeCN) gradient elution system. The

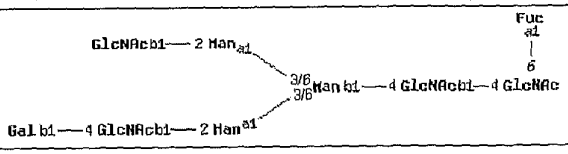
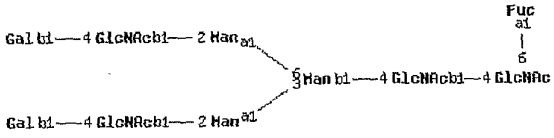
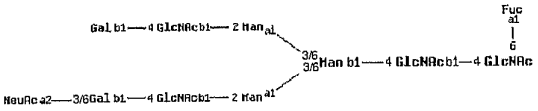
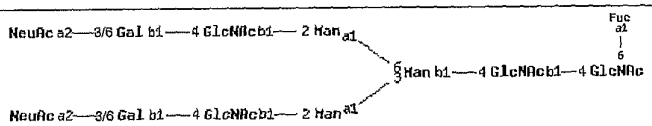
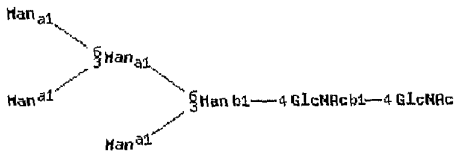
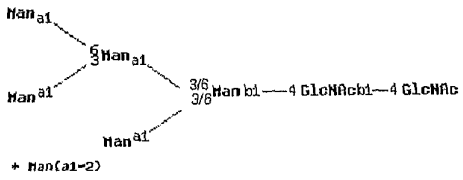
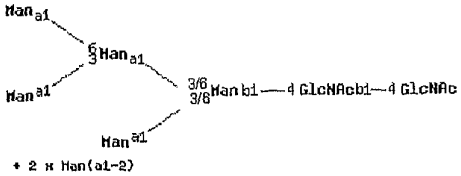
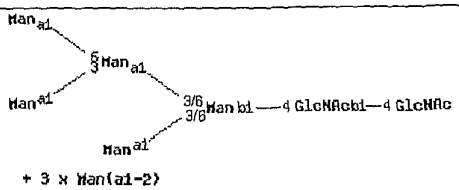
generated peak profile that was generated was a "fingerprint" of the oligosaccharides present on the isoforms. Furthermore, the mass spectrometry system simultaneously generates information on the mass of each of the sugars present in the sample which was used to identify their structure through pattern matching with the GlycoSuite database. In addition, individual mass peaks were fragmented multiple times to give MS^n spectra. These fragments allowed structural prediction using the GlycosidIQ software package (Tables 9 and 10).

The above separation, deglycosylation and analysis procedures were repeated using corresponding recombinant human IL-12 expressed in non-human cell systems, namely CHO cells (CellSciences) and *Sf*21 cells (R&D Systems) and the respective glyco mass fingerprints and corresponding structural predictions were found to be significantly different (Tables 9 and 10).

TABLE 9

Predicted structures of N-glycans present in the IL-12 of the present invention and recombinant human IL-12 expressed in CHO and *Sf*21cells

Predicted structure	MW (Da)	% of total oligosaccharides		
		IL-12 PI	CHO IL-12	*Sf*21 IL-12
	1056			64
	1259			3
	1462		7	

Predicted structure	MW (Da)	% of total oligosaccharides		
		IL-12 PI	CHO IL-12	*Sf*21 IL-12
	1624		7	
	1786		4	
	2077		19	
	2368	35	19	
	1234		11	0.3
	1396		9	4.7
	1558	22	9	5
	1720	16	8	21

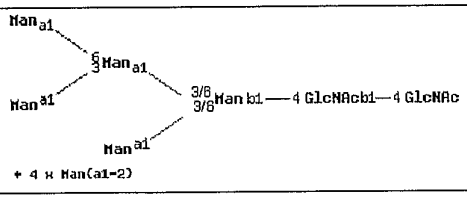
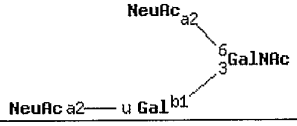
Predicted structure	MW (Da)	% of total oligosaccharides		
		IL-12 PI	CHO IL-12	*Sf*21 IL-12
	1882	27	7	2

TABLE 10

Predicted structures of O-glycans present in the IL-12 of the present invention and recombinant human IL-12 expressed in CHO cells using GlycosidIQ

Predicted structure	MW (Da)	% of total oligosaccharides	
		IL-12 PI	CHO IL-12
Gal b1—3 GalNAc + NeuAc(a2- 3/6)	674	60	47
	965	40	53

C-mannosylation was found in tryptic digests of both IL-12 of the present invention and recombinant human IL-12 expressed in CHO cells but not in an additional sample containing recombinant human IL-12 expressed in *Sf*21 cells (Table 11). The site of C-mannosylation was confirmed to be Trp-319 for both IL-12 of the present invention and recombinant human IL-12 expressed in CHO cells by digestion with trypsin and V8. Trp-321 was not found to be glycosylated.

TABLE 11

Confirmed sites of C-mannosylation in various recombinant human IL-12 molecules

Mannosylated tryptophan	IL-12 of the present invention	CHO expressed human IL-12	*Sf*21expressed human IL-12
Trp-319	yes	yes	no
Trp-321	no	no	no

At a structural level, such results indicate differing patterns of glycan structures present on the IL-12 of the present invention and the corresponding recombinant human IL-12 molecules expressed in non-human systems such as CHO and *Sf*21 cells.

EXAMPLE 6

Fluorophore Assisted Carbohydrate Electrophoresis

Oligosaccharide profiles of the IL-12 are derived using the fluorophore assisted carbohydrate electrophoresis protocols (FACE protocols). The oligosaccharides from the IL-12 are hydrolysed from the amino acid backbone using ammonium hydroxide and subsequently labelled using the fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS). Polyacrylamide gel electrophoresis is used to separate the species and standards used to identify an oligosaccharide profile that is typical of the target molecule. Further, the oligosaccharides are identified using matrix assisted laser desorption and ionisation – time of flight mass spectrometry (MALDI-TOF) relying on the fluorophore and a specific matrix to ionise each sugar. The mass of each sugar is determined and potential structures identified using the GlycoSuite database. The potential sugar structures are further characterised by tandem mass spectrometric techniques, which allows partial or complete characterisation of the oligosaccharides present and their relative amounts. Further, the process is repeated using the isoforms identified by 2D gel electrophoresis to generate a profile of the oligosaccharides present on each of the isoforms isolated.

EXAMPLE 7

QCM and SPR

The binding characteristics and activity of the IL-12 is determined using either quartz crystal microbalance (QCM) or surface plasmon resonance (SPR). In both cases a suitable receptor for the molecule is bound to a wafer using the chemistry described by the manufacturer. The target molecule is dissolved into a suitable biological buffer and allowed to interact with the receptor on the chip by passing the buffer over it. Changes in

the total protein mass on the surface of the wafer are measured either by change of oscillation frequency (in the case of QCM) or changes in the light scattering qualities of the chip (in the case of SPR). The chip is then treated with the biological buffer alone to observe the release of the target molecule back into solution. The rate at which the receptors reach saturation and complete disassociation is then used to calculate the binding curve of the target molecule.

EXAMPLE 8

Generation of a Transgenic Host Cell Line

(a) Transgenic Host Cell Line with alpha-2,6-sialyltransferase

The cDNA coding for alpha-2,6-sialyltransferase (alpha 2,6ST) is amplified by PCR from poly(A)-primed cDNA. The PCR product is ligated into a suitable vector, for instance pIRESpuro4 or pCEP4, to generate an alpha 2,6ST plasmid. The cloned cDNA is sequenced and its identity verified by comparison with the published alpha-2,6ST cDNA sequence. DNA sequencing is performed using known methods.

Mammalian host cells, including cell clones of the same lineage that express high levels of target molecule (cell line-target molecule) are transfected with the alpha 2,6ST plasmid, which also carries an antibiotic resistance marker. Selection of stably transfected cells is performed by incubaton of the cells in the presence of the antibiotic; colonies of antibiotic-resistant cells that appear subsequent to transfection are pooled and examined for intracellular alpha 2,6ST activity. To isolate individual cell clones expressing alpha 2,6ST, cell pools are cloned by a limiting dilution process as described by Kronman (*Gene 121*: 295–304, 1992). Individual cell clones are chosen at random, cells expanded and clones tested for alpha 2,6ST activity.

Cell pellets are washed, resuspended in lysis buffer and left on ice prior to sonication. The cell lysate is centrifuged and the clear supernatant is assayed for protein concentration (via known methods) and sialyltransferase activity. Sialyltransferase activity is assayed by

known methods, for example the method detailed by Datta *et al.* (*J Biol Chem 270:*1497–1500, 1995).

Expressed IL-12 is purified from high-expressing alpha 2,6ST cell line-target molecule cells and subjected to *in vitro* and/or *in vivo* half-life bioassays (see Example 10). IL-12 from high-expressing alpha 2,6ST cell displays an increased *in vitro* and/or *vivo* half-life in comparison to IL-12 derived from the same parent cell line without any subsequent transgene manuipulation or IL-12 derived from other cell lines.

(b) Transgenic Host Cell Line with fucosyltransferase

The cDNA coding for a fucosyltransferase (FT) such as FUT1, FUT2, FUT3, FUT4, FUT5, FUT6, FUT7, FUT8, FUT9, FUT10, FUT11 is amplified by PCR from poly(A)-primed cDNA. The PCR product is ligated into a suitable vector, for instance pIRESpuro4 or pCEP4, to generate an alpha 2,6ST plasmid. The cloned cDNA is sequenced and its identity verified by comparison with the published FT cDNA sequence. DNA sequencing is performed using known methods.

Human host cells, including cell clones of the same lineage that express high levels of IL-12 (cell line-target molecule) are transfected with the FT plasmid, which also carries an antibiotic resistance marker. Selection of stably transfected cells is performed by incubation of the cells in the presence of the antibiotic; colonies of antibiotic-resistant cells that appear subsequent to transfection are pooled and examined for intracellular FT activity. To isolate individual cell clones expressing FT, cell pools are cloned by a limiting dilution process as described by Kronman (*Gene 121*: 295–304, 1992); Individual cell clones are chosen at random, cells expanded and clones tested for FT activity.

Cell pellets are washed, resuspended in lysis buffer and left on ice prior to sonication. The cell lysate is centrifuged and the clear supernatant is assayed for protein concentration (via known methods) and FT activity. FT activity is assayed by known methods, for example the method detailed by Mas *et al.* (*Glycobiology 8(6)*: 605-13, 1998).

Expressed IL-12 is purified from high-expressing FT cell line-target molecule cells. A Lewis x-specific antibody, such as L5 and a sialyl Lewis x-specific antibody such as KM93, HECA493, 2H5 or CSLEX are used to test the presence of Lewis x or sialyl Lewis x structures according to methods known in the art, for example, as detailed in Lucka *et al.* (*Glycobiology 15(1)*:87, 2005). Alternatively, the presence of Lewis x or sialyl Lewis x structures may be detected by treating the sample with appropriate glycosidases and detecting the effect of the glycosidases on parameters such as mass using MS or retention time using HPLC. Glyco mass fingerprinting, as described in Example 5, may also be employed to predict the presence of Lewis x or sialyl Lewis x structures.

EXAMPLE 9

Differential Gene Expression

Differences in gene expression can be analyzed using a target cell line of the IL-12. The target cells are grown to the appropriate density and treated with a range of concentration of target molecule or buffer control for a number of hours, for instance, 72 hours.

At various time points RNA is harvested, purified, and reverse transcribed according to Affymetrix protocols. Labelled cRNA (e.g. biotin labelled) is then prepared and hybridised to expression arrays e.g. U133 GeneChips. Following washing and signal amplification, the GeneChips are scanned using a GeneChip scanner (Affymetrix) and the hybridisation intensities and fold change information at various time points is obtained using GeneChip software (Affymetrix).

The IL-12 induces unique gene expression and results in different mRNA profiles upon comparison with profiles induced by IL-12 produced from different sources e.g. *E. coli*, yeast or CHO cells.

EXAMPLE 10

Determining the Half-Life of the Target Molecule of the Present Invention

The half-life of IL-12 is determined in an *in vitro* system. Composition containing IL-12 is mixed into human serum/plasma and incubated at a particular temperature for a particular time (e.g. 37 degrees for 4h, 12h etc). The amount of target molecule remaining after this treatment is determined by ELISA methods or dot blot methods known in the art. The biological activity of the remaining target molecule is determined by performing a suitable bioassay chosen by a person skilled in the relevant art. The serum chosen may be from a variety of human blood groups (eg A, B, AB, O etc.).

The half-life of IL-12 is also determined in an *in vivo* system. Composition containing IL-12 is labelled by a radioactive tracer (or other means) and injected intravenously, subcutaneously, retro-orbitally, intramuscularly or intraperitonally into the species of choice for the study, for instance, mouse, rat, pig, primate or human. Blood samples are taken at time points after injection and assayed for the presence of IL-12 (either by ELISA methods, dot blot methods or by trichloroacetic acid (TCA)-precipitable label e.g. radioactive counts). A comparison composition consisting of IL-12 produced from other sources eg *E. coli*, yeast, or CHO cells can be run as a control.

EXAMPLE 11

In Vivo Studies using the IL-12 as Immunotherapy in the Eradication of Established Tumors

The individual subjects of the *in vivo* studies described herein are warm-blooded vertebrate animals, which includes humans.

The clinical trial is subjected to rigorous controls to ensure that individuals are not unnecessarily put at risk and that they are fully informed about their role in the study.

Preferably to account for the psychological effects of receiving treatments, the trial is conducted in a double-blinded fashion. Volunteers are randomly assigned to existing or IL-

12 treatment groups. Furthermore, the relevant clinicians are blinded as to the treatment regime administered to a given subject to prevent from being biased in their post-treatment observations. Using this randomization approach, each volunteer has the same chance of being given either the new treatment or the existing treatment. In particular, the "IL-12 treatment" comprises a combination of IL-12 of the present invention and cyclophosphamide.

Volunteers exhibit a confirmed diagnosis of metastatis exhibiting an immunogenic phenotype, for example, particular lung metastases, ascites tumors, mammary carcinomas, renal cell carcinomas and colorectal carcinomas. Existing (non-surgical) treatments for such disease states include the administration of 5FU, IFNa2b or cyclophosphamide (alone).

Volunteers receive either the IL-12 or existing treatments for an appropriate period with biological parameters associated with the disease state being measured at the beginning (baseline measurements before any treatment), end (after the final treatment), and at regular intervals during the study period.

Such measurements include the levels of IL-12 and other co-treatment molecules in body fluids, tissues or organs compared to pre-treatment levels. Other measurements include, but are not limited to, indices of the disease state or condition being treated, body weight, blood pressure, serum titres of pharmacologic indicators of disease such as specific disease indicators or toxicity as well as ADME (absorption, distribution, metabolism and excretion) measurements. Patients are seen by a clinician using a standard time-course, for example, every 2-3 months for 1 year and then every 6 months for 2 years or at any time when the physical examination or symptoms are suspicious for tumor progression.

Information recorded for each patient includes age (years), gender, height (cm), family history of disease state or condition (yes/no), motivation rating (some/moderate/great) and number and type of previous treatment regimens for the indicated disease or condition.

Volunteers taking part in this study are adults aged 18 to 65 years and roughly an equal number of males and females participate in the study. Volunteers with certain characteristics are equally distributed for existing and IL-12 treatments. In general, the volunteers treated with existing therapies have little or no response to treatment, whereas the volunteers treated with the IL-12 therapy show positive trends in their disease state or condition index at the conclusion of the study.

EXAMPLE 12

(a) Comparing the bioactivities of IL-12 and human IL-12 expressed using non-human systems using a lymphoblast proliferation assay

IL-12 has been reported to induce proliferation in PHA activated human peripheral blood lymphoblasts. Human peripheral blood mononuclear cells (PBMC) were isolated using the commercially available Lymphoprep solution (Axis-Shield, Norway). PBMCs are activated with 10ug/ml PHA for a total of 4 days, during which time the cells became lymphoblasts. On day 3, the cultures were split and 10 ng/ml IL-2 was added to promote lymphoblast proliferation. On day 4, the lymphoblasts were harvested, washed and prepared for IL-12 treatment.

In a 96-well plate, 40,000 lymphoblasts per well were treated with 0-500 ng/ml IL-12 of the present invention for 48 hours at 37°C.

Cell number was then measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega). In this assay a tetrazolium compound MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) in the presence of an electron coupling reagent (phenazine methosulfate) is bioreduced by cells into a formazan product. The concentration of the formazan was determined by reading the absorbance of the resultant solution at 490nm by a spectrophotometer (E max precision microplate reader, Molecular Devices).

ED_{50} was calculated after curve fitting the absorbance and the IL-12 concentration values using a 4 parameter equation.

The above assay was repeated using human IL-12 expressed in CHO cells (Peprotech Catalogue # 200-12), and the corresponding ED_{50}s were found to be significantly different. Specifically, the ED_{50} of IL-12 of the present invention was 0.05 – 0.2 ng/ml, up to 48-fold lower than the ED_{50} of the human IL-12 expressed in CHO cells (0.1 - 2.4 ng/ml; Figure 1). Thus, the IL-12 of the present invention displays up to 48-fold greater lympho-proliferative activity than human IL-12 expressed in CHO cells.

(b) Comparing the bioactivities of IL-12 and human IL-12 expressed using non-human systems to activate STAT4 in PHA-activated human peripheral lymphoblasts

IL-12 has been reported to induce activation of STAT4 in PHA activated human peripheral blood lymphoblasts. Human peripheral blood mononuclear cells (PBMC) were isolated using the commercially available Lymphoprep solution (Axis-Shield, Norway). PBMCs were activated with 10ug/ml PHA for a total of 4 days, during which time the cells became lymphoblasts. On day 3, the cultures were passaged and 10 ng/ml IL-2 (R&D Systems # 202-IL) was added to promote lymphoblast proliferation.

On day 4, the lymphoblasts were harvested, washed and prepared for IL-12 treatment.

Cells were incubated for 0-60 min with 50 ng/ml IL-12 of the present invention. Cells were then lysed and total protein extracts were separated by SDS-PAGE followed by western blotting according to standard chromatography techniques. The western blots were probed with an anti-phospho STAT4 antibody to measure STAT4 activation (Becton Dickinson, #612738), and a duplicate blot was probed with a STAT4 antibody (Santa Cruz, #sc-7959) to allow for normalisation against total STAT4 loading onto the SDS gel.

The above assay was repeated using 50 ng/ml recombinant human IL-12 expressed in CHO cells (Peprotech Catalogue # 200-12).

Treatment with IL-12 of the present invention induced a maximum of 2090-fold activation of STAT4, whereas rhIL-12 expressed in CHO cells only induced a maximum of 820-fold activation of STAT4 (Figure 2). Therefore IL-12 of the present invention is 2.6-fold more potent inducer of STAT4 signalling than IL-12 expressed in CHO cells.

(c) Comparing the ability of IL-12 and IL-12 expressed using non-human systems to stimulate differentiation of $CD34^+$ cells derived from human umbilical cord blood

Human umbilical cord blood, collected at the time of delivery, was processed using standard techniques to isolate $CD34^+$ human haematopoietic cells. $CD34^+$ cells were plated into multi-well plates at 2 $x10^4$ cells/ml in the presence of IL-12 of the present invention (20 ng/ml) in combination with recombinant human IL-3 (10 ng/ml; R&D systems Catalogue # 203-IL) and rhSCF (10 ng/ml; Peprotech Cat#300-07). Plates were incubated at 37 °C in a 5% CO_2 incubator for 7 days. Cells were harvested, counted and subjected to surface antigen analysis, including CD56 markers, by flow cytometry using standard methods.

The above assay was repeated using a recombinant human IL-12 expressed in CHO cells (Peprotech Catalogue # 200-12).

Treatment with IL-12 of the present invention yielded 25-fold more CD56 expression than that observed following treatment with rhIL-12 expressed in CHO cells (Figure 3), indicating that IL-12 of the present invention is a more potent inducer of differentiation of $CD34^+$ cord blood stem cells.

(d) Comparing the bioactivities of IL-12 and human IL-12 expressed using non-human systems to induce interferon-gamma production by PHA-activated human peripheral lymphoblasts

IL-12 is known to upregulate production of interferon-gamma by activated T-cells. Interferon-gamma is also upregulated by IL-12 treatment of PHA activated human peripheral blood lymphoblasts. Human peripheral blood mononuclear cells (PBMC) were

isolated using the commercially available Lymphoprep solution (Axis-Shield, Norway). PBMC were activated with 10ug/ml PHA for a total of 4 days, during which time the cells became lymphoblasts. On day 3, the cultures were split and 10 ng/ml IL-2 (R&D Systems # 202-IL) was added to promote lymphoblast proliferation. On day 4, the lymphoblasts were harvested, washed and prepared for IL-12 treatment.

200,000 lymphoblasts per well were plated in a 12-well plate and treated with 0-10 ng/ml IL-12 of the present invention for 48 hours at 37 °C. Cell culture media was then removed and assayed by ELISA using standard techniques to determine interferon-gamma production, as described below in Example 13.

The above assay was repeated using a recombinant human IL-12 expressed in CHO cells (Peprotech Catalogue # 200-12).

IL-12 of the present invention induced interferon-gamma production by PHA stimulated lymphoblasts at the full range of tested concentrations (Figure 4). For example, at a concentration of 2.5 ng/ml, IL-12 of the present invention stimulated 15-fold more interferon-gamma production than rhIL-12 expressed in CHO, and at a concentration of 5 ng/ml IL-12 of the present invention stimulated 4-fold more interferon-gamma production than rhIL-12 expressed in CHO. This indicates IL-12 of the present invention is a more potent activator of interferon-gamma production especially at lower concentrations where rhIL-12 expressed in CHO has little activity.

EXAMPLE 13

In vitro comparison of Immunoreactivity Profiles between IL-12 and human IL-12 expressed using non-human systems

Protein estimation of IL-12 of the present invention was determined using the A280 absorbance method using the calculated extinction coefficient (ε) and the measured molecular mass based on SDS-PAGE analysis.

IL-12 of the present invention, standardised using the standard protein assay results, was diluted and tested in a commercially available ELISA kit, an R&D Systems human IL-12 DuoSet® ELISA kit (Cat # DY1270) in accordance with the manufacturer's instructions. The above-mentioned ELISA kit was calibrated against a recombinant human IL-12 expressed in insect *Sf*21 cells. Additional recombinant human IL-12 preparations expressed in CHO cells (CellSciences) and insect *Sf*21 cells (R&D Systems; 219-IL, sold separate to the ELISA kit) were run in parallel.

The protein concentrations of IL-12 of the present invention and recombinant human IL-12 expressed in CHO cells, both determined by the commercially available ELISA kit, differed from that determined by a standard protein assay method as the capture and/or detection antibodies employed in the commercially available ELISA kit were raised against a non-human cell expressed human IL-12 protein (Figure 5).

At a structural level, such a result indicates different immunoreactivity profiles of IL-12 of the present invention and various non-human cell expressed human IL-12 molecules.

EXAMPLE 14

Further Purification of IL-12 and Peptide Mass Fingerprinting by ESI-MS/MS

In addition to the purification protocol as described in Example 2, purification of the IL-12 of the present invention is further performed by RP-HPLC, using a commercially available column. Eluting proteins are monitored by the absorbance at 215 or 280 nm and collected with correction being made for the delay due to tubing volume between the flow cell and the collection port.

A gel piece containing the protein sample from a 1D or 2D gel is digested in trypsin solution as described in Example 3. Alternatively, a solution containing the protein sample is digested with trypsin in an ammonium bicarbonate buffer (10-25 mM, pH 7.5-9). The solution is incubated at 37° C overnight. The reaction is then stopped by adding acetic acid until the pH is in the range 4-5. The peptide samples are concentrated and desalted using C18 Zip-Tips (Millipore, Bedford, MA) or pre-fabricated micro-columns containing Poros

R2 chromatography resin (Perspetive Biosystems, Framingham, MA) as described in Example 3.

The protein sample (2-5 μl) is injected onto a micro C18 precolumn and washed with 0.1% formic acid at 30 μl/min to concentrate and desalt. After a 3 min wash the pre-column is switched into line with the analytical column containing C18 RP silica (Atlantis, 75μm x 100mm, Waters Corporation). Peptides are eluted from the column using a linear solvent gradient, with steps, from H_2O:CH_3CN (95:5; + 0.1% formic acid) to H_2O:CH_3CN (20:80, + 0.1% formic acid) at 200 nl/min over a 40 min period. The LC eluent is subject to positive ion nanoflow electrospray analysis on a Micromass QTOF Ultima mass spectrometer (Micromass, Manchester, UK).

Tandem MS is performed using a Q-Tof hybrid quadrupole / orthogonal-acceleration TOF mass spectrometer (Micromass). The QTOF is operated in a data dependent acquisition mode (DDA). A TOFMS survey scan was acquired (m/z 400-2000, 1.0s), with the three largest multiply charged ions (counts >15) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 8 s (m/z 50-2000).

The LC/MS/MS data are searched using Mascot (Matrix Science, London, UK) and Protein Lynx Global Server ("PLGS") (Micromass). The protein sample is anticipated to be IL-12.

EXAMPLE 15

(a) Immunogenicity in non-human animals

(i) Animal immunization with target protein

Separate groups of non-human animals, for example, mice are immunized either sub-cutaneously, intramuscularly or intraperitoneally (IP) with 1-100ug of IL-12 of the present invention and the protein expressed in non-human cells, respectively. Animals receive a secondary immunization one month following immunization. Prior to immunization,

protein is emulsified in an adjuvant, for example, complete Freud's adjuvant for the primary immunization and incomplete Freud's adjuvant for the secondary immunization.

(ii) Detection of antibodies directed to target protein

For the detection of antibody response, animals from each group are bled from the tail and sera pooled. Protein-specific antibodies are detected by a solid phase ELISA using 50ng/well of protein of the present invention. Different immunoglobulin isotypes are detected by using labelled detection antibodies raised against IgG1, IgG2, IgG2b, IgG3, IgM, IgA, IgD. Alternatively, antibody response is measured against IL-12 of the present invention blotted onto a membrane either as a dot blot or Western blot. Detection of different immunoglobulin isotypes are detected as described above. It is anticipated that the IL-12 of the present invention will elicit an antibody response that is distinct to that of human IL-12 expressed in non-human cells.

(iii) T cell proliferation assay

Immunised animals are euthanised and spleen cells prepared. A suitable number of spleen cells, for example, 5 x 10^5 cells, from animals immunized with IL-12 of the present invention are cultured with various concentrations of IL-12 of the present invention while and equivalent number of spleen cells from animals immunized with human IL-12 expressed in non-human cells are cultured with various concentrations of human IL-12 expressed in non-human cells. For T cell proliferation assays, spleen cells are cultured for 96 hours and treated with 1μCi [^{3}H] thymidine (6-7 μCi/umol) during the final 16 hours. The cells are harvested onto filter strips and [^{3}H] thymidine incorporation determined using standard methods. It is anticipated that the IL-12 of the present invention will elicit a different proliferation response compared to the human IL-12 expressed in non-human cells.

(iv) IFN gamma assay

For the IFN gamma assay, culture supernatant from spleen cells incubated with either the IL-12 of the present invention or human IL-12 expressed in non-human cells are harvested at 96 hours and IFN gamma production is detected by a sandwich ELISA, for example, a R&D Systems anti-IFN gamma Quantikine® ELISA kit (Cat # DIF50) in accordance with the manufacturer's instructions. It is anticipated that IFN gamma production will be different in culture supernatant derived from cells incubated with IL-12 of the present invention compared with culture supernatant derived from cells incubated with human IL-12 expressed in non-human cells.

(b) In vitro Human Immunogenicity assays

(i) Human T-Cell response assay

Human dendritic cells and $CD4^+$ T cells are prepared from human blood as described in Stickler *et al. Toxicological Sciences 77:*280-289, 2004. Co-cultures of dendritic cells and $CD4^+$ T cells are plated out in 96 well plates containing 2×10^4 dendritic cells and 2×10^5 $CD4^+$ T cells. The IL-12 of the present invention and human IL-12 expressed in non-human cells undergo enzymatic digestion into peptide fragments using a suitable enzyme determined by cleavage site prediction software, for example, Peptide Cutter (http://au.expasy.org/tools/peptidecutter). The resulting peptide fragments are purified by a suitable technique, for example, liquid chromatography and added to the co-cultures to a final concentration of 5ug/ml. Cultures are incubated for 5 days and 0.5uCi ^3H thymidine is then added to each culture. The cells are harvested onto filter strips and cell proliferation is determined by [^3H] thymidine incorporation.

It is anticipated that the peptides derived from IL-12 of the present invention will elicit a weaker proliferation response compared to peptides derived from the human IL-12 expressed in non-human cells.

(ii) Human antibody response assay

Human donors undergoing treatment with human IL-12 expressed in non-human cells are bled and sera prepared. IL-12-specific antibodies are detected by a solid phase ELISA against both 50ng/well of IL-12 of the present invention and human IL-12 expressed in non-human cells. Different immunoglobulin isotypes are detected by using labelled detection antibodies raised against human IgG1, IgG2, IgG3, IgG4, IgM, IgA, IgD.

Alternatively, antibody response is measured against IL-12 of the present invention and human IL-12 expressed in non-human cells blotted onto a membrane either as a dot blot or Western blot. Detection of different immunoglobulin isotypes are detected as described above.

It is anticipated that the immunoglobulin present in the sera of people treated with human IL-12 expressed in non-human cells will bind to human IL-12 expressed in non-human cells while either binding weakly or not binding with IL-12 of the present invention.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to, or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS:

1. An isolated protein comprising a profile of measurable physiochemical parameters, wherein said profile is indicative of, associated with or forms the basis of one or more distinctive pharmacological traits, wherein said isolated protein comprises a physiochemical profile comprising a number of measurable physiochemical parameters, $\{[P_x]_1, [P_x]_2,\ldots[P_x]_n,\}$, wherein P_x represents a measurable physiochemical parameter and "n" is an integer ≥ 1, wherein each of $[P_x]_1$ to $[P_x]_n$ is a different measurable physiochemical parameter, wherein the value of any one of the measurable physiochemical characteristics or an array of values of more than one measurable physiochemical characteristics is indicative of, associated with, or forms the basis of, a distinctive pharmacological trait, T_y, or an array of distinctive physiochemical traits $\{[T_y]_1, [T_y]_2, \ldots.[T_y]_m\}$ wherein T_y represents a distinctive pharmacological trait and m is an integer ≥ 1 and each of $[T_y]_1$ to $[T_y]_m$ is a different pharmacological trait, wherein the isolated protein is IL-12.

2. The isolated protein of Claim 1, wherein said protein comprises one or more of the measurable physiochemical parameters set forth in Table 2.

3. The isolated protein of Claim 1 wherein said protein comprises one or more of the distinctive pharmacological traits set forth in Table 3.

4. A pharmaceutical composition comprising the isolated IL-12 of any one of Claims 1 to 3.

5. A method of treating or preventing a condition in a mammalian subject, wherein said condition can be ameliorated by increasing the amount or activity of a protein, said method comprising administering to said mammalian subject an effective amount of an isolated protein according to any one of Claims 1 to 3 or the pharmaceutical composition of Claim 4.

6. A nucleotide sequence selected from the list consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, or a nucleotide sequence having at least about 90% identity to any

one of the above-listed sequences or a nucleotide sequence capable of hybridizing to any one of the above sequences or their complementary forms under high stringency conditions.

7. An isolated IL-12 encoded by a nucleotide sequence selected from the list consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, or a nucleotide sequence having at least about 90% identity to any one of the above-listed sequence or a nucleotide sequence capable of hybridizing to any one of the above sequences or their complementary forms under high stringency conditions.

8. An isolated nucleic acid molecule encoding a protein or a functional part thereof comprising a sequence of nucleotides having at least 90% similarity SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or after optimal alignment and/or being capable of hybridizing to one or more of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or their complementary forms under high stringency conditions.

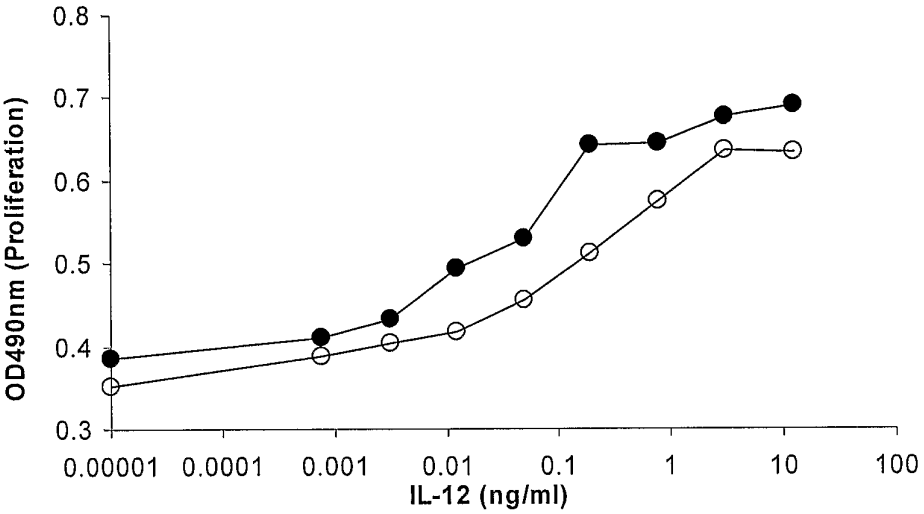
9. An isolated nucleic acid molecule comprising a sequence of nucleotides encoding a protein having an amino acid sequence substantially as set forth in one or more of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 21 or an amino acid sequence having at least about 90% similarity to one or more of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 21 after optimal alignment.

10. A kit for determining the level of human cell expressed human protein present in a biological preparation comprising (a) a solid phase support matrix; (b) one or more antibodies directed against a human protein according to any one of Claims 1 to 3; (c) a blocking solution; (d) one or more stock solutions of substrate; (e) a solution of substrate buffer; (f) a standard human protein sample; and (g) instructions for use.

11. The kit of Claim 10, wherein the standard human protein sample is a preparation of the isolated protein of any one of Claim 2 or 3.

12. The kit of Claim 10 or 11, wherein the or each antibody is derived from an immunization of a mammal with a preparation comprising the isolated protein of any one of Claims 2 or 3.

13. The kit of any of Claims 10 to 12, wherein the human cell expressed human protein is naturally occurring human IL-12.


0.8
0.7
0.6
0.5
0.4
0.3
OD490nm (Proliferation)
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0.0001
0.001
0.01
0.1
1
10
100
IL-12 (ng/ml)

FIGURE 1

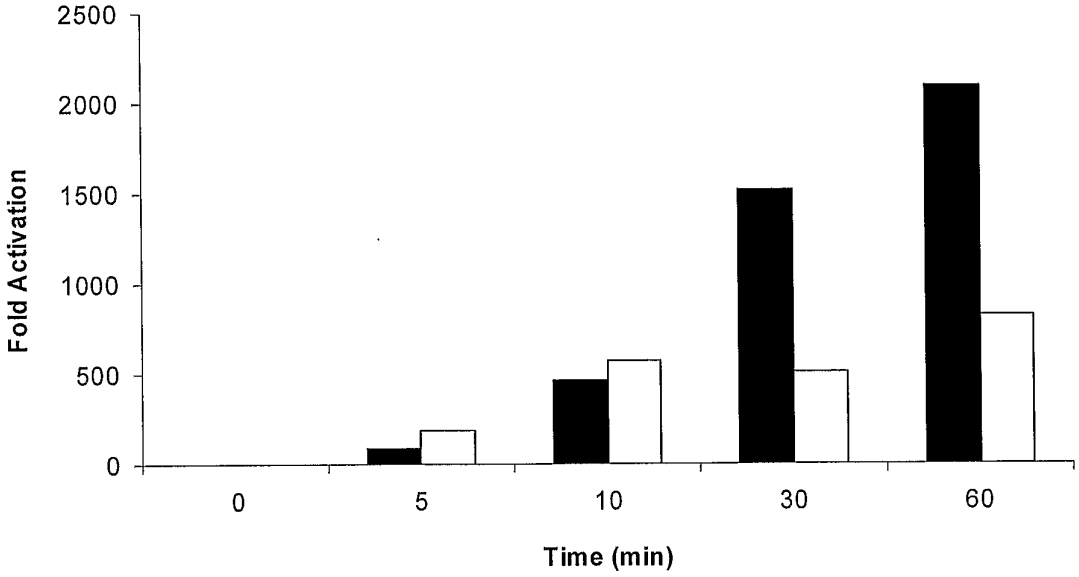
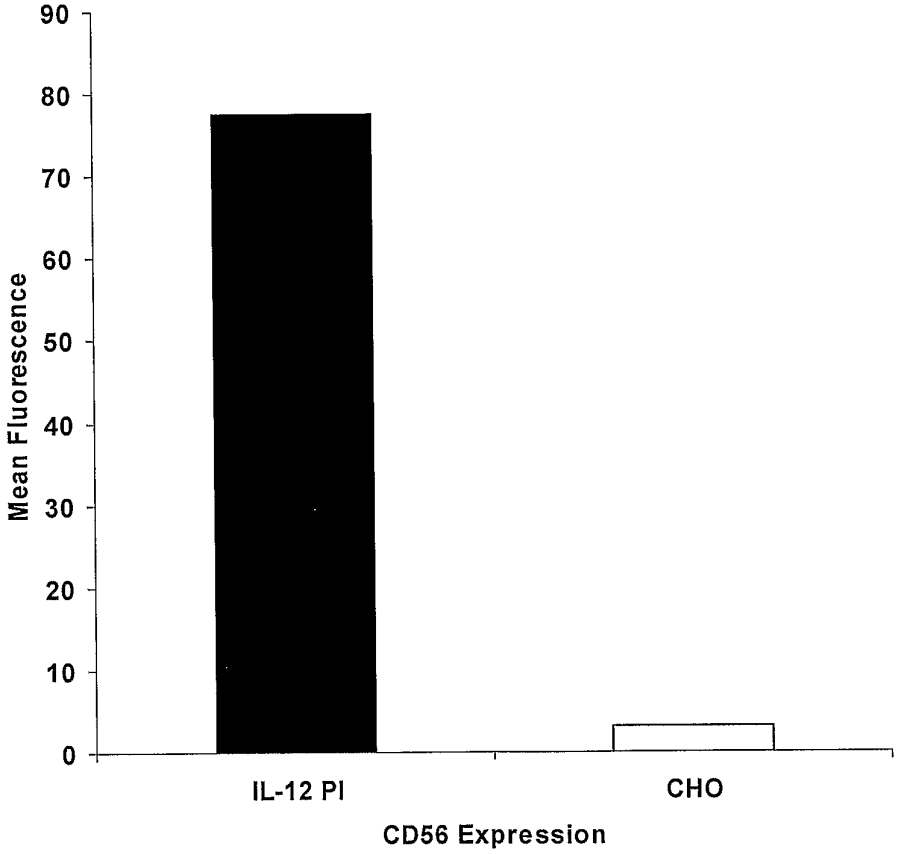


FIGURE 2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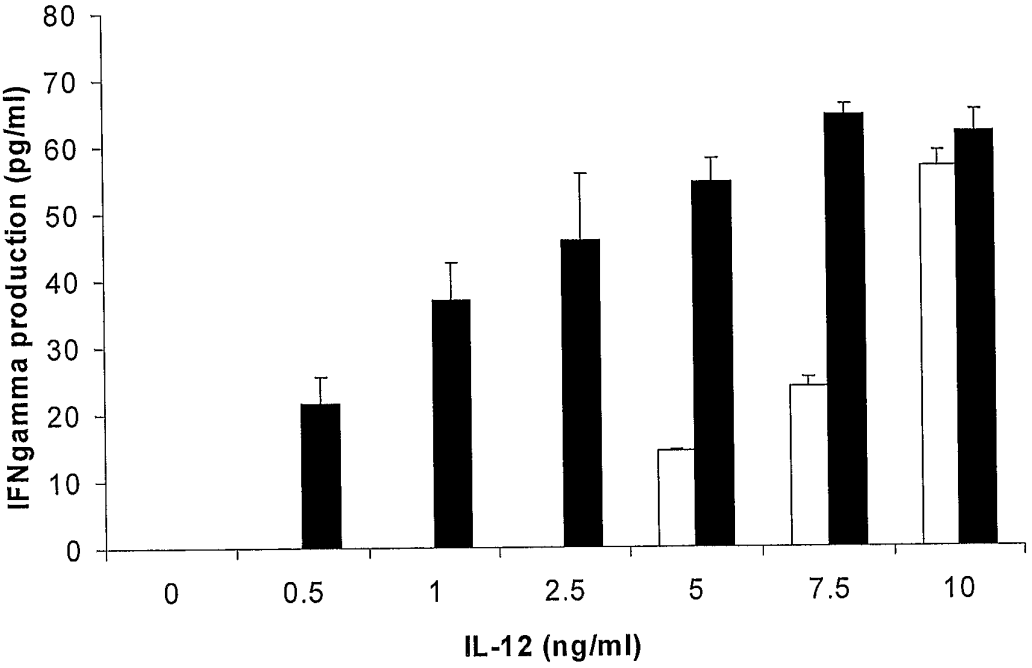
70
60
50
40
30
20
10
0
Mean Fluorescence
IL-12 PI
CHO
CD56 Expression

FIGURE 3


80
70
60
50
40
30
20
10
0
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0
0.5
1
2.5
5
7.5
10
IL-12 (ng/ml)

FIGURE 4

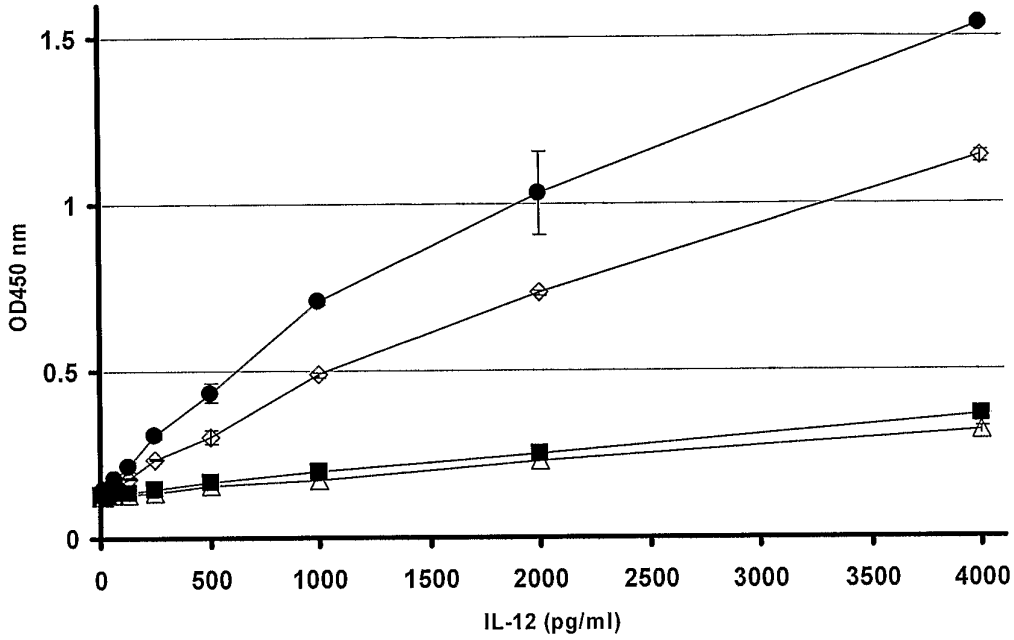
1.5
1
0.5
0
OD450 nm
0
500
1000
1500
2000
2500
3000
3500
4000
IL-12 (pg/ml)

FIGURE 5