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(57) Abstract: The invention provides compositions and methods for modulating the immunostimulatory properties and/or anti-inflammatory properties of IL-10. The present invention provides scIL-10 polypeptides of Formula 1. The polypeptides of the invention are optionally linked to a fusion partner.

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COMPOSITIONS AND METHODS FOR MODULATING IL-10 IMMUNOSTIMULATORY AND ANTI-INFLAMMATORY PROPERTIES

BACKGROUND OF THE INVENTION

IL-10 is considered a potent anti-inflammatory cytokine that strongly inhibits the production of inflammatory mediators. However, recent studies have suggested that IL-10 also has immunostimulatory properties on $CD4^+$, $CD8^+$ T cells, and/or NK cells, resulting in increased IFN-γ production which in turn may lead to related inflammatory responses in humans.

Despite encouraging pre-clinical data suggesting this cytokine as therapeutically valuable biological, results of clinical trials evaluating the merit of IL-10 administration in chronic inflammation have been preponderantly disappointing. Bulk of pre-clinical data and analysis of patients with IL-10 or IL-10 receptor defects clearly point to endogenously produced IL-10 as potent and significant anti-inflammatory determinant. However, thorough analysis further suggests that IL-10 has the potential to acquire sharply contrasting properties in an inflammatory environment *in vivo*. In recent years several studies have been performed in order to verify the human response upon IL-10 administration, particularly in view of its anti-inflammatory potential. Those clinically important studies disclosed perplexing pro-inflammatory functions of IL-10. However, the basis of IL-10 immunostimulatory action remains unclear.

On the other hand IL-10 has been explored for use in the treatment of proliferative disorders, e.g., cancer, tumors, etc. IL-10 induces cytotoxic activity of CD8 T-cells, antibody production of B-cell and suppresses macrophage activity and tumor promoting inflammation. IL-10 appears to increase the infiltration of CD8+ T cells to a tumor, as well as increasing the expression of inflammatory cytokines that play a role in tumor immunity. Treatment with IL-10 may provide a significant improvement for tumor treatment.

One drawback of using IL-10 and particularly any form of recombinant IL-10 in therapy is its short serum half-life. One strategy for increasing serum half-life of a therapeutic protein such as IL-10 is to attach the protein to an Fc (fragment crystallizable) domain of an antibody. Many such fusion proteins are capable of forming homodimers or heterodimers thereby forming antibody-like fusion protein molecules.

Depending on the therapeutic application, the ability to selectively enhance either the anti-inflammatory activity or the immunostimulatory activity of IL-10 would be desired. It would also be desirable to increase the half-life of recombinant IL-10.

SUMMARY OF THE INVENTION

The invention provides compositions and methods for modulating the immunostimulatory properties and/or anti-inflammatory properties of IL-10. The present invention provides scIL-10 polypeptides of Formula 1. The polypeptides of the invention are optionally linked to a fusion partner.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale emphasis instead being placed upon illustrating the principles of the invention.

FIG. 1 is a diagram of an Fc fusion protein homodimer of two polypeptide chains, wherein in each polypeptide chain comprises as X, scIL-10 which is then fused to the Fc region of an IgG1 antibody via an scCLCH1 linker.

FIG. 2 is a diagram of an Fc fusion protein homodimer of two polypeptide chains, wherein in each polypeptide chain comprises scIL-10 which is then fused to the Fc region of an IgG1 antibody via the novel scCH1CL linker.

FIG. 3 is an SDS-PAGE showing expression of an Fc fusion protein comprising scIL-10 fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker (left) or via the novel scCH1CL linker (right) under reducing and non-reducing conditions.

FIG. 4 is a chromatogram showing the characterization of the IL-10 fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker by analytical gel filtration.

FIG. 5 is a chromatogram showing the characterization of the IL-10 fused to the Fc region of an IgG1 antibody via the novel scCH1CL linker by analytical gel filtration.

FIG. 6 is a graph showing stimulation of mouse mast cell line MC/9 by the IL-10 single chain fusion proteins of the invention as compared to the scIL-10 direct Fc fusion protein used as a control.

FIG. 7 is a schematic of the effects of amino acid substitutions that disrupt either one or both of the two IL-10R1 interfaces (SEQ ID NOS: 20, 21 and 22).

FIG. 8 is a schematic of the effects of amino acid substitutions that disrupt either one or both of the two IL-10R2 interfaces (SEQ ID NOS: 23, 24 and 25).

FIG. 9 is a schematic of the effects of amino acid substitutions that simultaneously disrupt one of the IL-10R1 and one of the IL-10R2 interfaces. (SEQ ID NOS: 26-29).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

By "polypeptide" is meant any sequence of two or more amino acids, regardless of length, post-translation modification, or function. "Polypeptide," "peptide," and "protein" are used interchangeably herein. Polypeptides can include natural amino acids and non-natural amino acids. Polypeptides can also be modified in any of a variety of standard chemical ways (e.g., an amino acid can be modified with a protecting group; the carboxy-terminal amino acid can be made into a terminal amide group; the amino-terminal residue can be modified with groups to, e.g., enhance lipophilicity; or the polypeptide can be chemically glycosylated or otherwise modified to increase stability or in vivo half-life). Polypeptide modifications can include the attachment of another structure such as a cyclic compound or other molecule to the polypeptide and can also include polypeptides that contain one or more amino acids in an altered configuration (i.e., R or S; or, L or D).

As used herein, "antibody" and "immunoglobulin" are used interchangeably and refer to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically bind and recognize an antigen. Identified immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD, and IgE, respectively. Terms understood by those in the art of antibody technology are each given the meaning acquired in the art, unless expressly defined differently herein. Antibodies are known to have variable regions, a hinge region, and constant domains. Immunoglobulin structure and function are reviewed, for example, in Harlow et al, Eds., Antibodies: A Laboratory Manual, Chapter 14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, 1988).

"Percent (%) amino acid sequence identity" herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in a selected sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared.

The notations "mg/kg", or "mg per kg" refer to milligrams per kilogram. All notations are used interchangeably throughout the present disclosure.

The "half-life" of a polypeptide can generally be defined as the time taken for the serum concentration of the polypeptide to be reduced by 50%, *in vivo*, for example due to degradation of the polypeptide and/or clearance or sequestration of the polypeptide by natural mechanisms. The half-life can be determined in any manner known per se, such as by pharmacokinetic analysis. Suitable techniques will be clear to the person skilled in the art, and may, for example, generally involve the steps of administering a suitable dose of a polypeptide to a rodent or primate; collecting blood samples or other samples from a rodent or primate at regular intervals; determining the level or concentration of the polypeptide in said blood sample; and calculating, from (a plot of) the data thus obtained, the time until the level or concentration of the polypeptide has been reduced by 50% compared to the initial level upon dosing. Methods for determining half-life may be found, for example, in Kenneth et al., Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists (1986); Peters et al, Pharmacokinete analysis: A Practical Approach (1996); and "Pharmacokinetics", M Gibaldi & D Perron, published by Marcel Dekker, 2nd Rev. edition (1982).

The half-life of a fusion polypeptide is increased if presence in a biological matrix (blood, serum, plasma, tissue) persists, *in vivo*, for a longer period as compared to an appropriate control. Half-life may be increased by 10%, 20%, 30%, 40%, 50% or more as compared to an appropriate control.

Half-life can be expressed using parameters such as the $t_{1/2\text{-alpha}}$, $t_{1/2\text{-beta}}$, and HL_Lambda_z. In the present specification, an "increase in half-life" refers to an increase in any one of these parameters, any two of these parameters, or all three of these parameters. An "increase in half-life" in particular refers to an increase in the $t_{1/2}$-beta and/or

HL_Lambda_z, either with or without an increase in the $t_{1/2}$-alpha. Other PK parameters that can be assessed include volume of distribution (VD), clearance (CL), and mean residence time (MRT), and the area under the curve (AUC). In the present specification, a "change in pharmacokinetics" refers to changes in any one of these parameters, any two of these parameters, any three of these parameters, or all four of these parameters, in the presence or absence of changes in the half-life parameters listed above.

"Activity" for the purposes herein refers to an action or effect of a component of a fusion protein consistent with, but not necessarily identical to, that of the corresponding native active protein, wherein "biological activity" or "bioactivity" refers to an *in vitro* or *in vivo* biological function or effect, including but not limited to receptor binding, antagonist activity, agonist activity, or a cellular or physiologic response.

As used herein, a "dimer complex" comprises two single chains of sc-IL-10, or sc-IL-10 fused to an appropriate fusion partner such as, for example, the scIL-10-L1-HINGE-Fc fusion proteins of the invention, wherein the two single chain polypeptides are associated together under appropriate conditions via either non-covalent binding or covalent binding, for example, by a disulfide bridge. A "heterodimeric protein", "heterodimerized complex", or "heterodimer" as used interchangeably herein refers to a protein that is made of two single chain scIL-10-L1-HINGE-Fc polypeptides forming a dimer complex, wherein said two single chain polypeptides have different amino acid sequences. For example, one single chain peptide of the heterodimer has an scIL-10 based on Formula 1with at least one amino acid substitution and the other single chain peptide of the heterodimer has an scIL-10 sequence based on Formula 1 with no amino acid substitutions. A "homodimeric protein" "homodimerized complex" or "homodimer" as used interchangeably herein, refers to a protein that is made of two identical or substantially identical polypeptides forming the dimer complex, wherein said two single chain polypeptides preferably share 100% identity. There are circumstances, especially with regard to larger polypeptides wherein a homodimer comprises two substantially identical polypeptides having at least about 95% or at least about 99% identity, wherein any amino acid differences between the two polypeptide chains comprise amino acid substitutions, additions or deletions which do not affect the functional and physical properties of the polypeptide compared to its partner polypeptide of the homodimer such as, for example, conservative amino acid substitutions.

As used herein, a protein is "soluble" when it lacks any transmembrane domain or protein domain that anchors or integrates the polypeptide into the membrane of a cell expressing such polypeptide.

As used herein, "Fc domain", "Fc region" or "Fc portion" as those terms may be used interchangeably herein to describe an scIL-10-L1-HINGE-Fc fusion protein of the invention, encompasses domains derived from the constant region of an immunoglobulin, preferably a human immunoglobulin, including a fragment, analog, variant, mutant or derivative of the constant region. Suitable immunoglobulins include IgG1, IgG2, IgG3, IgG4, and other classes such as IgA, IgD, IgE and IgM. The constant region of an immunoglobulin is defined as a naturally-occurring or synthetically-produced polypeptide homologous to the immunoglobulin C-terminal region, and can include a CH1 domain, a hinge, a CH2 domain, a CH3 domain, or a CH4 domain, separately or in combination.

As used herein, "treatment" or "treating," or "palliating" or "ameliorating" is used interchangeably herein. These terms refer to an approach for obtaining beneficial or desired results including but not limited to a therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant eradication or amelioration of the underlying disorder being treated. Also, a therapeutic benefit is achieved with the eradication or amelioration of one or more of the physiological symptoms associated with the underlying disorder such that an improvement is observed in the subject, notwithstanding that the subject may still be afflicted with the underlying disorder.

For prophylactic benefit, the compositions may be administered to a subject at risk of developing a particular disease, or to a subject reporting one or more of the physiological symptoms of a disease, even though a diagnosis of this disease may not have been made.

A "therapeutic effect", as used herein, refers to a physiologic effect, including but not limited to the cure, mitigation, amelioration, or prevention of disease in humans or other animals, or to otherwise enhance physical or mental well-being of humans or animals, caused by a fusion protein of the invention.

The terms "therapeutically effective amount" and "therapeutically effective dose", as used herein, refers to an amount of an active protein, either alone or as a part of a fusion protein composition, that is capable of having any detectable, beneficial effect on any symptom, aspect, measured parameter or characteristics of a disease state or condition when administered in one or repeated doses to a subject. Such effect need not be absolute to be beneficial.

The term "therapeutically effective dose regimen", as used herein, refers to a schedule for consecutively administered doses of an active protein, either alone or as a part of a fusion protein composition, wherein the doses are given in therapeutically effective amounts to

result in sustained beneficial effect on any symptom, aspect, measured parameter or characteristics of a disease state or condition.

As used herein the "anti-inflammatory window" is defined as the range of scIL-10 concentrations that produce anti-inflammatory effects on PBMCs/macrophages, while not inducing immunostimulatory effects (on CD8 T cells, NK cells, etc...). For example, two assays are used in the Examples to define the potencies of those two bioactivities:

1) PBMC cytokine release assay: yields an IC50 value (usually in the low picomolar range) for the concentration at which anti-inflammatory effects occur as measured by inhibition of release of TNF-alpha (TNFα); and
2) MC/9 proliferation assay: yields an EC50 value (usually in the high picomolar to nanomolar range) for the concentration at which immunostimulation effects occur.

The ratio in Tables 11 and 12 is the ratio of (MC/9 EC50) / (PBMC IC50) values. These two assays represent an approximation of the two types of activities. IL-10 targets cell populations within PBMCs to suppress their release of pro-inflammatory cytokines upon LPS stimulation, and IL-10 drives the proliferation of MC/9 cells at concentrations relevant to its immunostimulatory effects. There are many other potential assays that may be used to address the anti-inflammatory window size of the molecules of the invention. However, it is understood that both the immunostimulatory and anti-inflammatory effects of scIL-10 occur in a wider number of cell types.

scIL-10.

Human wild-type IL-10 (wtIL-10) is a non-covalently linked dimer protein comprising two identical monomer subunits. Each identical monomer subunit of human wild type IL-10 (wtIL-10) has the following amino acid sequence (absent the leader sequence): SPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFK GYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPC ENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN (SEQ ID NO: 1) (UniProtKB- P22301[chain 19-178] of IL 10, Interleukin-10, Homosapiens). SEQ ID NO: 1 is also referred to herein as an "unsubstituted IL-10 monomer subunit". Amino Acid sequences based on SEQ ID NO: 1 that comprise at least one amino acid substitution are referred to herein as "substituted IL-10 monomer subunits".

The polypeptides of Formula 1 are referred to herein as "scIL-10" polypeptides and comprise an amino acid sequence arrangement from N-terminus to C-terminus in accordance with Formula 1:

(first monomer subunit)-LINKER-(second monomer subunit)

Formula 1

wherein the first monomer subunit, the second monomer subunit or both the first and second monomer subunits may be independently selected from: an unsubstituted IL-10 monomer subunit; or a substituted IL-10 monomer subunit comprising at least one amino acid substitution; and

wherein LINKER is any amino acid linker of at least 1-100 amino acids in length.

Preferably, LINKER has a length of between at least 2 amino acid and less than 100 amino acids, such as for example between at least 2 amino acids and less than 75 amino acids, more preferably between at least 3 amino acids and less than 50 amino acids, such as for example between at least 4 amino acids and less than 25 amino acids, such as for example between at least 5 amino acids and less than 20 amino acids and even more preferably between at least 6 amino acids and less than 15 amino acids. More preferably, the linker has a length of between at least 3 amino acids and less than 10 amino acids. Most preferably, the linker has a length of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acids. Preferably, the linker is a flexible linker. Preferably, the flexible linker comprises or consists of the amino acids glycine, asparagine and/or serine. More preferably, the flexible linker comprises or consists of the amino acids glycine and serine.

Preferably the first monomer subunit and the second monomer subunit of Formula 1 are both unsubstituted IL-10 monomer subunits and each have the amino acid sequence of SEQ ID NO: 1. These peptides are also referred to herein as "unsubstituted scIl-10".

Preferably, scIL-10 peptides of Formula 1 comprise at least one amino acid substitution in either the first monomer subunit of Formula 1, the second monomer subunit of Formula 1, or in both the first and second monomer subunits of Formula 1. These scIl-10 proteins comprising substituted monomer subunits as compared to human wtscIL-10 of SEQ ID NO: 1 are also referred to herein as "scIL-10 variants".

A preferred scIL-10 peptide of the invention is referred to herein as "unsubstituted scIL-10 (10aa linker)" and has the following amino acid sequence:

SPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFK
GYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPC
ENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRNGGSGGGGSGGS
PGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKG

YLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPCE NKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRN (SEQ ID NO: 29 3501) or a sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to (SEQ ID NO: 2). The ten amino acid linker between the two IL-10 subunits at amino acids 179-188 is indicated by underlining. It is understood that other covalently linked IL-10 dimer proteins may include any suitable flexible peptide linker and may also be longer or shorter than the underlined sequence of SEQ ID NO: 2.

scIL-10 (10aa linker) as represented by SEQ ID NO: 2 comprises two unsubstituted scIL-10 monomer subunits each comprising the amino acid sequence of SEQ ID NO: 1 and as per Formula 1, a LINKER, wherein LINKER is 10 amino acids in length having the sequence: GGSGGGGSGG (SEQ ID NO: 3). Preferably LINKER of scIL-10 is not SEQ ID NO: 3.

Other preferred unsubstituted scIL-10 peptides of Formula 1 include peptides wherein LINKER is a 5 amino acid linker also referred to herein as "unsubstituted sc-IL10 (5aa linker)". One preferred five, amino acid linker is the sequence: GGSGG (SEQ ID NO: 4).

Other preferred unsubstituted scIL-10 peptides of Formula 1 include peptides wherein LINKER is a three amino acid linker also referred to herein as "unsubstituted sc-IL10 (3aa linker)". One preferred three, amino acid linker is the sequence is the sequence GGG.

The present invention is based in part on the discovery that fusion proteins comprising unsubstituted scIL-10 as represented by Formula 1 and scIL-10 comprising at least one amino acid substitution ("scIL-10 variants") also represented by Formula 1, possess a broad anti-inflammatory window. The present invention is also based in part on the discovery that certain amino acid substitutions of unsubstituted scIL-10 further increase the immunostimulatory EC_{50}. The ability to increase the immunostimulatory EC_{50} while maintaining a low anti-inflammatory IC_{50} provides several orders of magnitude increase in the anti-inflammatory window size as compared to, for example, wild-type IL-10 or other fusion proteins comprising IL-10 that are not modified in accordance with the invention.

Without being limited to any theory, it is believed that amino acid substitutions at the interface of scIL-10 with the IL-10R1 and/or IL-10 R2 receptor resulted in modulation of IL-10's immunostimulatory properties, anti-inflammatory properties or both.

It was found that an amino acid substitution at aspartic acid at position 41 (based on SEQ ID NO: 1) in the first monomer subunit or at aspartic acid at position 41 (based on SEQ ID NO: 1) of the second monomer subunit of scIL-10 of Formula 1 disrupts at least one of the scIL-10 interfaces with its IL-10R1 receptor thereby slightly weakening the anti-

inflammatory potency while significantly weakening the immunostimulatory potency of scIL-10 resulting in an increase in the anti-inflammatory window. It was also found that mutations that disrupt scIL-10 at one interface with IL-10R1 on one of either the first or second monomer subunit and also disrupts scIL-10 at one interface with IL-10R2, (for example at the methionine at position 22 of SEQ ID NO: 1) on either the first or second monomer subunit that is not the same as the mutation that disrupts the IL-10 R1 interface provides an extremely large anti-inflammatory window.

It was also discovered that an amino acid substitution of isoleucine at position 87 (based on SEQ ID NO: 1) and which is believed to affect the binding to both IL-10R1 and IL-10R2 appears to have a similar effect as when scIL-10 is designed to disrupt IL-10R1 in one subunit and disrupt IL-10R2 in the other subunit. Without being limited to any theory, it is believed that the isoleucine at position 87 in human wtIL-10 modulates the interaction with both IL-10 receptors although it is not clear how such interaction takes place.

Preferably, the invention provides scIL-10 variants wherein at least one amino acid substitution (as compared to human wild type IL-10 of SEQ ID NO: 1) is introduced in the first and/or second monomer subunit of Formula 1. Preferably scIL-10 comprises at least one amino acid substitution at the interface of the IL-10R1 interface on only one of the first or second monomer subunits of Formula 1 but not both of the first or second monomer subunits of Formula 1. Even more preferably scIL-10 comprises at least one amino acid substitution at the interface of the IL-10R1 interface of only one of the first or second monomer subunits of Formula 1 and also comprises at least one amino acid substitution at an IL-10R2 interface on only one of the first or second monomer subunits of Formula that is not the same monomer subunit as the amino acid substitution at the IL-1-R1 interface.

Preferred amino acid substitutions for scIL-10 variants are based on the numbering of amino acids of SEQ ID NO: 1 and include the following mutations: methionine at position 22 and aspartic acid at position 41.

Preferably the invention provides scIL-10 variants wherein at least one amino acid is substituted at position 41 in the first or second monomer subunit of Formula 1 and at least one amino acid is substituted at position 22 in the first or second monomer subunit that is not the same subunit that comprises the amino acid substitution at position 41.

Preferably the invention provides scIL-10 variants wherein at least one amino acid is substituted at the isoleucine at position 87 of only the first monomer subunit or the second monomer subunit of Formula 1 but not at both monomer subunits.

Amino acid substitutions of methionine at position 22, aspartic acid at position 41 and isoleucine at position 87 may include substitution with any other amino acid. Either conservative or non-conservative amino acid substitutions can be made at one or more amino acid residues. Both conservative and non-conservative substitutions can be made. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic=aspartate, glutamate; (2) basic=lysine, arginine, histidine; (3) nonpolar (hydrophobic)=cysteine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, glycine, tyrosine; and (4) uncharged polar=asparagine, glutamine, serine, threonine. Non-polar may be subdivided into: strongly hydrophobic=alanine, valine, leucine, isoleucine, methionine, phenylalanine and moderately hydrophobic=glycine, proline, cysteine, tyrosine, tryptophan. In alternative fashion, the amino acid repertoire can be grouped as (1) acidic=aspartate, glutamate; (2) basic=lysine, arginine, histidine, (3) aliphatic=glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic=phenylalanine, tyrosine, tryptophan; (5) amide=asparagine, glutamine; and (6) sulfur-containing=cysteine and methionine.

Preferred amino acid substitutions for the first monomer subunit and/or the second monomer subunit in accordance with Formula 1 include the following substitutions: the methionine at position 22 to alanine (M22A); aspartic acid at position 41 to asparagine (D41N); aspartic acid at position 41 to alanine (D41A); aspartic acid at position 41 to phenylalanine (D41F); isoleucine at position 87 to alanine (I87A).

The invention is also based in part on the discovery that the immunostimulatory or anti-inflammatory activities of scIL-10 and scIL-10 variants can be further modulated by fusing scIL-10 or scIL-10 variants to fusion partners including, but not limited to, Fc polypeptides and modified Fc polypeptides such as single chain Fc fusion proteins, mucin linker Fc fusions, Fc polypeptides with truncated hinge regions. Other fusion partners include, but are not limited to: mucin domain polypeptides, albumin fusion proteins, transferrin proteins and other fusion partners not comprising an Fc domain.

Single Chain Fc Fusion Proteins of sc-IL10

Single chain Fc fusion proteins of the invention have the following arrangement from amino-terminus (N-terminus) to carboxy-terminus (C-terminus) as shown in Formula 2:

(scIL-10)-L1-HINGE:Fc

(Formula 2)

wherein, scIL-10 has the amino acid sequence of Formula 1;

L1 is a linker having the following arrangement from amino-terminus to carboxy-terminus:

L2-CL-L3-CH1-L4 (Formula 3) or L2-CH1-L3-CL-L4 (Formula 4)

wherein,

L2 and L4 are independently polypeptide linkers or are independently absent,

L3 is a polypeptide linker;

CL is a constant region polypeptide from an immunoglobulin light chain; and

CH1 a constant region polypeptide from a CH1 domain of an immunoglobulin heavy chain;

HINGE is a hinge sequence of an immunoglobulin or is absent with the proviso that if HINGE is absent, L4 is present; and

Fc is the carboxy-terminus of an immunoglobulin or any active fragment or derivative thereof.

In accordance with the invention, an scIL-10 of Formula 1 is fused to the N-terminal region of an immunoglobulin Fc region via a novel linker (L1) that is derived from the CL and CH1 domains of an immunoglobulin arranged as a single chain (sc) also referred to herein as "scCLCH1 linkers" (Formula 3).

The C-terminus of the CL region may be linked to the N-terminal region of a CH1 region via polypeptide linker L3. The N-terminus of the CL region may be fused to the C-terminus of scIL-10 of Formula 1 via an optional polypeptide linker L2. The C-terminus of the CH1 domain is linked to the Fc domain via an immunoglobulin hinge region (HINGE) or a polypeptide linker (L4) or both a hinge (HINGE) and a polypeptide linker (L4).

The C-terminus of the CH1 domain may also be linked to the N-terminus of a CL region via polypeptide linker L3. The N-terminus of the CH1 region may be fused to the C-terminus of scIL-10 of Formula 1 via an optional polypeptide linker L2. The C-terminus of the CL region is linked to the Fc region via an immunoglobulin hinge region (HINGE) or a polypeptide linker (L4) or both a hinge (HINGE) and a polypeptide linker (L4).

Preferably, L3 is selected from artificial flexible domains comprising amino acids selected from Gly (G), and/or Ser (S). Preferably, the linker is comprised of polypeptide of the general formula (Gly-Gly-Gly-Ser (SEQ ID NO: 5))n or (Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 6))n wherein n is an integer from 1 to 10. Preferably, each linker is a polypeptide comprising from about 1 to about 100 amino acids, preferably about 1-50 amino acids, preferably about 1-25 amino acids, preferably about 1-15 amino acids preferably about 1-10 amino acids, preferably about 4-24 amino acids, preferably about 5-20 amino acids preferably about 5-15 amino acids and preferably about 5-10 amino acids. Preferably, the linker is (Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 6)) n wherein n is 2 or 4.

L2 and L4 are independently selected from artificial flexible domains comprising amino acids selected from, for example, Gly (G), and Ser (S). Preferably, the linker is comprised of polypeptide of the general formula (Gly-Gly-Gly-Ser (SEQ ID NO: 5))n or (Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 6))n wherein n is an integer from 1 to 10. Preferably, each linker is a polypeptide comprising from about 1 to about 100 amino acids, preferably about 1-50 amino acids, preferably about 1-25 amino acids, preferably about 1-15 amino acids preferably about 1-10 amino acids, preferably about 4-24 amino acids, preferably about 5-20 amino acids preferably about 5-15 amino acids and preferably about 5-10 amino acids. Preferably, the linker is (Gly-Gly-Gly-Gly-Ser(SEQ ID NO: 6))n wherein n is 2 or 4.

L2, L3 and L4, may further comprise amino acids such as, for example, Lys (K), Thr (T), Glu (E), and Asp (D).

The CL region of the novel scCLCH1 linker (L1) may be substantially identical to the corresponding CL region of native immunoglobulins belonging to any of the immunoglobulin classes, i.e., IgA, IgD, IgE, IgG, or IgM or any of the IgG antibody subclasses, i.e., IgG1, IgG2, IgG3, and IgG4. The CL region (L1) may have amino acid sequence that is at least 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the corresponding CL region of native immunoglobulins belonging to any of the immunoglobulin classes, i.e., IgA, IgD, IgE, IgG, or IgM or any of the IgG antibody subclasses, i.e., IgG1, IgG2, IgG3, and IgG4. If the CL region of L1 is a modified derivative or variant of a native CL region such modifications include, but are not limited to, amino acid insertions, deletions, substitutions and rearrangements. Preferably, the amino acid sequence of the CL region in accordance with the invention, is at least 80%, more preferably at least 85%, more preferably at least 90%, and more preferably at least 95% identical to the corresponding CL region of native immunoglobulins belonging to any of the immunoglobulin classes, i.e., IgA, IgD, IgE, IgG, or IgM or any of the IgG antibody subclasses, i.e., IgG1, IgG2, IgG3, and IgG4.

The CH1 region of the novel scCLCH1 linker (L1) may be substantially identical to the corresponding CH1 region of native immunoglobulins belonging to any of the immunoglobulin classes, i.e., IgA, IgD, IgE, IgG, or IgM or any of the IgG antibody subclasses, i.e., IgG1, IgG2, IgG3, and IgG4. The CH1 region of L1 may have amino acid sequence that is at least 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the corresponding CH1 region of native immunoglobulins belonging to any of the immunoglobulin classes, i.e., IgA, IgD, IgE, IgG, or IgM or any of the IgG antibody subclasses, i.e., IgG1, IgG2, IgG3, and IgG4. If the CH1 region of the L1 linker is a modified derivative or variant of a native CH1 immunoglobulin region such modifications include, but are not limited to, amino acid insertions, deletions, substitutions and rearrangements. Preferably, the amino acid sequence of the CH1 region is at least 80%, more preferably at least 85%, more preferably at least 90%, and more preferably at least 95% identical to the corresponding CH1 region of native immunoglobulins belonging to any of the immunoglobulin classes, i.e., IgA, IgD, IgE, IgG, or IgM or any of the IgG antibody subclasses, i.e., IgG1, IgG2, IgG3, and IgG4.

The CH1 region and CL regions of L1 do not need to be identical to or a variant of, the corresponding regions of the same immunoglobulin class. For example, the CL region may be derived from the corresponding region of IgE and the CH1 region may be derived from the corresponding region of IgG.

Preferably, CL and CH1 of the scCLCH1 linker are derived from the corresponding CL and CH1 regions of IgG1, preferably human IgG1.

An exemplary CL region corresponding to the CL region of a human IgG1 (hIgG1) includes:

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVT EQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGES (SEQ ID NO: 7).

An exemplary CH1 region corresponding to the CH1 region of hIgG1 includes:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRV (SEQ ID NO: 8).

The single chain Fc fusion proteins disclosed herein comprise an Fc region that includes at least a portion of the carboxy-terminus of an immunoglobulin heavy chain. For example, the Fc portion may comprise: a CH2 domain, a CH3 domain, a CH4 domain, a CH2-CH3 domain, a CH2-CH4 domain, a CH2-CH3-CH4 domain, a hinge-CH2 domain, a hinge-CH2-CH3 domain, a hinge-CH2-CH4 domain, or a hinge-CH2-CH3-CH4 domain. The

Fc domain may be derived from antibodies belonging any of the immunoglobulin classes, i.e., IgA, IgD, IgE, IgG, or IgM or any of the IgG antibody subclasses, i.e., IgG1, IgG2, IgG3, and IgG4. Preferably, the Fc region is derived from IgG1 preferably human IgG1.

The Fc domain may be a naturally occurring Fc sequence belonging any of the immunoglobulin classes, i.e., IgA, IgD, IgE, IgG, or IgM or any of the IgG antibody subclasses, i.e., IgG1, IgG2, IgG3, and IgG4, including natural allelic or splice variants. Alternatively, the Fc domain may be a hybrid domain comprising a portion of an Fc domain from two or more different Ig isotypes, for example, an IgG2/IgG4 hybrid Fc domain. Preferably, the Fc domain is derived from a human immunoglobulin molecule. Alternatively, the Fc domain may be a humanized or deimmunized (removal of T cell epitopes which can activate helper T cells) version of an Fc domain from a non-human animal, including but not limited to mouse, rat, rabbit, and monkey.

The Fc domain may be a variant Fc sequence, e.g., an Fc sequence that has been modified (e.g., by amino acid substitution, deletion and/or insertion) relative to a parent Fc sequence (e.g., an unmodified Fc polypeptide that is subsequently modified to generate a variant), to provide desirable structural features and/or biological activity. For example, one may make modifications in the Fc region in order to generate an Fc variant that (a) has increased or decreased antibody-dependent cell-mediated cytotoxicity (ADCC), (b) increased or decreased complement mediated cytotoxicity (CDC), (c) has increased or decreased affinity for C1q and/or (d) has increased or decreased affinity for a Fc receptor relative to the parent Fc. Such Fc region variants will generally comprise at least one amino acid modification in the Fc region. Combining amino acid modifications is thought to be particularly desirable. For example, the variant Fc region may include two, three, four, five, etc. substitutions therein, e.g. of the specific Fc region positions identified herein.

The hinge region of the Fc fusion proteins of the invention may be derived from antibodies belonging to any of the immunoglobulin classes, i.e., IgA, IgD, IgE, IgG, or IgM. The hinge region may be derived from any of the IgG antibody subclasses, i.e., IgG1, IgG2, IgG3, and IgG4. The hinge region may naturally contain a cysteine residue or may be engineered to contain one or more cysteine residues.

Preferably, the hinge region may have an amino acid sequence that is at least 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the corresponding hinge region of native immunoglobulins belonging to any of the immunoglobulin classes, i.e., IgA, IgD, IgE, IgG, or IgM or any of the IgG antibody subclasses, i.e., IgG1, IgG2, IgG3, and IgG4. Preferably, the amino acid sequence of the hinge region is at least 80%, more

preferably at least 85%, more preferably at least 90%, and more preferably at least 95% identical to the corresponding hinge region of human IgG1.

Shown below is the sequence of a human IgG1 immunoglobulin constant region, and the relative position of the hinge region is indicated by solid underlining:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCP**AP**
ELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHN
AKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO: 9). The CH1 region is indicated by underlining with a dotted line, and the CH2 and CH3 regions are indicated by bold lettering. The C-terminal lysine of an IgG sequence may be removed or replaced with a non-lysine amino acid, such as alanine, to further increase the serum half-life of the Fc fusion protein.

The hinge sequence may include substitutions that confer desirable pharmacokinetic, biophysical, and/or biological properties. An exemplary hinge region of the invention comprises an amino acid sequence that is at least 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the following: EPKSSDKTHTCPPCP (SEQ ID NO: 51).

The Fc domain and the hinge region may be derived from one antibody class or subclass. For example, the hinge region and the Fc domain may be derived from IgG1. The Fc domain and hinge region may correspond to different antibody classes or subclasses. For example, the Fc domain may correspond to the Fc region of IgG2 or IgG4 and the hinge region may correspond to IgG1.

Preferably, all immunoglobulin domains of the Fc fusion proteins of the invention are derived from IgG1, preferably human IgG1. Preferably, the combined hinge region and Fc region of the fusion proteins of the invention comprise an amino acid sequence that is at least 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to:
EPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL
PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS
LSPGK (SEQ ID NO: 10). Preferably, the combined hinge region and Fc region of the fusion proteins of the invention comprise an amino acid sequence that is at least 50%, 60%,

75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to:
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPQV
KFNWYVDGVQVHNAKTKPREQQYNSTYRVVSVLTVLHQNWLDGKEYKCKVSNKA
LPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNG
QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSL
SLSPGK (SEQ ID NO: 11).

It may be desirable to have a hinge sequence and/or Fc region of the single chain fusion proteins of the invention comprising a free cysteine residue in order to permit the formation of a disulfide bond between the hinge and or Fc regions thereby forming dimers of the Fc fusion proteins of the invention. It may be desirable to alter the hinge and/or Fc region sequences to remove free cysteine residues, e.g., by mutating one or more cysteine residues in a linker to another residue, such as a serine, alanine or glycine. The hinge region of the single chain fusion proteins of the invention may comprise one or more free cysteine residues capable of forming one or more disulfide bonds with a second single chain fusion protein of the invention thereby forming a dimer complex.

Preferably, the (scIL-10)-L1-HINGE-Fc fusion proteins of the invention are dimer complexes comprising two monomeric single chain (scIL-10)-L1-HINGE-Fc fusion proteins of the invention linked via a disulfide bond to the hinge region or in the Fc region of the other monomer. The dimer complexes may be homodimeric (e.g. both monomeric fusion proteins are identical) or heterodimeric (e.g. scIL-10 may be different for each monomeric fusion protein). Preferably, the dimer complexes are homodimers thereby forming a homodimeric complex that provides an antibody configuration that resembles that of a native antibody.

Without being limited to any one theory, it is believed that the homodimeric fusion proteins of the invention increase half-life due to the presence of a dimerized Fc region which more closely resembles the native antibody structure as compared to traditional Fc fusion proteins. This is particularly true when the fusion protein has the configuration of Formula 3. A more native Fc domain antibody configuration is believed to enable better binding to the FcRn receptor and therefore increase the circulating half-life of the of the scIL-10-L1-HINGE-Fc dimer complex.

Another improved property associated with scIL-10-L1-HINGE-Fc dimer complexes is that bioactivity is increased versus a traditional Fc fusion proteins based on the use of the scCLCH1 linker which imparts flexibility to relieve steric hindrance caused by the dimerization through the Fc in the hinge region.

Preferably the invention provides (scIL-10)-L1-HINGE-Fc fusion wherein scIL-10 of Formula 1 is unsubstituted scIL-10 (10aa linker). Preferably the invention provides (scIL-10)-L1-HINGE-Fc fusion wherein the scIL-10 of Formula 1 is an sc-IL-10 variant comprising at least one amino acid substitution in the first monomer subunit or the second monomer subunit as per Formula 1 selected from the methionine at position 22, the aspartic acid at position 41, and the isoleucine at position 87 or any combination thereof. Preferably there is at least one amino acid substitution at position 41 in the first or second monomer subunit of Formula 1 and at least one amino acid is substituted at position 22 in the first or second monomer subunit that is not the same subunit that comprises the amino acid substitution at position 41.

A preferred scIL10-L1-HINGE-Fc fusion protein of the invention comprises an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 12 wherein scIL-10 is unsubstituted scIL-10 (10aa linker).

Preferred scIL-10-L1-HINGE-Fc fusion proteins of the invention comprise an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NOs: 20-21 and 37-44 all as shown in Table 4.

Preferred scIL-10-L1-HINGE-Fc fusion proteins of the invention comprise an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NOs: 17-19 as shown in Table 4 wherein scIL-10 is an scIL-10 variant.

The invention also provides nucleic acids encoding any of the various fusion proteins disclosed herein. Codon usage may be selected so as to improve expression in a cell. Such codon usage will depend on the cell type selected. Specialized codon usage patterns have been developed for *E. coli* and other bacteria, as well as mammalian cells, plant cells, yeast cells and insect cells. See for example: Mayfield et al., *Proc. Natl. Acad. Sci. USA,* 100(2):438-442 (Jan. 21, 2003); Sinclair et al., *Protein Expr. Purif.,* 26(I):96-105 (October 2002); Connell, N.D., *Curr. Opin. Biotechnol.,* 12(5):446-449 (October 2001); Makrides et al., *Microbiol Rev.,* 60(3):512-538 (September 1996); and Sharp et al., *Yeast,* 7(7):657-678 (October 1991).

General techniques for nucleic acid manipulation are described for example in Sambrook et al., *Molecular Cloning: A Laboratory Manual,* 2nd Edition, Vols. 1-3, Cold Spring Harbor Laboratory Press (1989), or Ausubel, F. et al., *Current Protocols in Molecular Biology*, Green Publishing and Wiley-Interscience, New York (1987) and periodic updates, herein incorporated by reference. Generally, the DNA encoding the polypeptide is operably linked to suitable transcriptional or translational regulatory elements derived from

mammalian, viral, or insect genes. Such regulatory elements include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences that control the termination of transcription and translation. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants is additionally incorporated.

The fusion proteins described herein may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. An exemplary N-terminal leader sequence for production of polypeptides in a mammalian system is MYRMQLLSCIALSLALVTNS (SEQ ID NO: 48), which is removed by the host cell following expression.

For prokaryotic host cells that do not recognize and process a native signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, or heat-stable enterotoxin II leaders.

For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, a factor leader (including *Saccharomyces* and *Kluyveromyces* alpha-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in U.S. Pat. No. 5,631,144. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available. The DNA for such precursor regions may be ligated in reading frame to DNA encoding the protein.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 micron plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the

origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the nucleic acid encoding the protein disclosed herein, e.g., a fibronectin-based scaffold protein. Promoters suitable for use with prokaryotic hosts include the phoA promoter, beta-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tan promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the protein disclosed herein. Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT (SEQ ID NO: 49) region where N may be any nucleotide. At the 3′ end of most eukaryotic genes is an AATAAA (SEQ ID NO: 50) sequence that may be the signal for addition of the poly A tail to the 3′ end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Transcription from vectors in mammalian host cells can be controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding proteins disclosed herein by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature,* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5′ or 3′ to the peptide-encoding sequence, but is preferably located at a site 5′ from the promoter.

Expression vectors used in eukaryotic host cells (e.g., yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5′ and, occasionally 3′, untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of mRNA encoding the protein disclosed herein. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO 94/11026 and the expression vector disclosed therein.

The recombinant DNA can also include any type of protein tag sequence that may be useful for purifying the protein. Examples of protein tags include but are not limited to a histidine tag, a FLAG tag, a myc tag, an HA tag, or a GST tag. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts can be found in *Cloning Vectors: A Laboratory Manual*, (Elsevier, New York (1985)), the relevant disclosure of which is hereby incorporated by reference.

The expression construct is introduced into the host cell using a method appropriate to the host cell, as will be apparent to one of skill in the art. A variety of methods for introducing nucleic acids into host cells are known in the art, including, but not limited to, electroporation; transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (where the vector is an infectious agent).

Suitable host cells include prokaryotes, yeast, mammalian cells, or bacterial cells. Suitable bacteria include gram negative or gram positive organisms, for example, *E. coli* or *Bacillus* spp. Yeast, preferably from the *Saccharomyces* species, such as *S. cerevisiae*, may

also be used for production of polypeptides. Various mammalian or insect cell culture systems can also be employed to express recombinant proteins. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow et al. (*Bio/Technology,* 6:47 (1988)). Examples of suitable mammalian host cell lines include endothelial cells, COS-7 monkey kidney cells, CV-1, L cells, C127, 3T3, Chinese hamster ovary (CHO), human embryonic kidney cells, HeLa, 293, 293T, and BHK cell lines. Purified polypeptides are prepared by culturing suitable host/vector systems to express the recombinant proteins. For many applications, the small size of many of the polypeptides disclosed herein would make expression in *E. coli* as the preferred method for expression. The protein is then purified from culture media or cell extracts.

In other aspects, the invention provides host cells containing vectors encoding the fusion proteins described herein, as well as methods for producing the fusion proteins described herein. Host cells may be transformed with the herein-described expression or cloning vectors for protein production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Host cells useful for high-throughput protein production (HTPP) and mid-scale production include the HMS 174-bacterial strain. The host cells used to produce the proteins disclosed herein may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma)), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma)) are suitable for culturing the host cells. In addition, many of the media described in various scientific literature may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as Gentamycin drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The fusion proteins provided herein can also be produced using cell-translation systems. For such purposes the nucleic acids encoding the fusion protein must be modified to allow *in vitro* transcription to produce mRNA and to allow cell-free translation of the mRNA

in the particular cell-free system being utilized (eukaryotic such as a mammalian or yeast cell-free translation system or prokaryotic such as a bacterial cell-free translation system).

The fusion proteins disclosed herein can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis,* 2nd Edition, The Pierce Chemical Co., Rockford, Ill. (1984)). Modifications to the fusion proteins can also be produced by chemical synthesis.

The fusion proteins disclosed herein can be purified by isolation/purification methods for proteins generally known in the field of protein chemistry. Non-limiting examples include extraction, recrystallization, salting out (e.g., with ammonium sulfate or sodium sulfate), centrifugation, dialysis, ultrafiltration, adsorption chromatography, ion exchange chromatography, hydrophobic chromatography, normal phase chromatography, reversed-phase chromatography, get filtration, gel permeation chromatography, affinity chromatography, electrophoresis, countercurrent distribution or any combinations of these. After purification, polypeptides may be exchanged into different buffers and/or concentrated by any of a variety of methods known to the art, including, but not limited to, filtration and dialysis.

The purified fusion protein is preferably at least 85% pure, or preferably at least 95% pure, and most preferably at least 98% pure. Regardless of the exact numerical value of the purity, the fusion protein is sufficiently pure for use as a pharmaceutical product.

Other Fusion Partners.

Other appropriate fusion partners for scIL-10 proteins of the invention include but are not limited to proteins comprising an Fc region of all other types.

For example, scIL-10 proteins may be fused directly to the hinge region of a native immunoglobulin containing an Fc region, for example IgG1. SEQ ID NO: 13 is an example of unsubstituted scIL-10 (5aa linker) fused to the hinge region of an IgG1 molecule. The IgG1 molecule may be modified, by, for example, by shortening the hinge region of IgG1. SEQ ID NO: 14 is an example of scIL-10 (5aa linker) fused to the hinge region of IgG1 wherein in the hinge region of the native IgG1 has been shortened by 4 amino acids. SEQ ID NO: 15 is an example of scIL-10 fused to the hinge region of IgG1 wherein in the hinge region of the native IgG1 has been shortened by 7 amino acids. SEQ ID NO: 16 is an example of scIL-10 fused to the hinge region of IgG1 wherein in the hinge region of the native IgG1 has been shortened by 10 amino acids.

A preferred fusion partner comprises an Fc region further comprising a mucin-domain polypeptide linker as is described in WO2013/184938 incorporated herein by reference. A "mucin-domain polypeptide linker" is defined herein as any protein comprising a "mucin domain" capable of being linked to one or more fusion polypeptide partners. A mucin domain is rich in potential glycosylation sites, and has a high content of serine and/or threonine and proline, which can represent greater than 40% of the amino acids within the mucin domain. A mucin domain is heavily glycosylated with predominantly O-linked glycans. A mucin-domain polypeptide has at least about 60%, at least 70%. at least 80%, or at least 90% of its mass due to the glycans. Mucin domains may comprise tandem amino acid repeat units (also referred to herein as TR) that may vary in length from about 8 amino acids to 150 amino acids per each tandem repeat unit. The number of tandem repeat units may vary between 1 and 25 in a mucin-domain polypeptide of the invention.

Mucin-domain polypeptide linkers of the invention include, but are not limited to, all or a portion of a mucin protein. A "portion thereof" is meant that the mucin polypeptide linker comprises at least one mucin domain of a mucin protein. Mucin proteins include any protein encoded for by a MUC gene (e.g., MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC19, MUC20, MUC21). The mucin domain of a mucin protein is typically flanked on either side by non-repeating amino acid regions. A mucin-domain polypeptide may comprise all or a portion of a mucin protein (e.g. MUC20). A mucin-domain polypeptide may comprise all or a portion of a mucin protein of a soluble mucin protein. Preferably the mucin-domain polypeptide comprises the extracellular portion of a mucin protein.

Preferably, an scIL-10 protein of Formula 1 is covalently linked to a molecule comprising an Fc region via a mucin-domain polypeptide linker. SEQ ID NO: 52 is an example of unsubstituted scIL-10 fused to mucin linker which is in turn fused to the hinge of a native IgG1 Fc region.

A preferred fusion partner is a mucin domain polypeptide (not including an Fc region) as is described in WO 2013/184939.

A preferred fusion partner comprises serum albumin or a domain of serum albumin. Human serum albumin is preferred when the fusion proteins of the invention are used for treating humans. In another embodiment, fusion partners comprise human transferrin.

Uses of scIL-10 proteins

In one aspect, the invention provides scIL-10 (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) that are useful as diagnostic or therapeutic agents. In one aspect, the invention provides proteins useful in the treatment of disorders.

The invention also provides a method for achieving a beneficial effect in a subject comprising the step of administering to the subject a therapeutically or prophylactically-effective amount of scIL-10 (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) of the invention. The effective amount can produce a beneficial effect in helping to treat a disease or disorder. In some cases, the method for achieving a beneficial effect can include administering a therapeutically effective amount of a fusion protein composition to treat a subject for diseases and disease categories wherein a therapeutic protein or peptide does not exist.

Preferably scIL-10 is not linked to any fusion partner.

Preferably, scIL-10 is covalently linked to an appropriate fusion partner such as scIL-10-L1-HINGE-Fc. Preferably, the invention provides dimer complexes of scIL-10 fused to an appropriate fusion partner.

Preferably scIL-10 (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) are used to treat patients who suffer from, for example, autoimmune disorders, fibrotic diseases, inflammatory diseases, ischemic diseases, neurodegenerative diseases, neuropathic diseases, pain disorders, auditory disorders, psychiatric disorders, cancer and trauma and injury.

Examples of autoimmune disorders include, but are not limited to: acute disseminated encephalomyelitis (ADEM), acute necrotizing hemorrhagic leukoencephalitis, Addison's disease, agammaglobulinemia, alopecia areata, amyloidosis, ankylosing spondylitis, anti-GBM/anti-TBM nephritis, antiphospholipid syndrome (APS), autoimmune angioedema, autoimmune aplastic anemia, autoimmune dysautonomia, autoimmune hepatitis, autoimmune hyperlipidemia, autoimmune immunodeficiency, autoimmune inner ear disease (AIED), autoimmune lymphoproliferative syndrome (ALPS), autoimmune myocarditis, autoimmune oophoritis, autoimmune pancreatitis, autoimmune retinopathy, autoimmune thrombocytopenic purpura (ATP), autoimmune thyroiditis, autoimmune urticaria, axonal & neuronal neuropathies, Balo disease, Behcet's disease, cardiomyopathy, Castleman disease, celiac disease, Chagas disease, chronic fatigue syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP), chronic recurrent multifocal ostomyelitis (CRMO),

cicatricial pemphigoid/benign mucosal pemphigoid, Cogans syndrome, cold agglutinin disease, congenital heart block, Coxsackie myocarditis, CREST disease, Crohn's disease, demyelinating neuropathies, dermatitis herpetiformis, dermatomyositis, Devic's disease (neuromyelitis optica), discoid lupus, Dressler's syndrome, endometriosis, eosinophilic esophagitis, eosinophilic fasciitis, erythema nodosum, essential mixed cryoglobulinemia, Evans syndrome, experimental allergic encephalomyelitis, fibromyalgia, fibrosing alveolitis, giant cell arteritis (temporal arteritis), giant cell myocarditis, glomerulonephritis, Goodpasture's syndrome, granulomatosis with Polyangiitis (GPA) (formerly called Wegener's Granulomatosis), Grave's disease, Guillain-Barre syndrome, Hashimoto's encephalitis, Hashimoto's thyroiditis, hemolytic anemia, Henoch-Schonlein purpura, herpes gestationis, hypogammaglobulinemia, idiopathic pulmonary fibrosis, idiopathic thrombocytopenic purpura (ITP), IgA nephropathy, IgG4-related sclerosing disease, immunoregulatory lipoproteins, inclusion body myositis, interstitial cystitis, juvenile arthritis, juvenile diabetes (Type 1 diabetes), juvenile myositis, Kawasaki disease, Lambert-Eaton syndrome, leukocytoclastic vasculitis, lichen planus, lichen sclerosus, ligneous conjunctivitis, linear IgA disease (LAD), Lupus (systemic lupus erythematosus), Lyme disease, chronic, Meniere's disease, microscopic polyangiitis, mixed connective tissue disease (MCTD), Mooren's ulcer, Mucha-Habermann disease, multiple sclerosis (MS), myasthenia gravis, myositis, narcolepsy, neuromyelitis optica (Devic's), neutropenia, ocular cicatricial pemphigoid, optic neuritis, palindromic rheumatism, PANDAS (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcus), paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Pars planitis (peripheral uveitis), Parsonnage-Turner syndrome, pemphigus, peripheral neuropathy, perivenous encephalomyelitis, pernicious anemia, POEMS syndrome, polyarteritis nodosa, polymyalgia rheumatica, polymyositis, postmyocardial infarction syndrome, postpericardiotomy syndrome, primary biliary cirrhosis, primary sclerosing cholangitis, progesterone dermatitis, psoriasis, psoriatic arthritis, pure red cell aplasia, pyoderma gangrenosum, Raynauds phenomenon, reactive Arthritis, reflex sympathetic dystrophy, Reiter's syndrome, relapsing polychondritis, restless legs syndrome, retroperitoneal fibrosis, rheumatic fever, rheumatoid arthritis (RA), rheumatoid arthritis, sarcoidosis, Schmidt syndrome, scleritis, scleroderma, Sjogren's syndrome, sperm & testicular autoimmunity, stiff person syndrome, subacute bacterial endocarditis, Susac's syndrome, sympathetic ophthalmia, Takayasu's arteritis, Temporal arteritis/Giant cell arteritis, thrombocytopenic purpura, Tolosa-Hunt syndrome, transverse myelitis, type 1 diabetes, type I, II, & III autoimmune polyglandular syndromes,

ulcerative colitis, undifferentiated connective tissue disease (UCTD), uveitis, vasculitis, vesiculobullous dermatosis, vitiligo, and Wegener's granulomatosis.

Examples of fibrotic diseases which may be treated by the scIL-10 and scIL-10 variant peptides (including fusions of each to an appropriate fusion partner) of the invention include, but are not limited to: adhesive capsulitis, arthrofibrosis, atrial fibrosis, chronic kidney disease, cirrhosis of the liver, cystic fibrosis (CF), Dupuytren's contracture, endomyocardial fibrosis, glial scar, idiopathic pulmonary fibrosis, keloid, macular degeneration, mediastinal fibrosis, myelofibrosis, NAFLD/NASH, nephrogenic systemic fibrosis, Peyronie's disease, progressive massive fibrosis (lungs), proliferative vitreoretinopathy, pulmonary fibrosis, retroperitoneal fibrosis, scar tissue formation resulting from strokes, scleroderma, systemic sclerosis, tissue adhesion.

Examples of inflammatory diseases include, but are not limited to: allergic enteritis, alpha-1-antitrypsin deficiency, ankylosing spondylitis, asthma, Barrett's esophagus, Behcet's disease, chronic fatigue syndrome (CFS / CFIDS / ME), chronic Lyme disease (borreliosis), cocaine-associated vasculitis, Crohn's disease, deficiency of the Interleukin-1 Receptor Antagonist (DIRA), depression, diabetes , Familial Mediterranean Fever (FMF), fibromyalgia (FM), gastroesophageal reflux disease (GERD), glomerulonephritis, graft versus host disease, granulomatous angiitis, Hashimoto's thyroiditis, hypertension, hyperthyroidism, hypothyroidism, inflammatory bowel disease (IBD), inflammatory myopathies (polymyositis, inclusion body myositis, dermatomyositis), interstitial cystitis (IC), irritable bowel syndrome (IBS), ischemic colitis, kidney stones, Löfgren's syndrome, Lupus erythematosis, methamphetamine-associated vasculitis, migraine headache, Morgellon's, multiple chemical sensitivity (MCS), multiple sclerosis (MS), neonatal onset multisystem inflammatory disease (NOMID), optic neuritis, osteoarthritis, pemphigus vulgaris, polymyalgia rheumatica, prostatitis, psoriasis, psoriatic arthritis, radiation colitis, Raynaud's syndrome/phenomenon, reactive arthritis (Reiter syndrome), reflex sympathetic dystrophy (RSD), restless leg syndrome, rheumatoid arthritis (RA), sarcoidosis, scleroderma, seasonal affective disorder (SAD), septic shock, sinusitis, Sjögren's syndrome, temporal arteritis, tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), ulcerative colitis, uveitis, vasculitis, and vertigo.

Examples of ischemic diseases include, but are not limited to: acute coronary syndrome, angina pectoris, angor animi, copeptin, coronary artery disease, coronary ischemia, hibernating myocardium, ischemic stroke, management of acute coronary syndrome, meldonium, myocardial infarction, myocardial infarction complications,

myocardial infarction diagnosis, myocytolysis, post-anoxic encephalopathy, Prinzmetal's angina, Sgarbossa's criteria, stroke, TIMI, transient ischemic attack (TIA) and unstable angina.

Examples of neurodegenerative diseases include, but are not limited to: ataxia telangiectasia, autosomal dominant cerebellar ataxia, Baggio–Yoshinari syndrome, Batten disease, estrogen and neurodegenerative diseases, hereditary motor and sensory neuropathy with proximal dominance, Infantile Refsum disease, JUNQ and IPOD, locomotor ataxia, Lyme disease, Machado–Joseph disease, mental retardation and microcephaly with pontine and cerebellar hypoplasia, multiple system atrophy, neuroacanthocytosis, neuronal ceroid lipofuscinosis, Niemann–Pick disease, pontocerebellar hypoplasia, protein aggregation, pyruvate dehydrogenase deficiency, radiation myelopathy, Refsum disease, retinitis pigmentosa, Sandhoff disease, Shy-Drager syndrome, spinal muscular atrophy, spinocerebellar ataxia, subacute combined degeneration of spinal cord, subacute sclerosing panencephalitis, Tabes dorsalis, Tay–Sachs disease, toxic encephalopathy, toxic leukoencephalopathy and Wobbly Hedgehog Syndrome.

Examples of neuropathic diseases include, but are not limited to: Bell's Palsy, campylobacter-associated motor axonopathies, Charcot-Marie-Tooth, chronic inflammatory demyelinating polyneuropathy, diabetic amyotrophy avulsion, diabetic neuropathies, Guillain Barre Syndrome and vasculitis.

Examples of pain disorders include, but are not limited to: Amplified musculoskeletal pain syndromes, Anterior cutaneous nerve entrapment syndrome, central pain syndrome, chronic functional abdominal pain, chronic pain, chronic prostatitis/chronic pelvic pain syndrome, chronic wound pain, degenerative disc disease, dentomandibular sensorimotor dysfunction, failed back syndrome, fibromyalgia, interstitial cystitis, irritable bowel syndrome (IBS), myofascial pain syndrome, pelvic pain, post-vasectomy pain syndrome, reflex neurovascular dystrophy, sickle-cell disease, theramine, and vulvodynia.

Examples of auditory disorders include, but are not limited to: conductive hearing loss, sensorineural hearing loss (SNHL), mixed hearing loss.

Examples of psychiatric disorders include, but are not limited to: major depressive disorder, treatment-refractory depression, treatment-resistant depression.

Examples of trauma and injury include, but are not limited to: including central nervous system (CNS) injuries, traumatic brain injury, spinal cord injury, crush injuries, shock, tendon damage, wounds to the cornea, wounds to the eye, skin wounds.

Preferably, an scIL-10 proteins (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) of the invention may be used to treat patients who suffer from, for example, autoimmune disorders including autoimmune lymphoproliferative syndrome (ALPS), autoimmune thyroiditis, Crohn's disease, Grave's disease, Hashimoto's thyroiditis , Kawasaki disease, Lupus (systemic lupus erythematosus), multiple sclerosis (MS), myasthenia gravis, psoriasis, rheumatoid arthritis, Sjogren's syndrome, type 1 diabetes, ulcerative colitis; fibrotic diseases including Chronic Kidney Disease, cirrhosis of the liver, macular degeneration, NAFLD/NASH, proliferative vitreoretinopathy, pulmonary fibrosis, scar tissue formation resulting from strokes, tissue adhesion; including inflammatory diseases including allergic enteritis, alpha-1-antitrypsin deficiency, asthma, Behcet's disease, cocaine-associated vasculitis, glomerulonephritis, Graft Versus Host Disease, granulomatous angiitis, inflammatory bowel disease, inflammatory myopathies (polymyositis, inclusion body myositis, dermatomyositis), ischemic colitis, methamphetamine-associated vasculitis, optic neuritis, pemphigus vulgaris, radiation colitis, sarcoidosis, Septic Shock, temporal arteritis, vasculitis; ischemic diseases including myocardial infarction, post-anoxic encephalopathy, stroke; neurodegenerative diseases including neuronal ceroid lipofuscinosis, radiation myelopathy, retinitis pigmentosa, spinal muscular atrophy; neuropathic diseases including campylobacter-associated motor axonopathies, Charcot-Marie-Tooth, chronic inflammatory demyelinating polyneuropathy, diabetic amyotrophy avulsion, diabetic neuropathies, Guillain Barre Syndrome; auditory disorders including Conductive hearing loss, Sensorineural hearing loss (SNHL), Mixed hearing loss; psychiatric disorders including major depressive disorder, treatment-refractory depression, treatment-resistant depression; trauma and injury including central nervous system (CNS) injuries, traumatic brain injury, spinal cord injury, crush injuries, shock, tendon damage, wounds to the cornea, wounds to the eye, skin wounds.

Most preferably, scIL-10 proteins (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) in accordance with the invention may be used to treat patients who suffer from, for example, autoimmune disorders including autoimmune lymphoproliferative syndrome (ALPS), autoimmune thyroiditis, Crohn's disease, Grave's disease, Hashimoto's thyroiditis , Kawasaki disease, Lupus (systemic lupus erythematosus), multiple sclerosis (MS), myasthenia gravis, psoriasis, rheumatoid arthritis, Sjogren's syndrome, type 1 diabetes, ulcerative colitis; fibrotic diseases including Chronic Kidney Disease, cirrhosis of the liver, macular degeneration, NAFLD/NASH, proliferative vitreoretinopathy, pulmonary fibrosis, scar tissue formation resulting from strokes, tissue adhesion; inflammatory diseases including allergic enteritis, alpha-1-antitrypsin deficiency,

asthma, Behcet's disease, cocaine-associated vasculitis, glomerulonephritis, Graft Versus Host Disease, granulomatous angiitis, inflammatory bowel disease, inflammatory myopathies (polymyositis, inclusion body myositis, dermatomyositis), ischemic colitis, methamphetamine-associated vasculitis, optic neuritis, pemphigus vulgaris, radiation colitis, sarcoidosis, Septic Shock, temporal arteritis, vasculitis; ischemic diseases including myocardial infarction, post-anoxic encephalopathy, stroke; neurodegenerative diseases including neuronal ceroid lipofuscinosis, radiation myelopathy, retinitis pigmentosa, spinal muscular atrophy; neuropathic diseases including campylobacter-associated motor axonopathies, Charcot-Marie-Tooth, chronic inflammatory demyelinating polyneuropathy, diabetic amyotrophy avulsion, diabetic neuropathies, Guillain Barre Syndrome; auditory disorders including Conductive hearing loss, Sensorineural hearing loss (SNHL), Mixed hearing loss; psychiatric disorders including major depressive disorder, treatment-refractory depression, treatment-resistant depression; trauma and injury including central nervous system (CNS) injuries, traumatic brain injury, spinal cord injury, crush injuries, shock, tendon damage, wounds to the cornea, wounds to the eye, skin wounds.

Preferably scIl-10 proteins (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) of the invention may be used to treat patients who suffer from, for example cancer of the uterus, cervix, breast, ovaries, prostate, testes, penis, gastrointestinal tract, esophagus, oropharynx, stomach, small or large intestines, colon, or rectum, kidney, renal cell, bladder, bone, bone marrow, skin, head or neck, skin, liver, gall bladder, heart, lung, pancreas, salivary gland, adrenal gland, thyroid, brain, gliomas, ganglia, central nervous system (CNS) and peripheral nervous system (PNS), and immune system, spleen or thymus, papilloma virus-induced cancers, epithelial cell cancers, endothelial cell cancers, squamous cell carcinomas, adenocarcinomas, carcinomas, melanomas, sarcomas, teratocarcinomas, immunogenic tumors, non-immunogenic tumors, dormant tumors, lymphomas, leukemias, myelomas, chemically-induced cancers, metastasis, and angiogenesis, and Tuberous sclerosis.

Preferably, scIL-10 fusion proteins (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) in accordance with the invention may be used to treat patients who suffer from auditory disorders, renal cell carcinoma, melanoma, psoriasis, fibrosis, depression, and inflammatory bowel disease (IBD).

Preferably, scIL-10 fusion proteins (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) in accordance with the invention may also be used in the manufacture of a medicament to treat patients to diseases as set forth above,

auditory disorders, auditory disorders, renal cell carcinoma, melanoma, psoriasis, fibrosis, depression, and inflammatory bowel disease (IBD).

The application further provides pharmaceutically acceptable compositions comprising scIL-10 proteins (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) described herein. Therapeutic formulations comprising scIL-10 proteins are prepared for storage by mixing the described proteins having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of aqueous solutions, lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulations herein may also contain more than one active compounds as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The Preferably, scIL-10 proteins (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) in accordance with the invention may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the fibronectin based scaffold proteins described herein, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinyl alcohol)), polylactides, copolymers of lactide and glycolide, copolymers of L-glutamic acid and y ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable sustained release of, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

While the skilled artisan will understand that the dosage of each scIL-10 protein (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) in accordance with the invention will be dependent on the patient's particular circumstances. The dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg, of the host body weight. For example, dosages can be 0.3 mg/kg body weight, 1 mg/kg body weight, 3 mg/kg body weight, 5 mg/kg body weight or 10 mg/kg body weight or within the range of 1-30 mg/kg. An exemplary treatment regime entails administration once per week, once every two weeks, once every three weeks, once every four weeks, once a month, once every 3 months or once every three to 6 months. Dosage regimens include 1 mg/kg body weight or 3 mg/kg body weight by intravenous administration, with the protein being given using one of the following dosing schedules: every four weeks for six dosages, then every three months; every three weeks; 3 mg/kg body weight once followed by 1 mg/kg body weight every three weeks. Preferably, scIL-10 fusion proteins (including fusions of each to an appropriate fusion partner and dimerized complexes thereof in accordance with the invention is usually administered on multiple occasions. Intervals between single dosages can

be, for example, weekly, monthly, every three months or yearly. Intervals can also be irregular as indicated by measuring blood levels of the soluble protein in the patient. In some methods, dosage is adjusted to achieve a plasma concentration of soluble protein of about 0.1-1000 pg/ml and in some methods about 5- 300 mg/ml.

For therapeutic applications, scIL-10 proteins (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) in accordance with the invention are administered to a subject, in a pharmaceutically acceptable dosage form. They can be administered intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, subcutaneous, intra-ocular, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The protein may also be administered by intratumoral, peritumoral, intralesional, or perilesional routes, to exert local as well as systemic therapeutic effects. Suitable pharmaceutically acceptable carriers, diluents, and excipients are well known and can be determined by those of skill in the art as the clinical situation warrants. Examples of suitable carriers, diluents and/or excipients include: (1) Dulbecco's phosphate buffered saline, pH about 7.4, containing about 1 mg/ml to 25 mg/ml human serum albumin, (2) 0.9% saline (0.9% w/v NaCl), and (3) 5% (w/v) dextrose. The methods of the present invention can be practiced *in vitro*, *in vivo*, or *ex vivo*.

Administration of scIL-10 proteins (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof), and one or more additional therapeutic agents, whether co-administered or administered sequentially, may occur as described above for therapeutic applications. Suitable pharmaceutically acceptable carriers, diluents, and excipients for co-administration will be understood by the skilled artisan to depend on the identity of the particular therapeutic agent being co-administered.

When present in an aqueous dosage form, rather than being lyophilized, scIL-10 (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) typically will be formulated at a concentration of about 0.1 mg/ml to 100 mg/ml, although wide variation outside of these ranges is permitted. For the treatment of disease, the appropriate dosage of scIL-10 (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) will depend on the type of disease to be treated, the severity and course of the disease, whether the scIL-10 proteins (including fusions of scIL-10 to an appropriate fusion partner and dimerized complexes thereof) are administered for preventive or therapeutic purposes, the course of previous therapy, the patient's clinical history and response to the scIL-10 protein (including fusions of scIL-10

to an appropriate fusion partner and dimerized complexes thereof), and the discretion of the attending physician. The scIL-10 protein is suitably administered to the patient at one time or over a series of treatments.

EXAMPLES

Example 1: unsubstituted scIL-10

Design of scIL-10:C_L:C_H1:Fc and scIL-10:C_{H1}:C_L:Fc

The scIL-10 single chain fusion body molecule contains a covalently linked IL-10 homodimer fusion protein linked to the CL-CH1-Fc (Formula 3) domain or the CH1-CL-Fc of the IgG1 heavy chain (FIGs.1 and 2). The amino acid sequences of each molecule synthesized is found in Table 1.

Table 1

Protein	Sequence
Unsubstituted scIL-10 (10aa linker):CL:CH1: Fc	MYRMQLLSCIALSLALVTNSSPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNL LLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFL PCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRNGGSGGGGSGGSPGQGTQS ENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLE EVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKA MSEFDIFINYIEAYMTMKIRNGGGSGGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREA KVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFN RGECGGGGSGGGGSGGGGSGGGGSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCP PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGK (SEQ ID NO: 23)
Unsubstituted scIL-10 (10aa linker):CH1:CL: Fc	MYRMQLLSCIALSLALVTNSSPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNL LLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFL PCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRNGGSGGGGSGGSPGQGTQS ENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLE EVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKA MSEFDIFINYIEAYMTMKIRNGGGSGGGGSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVGGGGSG GGGSGGGGSGGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNS QESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSGGEPKSCDK THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT KPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 24)
Unsubstituted scIL-10 (5aa linker):Fc (Control)	MYRMQLLSCIALSLALVTNSSPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNL LLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFL PCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRNGGSGGSPGQGTQSENSCTH FPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQA ENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFI NYIEAYMTMKIRNEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT ISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 25)
	The final protein used in experiments does not include the leader sequence MYRMQLLSCIALSLALVTNS (SEQ ID NO: 48)

Expression of scIL-10:C_L:C_H1:Fc and scIL-10:C_{H1}:C_L:Fc

The genes were synthetically synthesized and supplied in pcDNA3.1 expression vector (GeneArt), and transiently expressed in HEK293 cells using the Expi293 expression

system (Life Technologies). Proteins were purified using Protein A (GE Healthcare) with low pH elution and dialyzed against 2L 1X PBS 2 times.

The molecules were analyzed by SDS PAGE gel under reducing and non-reducing conditions (FIG. 3). For reducing and non-reducing conditions, 2.5ug of protein was loaded onto an Any kD gel (Invitrogen) with a Precision Plus Protein Kaleidoscope standard (Invitrogen) (MW range 10kD – 250 kD). The molecule was characterized by analytical gel filtration on an XBridge Protein BEH SEC column, 200Å, 3.5 µm, 7.8 mm X 150 mm (Waters). The column was equilibrated and run at 0.9 ml/min with 100mM sodium phosphate pH 7.0 as a running buffer for all analyses. Purified samples (0.5mg/ml) were injected (15ul) and eluted with a run time of 15 min (FIGs. 4 and 5).

Bioactivity of scIL-10:C_L:C_H1:Fc and scIL-10:C_{H1}:C_L:Fc

In vitro bioactivity was assessed by evaluating the ability of scIL-10:C_L:C_H1:Fc and scIL-10:C_{H1}:C_L:Fc to stimulate proliferation of the mouse mast cell line MC/9 (ATCC CRL-8306). The scIL-10 direct Fc fusion protein (scIL-10:Fc) was used as a control. For the assay, MC/9 cells were plated at 10,000 cells/well in DMEM media containing 10% heat inactivated fetal bovine serum, 2 mM glutamine and 0.05 mM 2-mercaptoethanol. Cells were incubated for 72 hours at 37°C, 5% CO_2 with varying concentrations of human IL-10 (R&D Systems), scIL-10:C_L:C_H1:Fc, scIL-10:C_{H1}:C_L:Fc or scIL-10:Fc. After 72 hours, the cells were stained with CellTiter-Blue (Promega) for 4 hours at 37°C, 5% CO_2 according to the manufacturer's protocol. Fluorescent measurements were taken at 560/590 nm. IL-10 (EC_{50} = 75 pM), scIL-10:C_L:C_H1:Fc (EC_{50} = 79 pM), scIL-10:C_{H1}:C_L:Fc (EC_{50} = 93 pM) and scIL-10:Fc (EC_{50} = 493 pM) were active in a dose dependent fashion (FIG. 6).

Mouse PK of scIL-10:C_L:C_H1:Fc and scIL-10:C_{H1}:C_L:Fc

scIL-10:C_L:C_H1:Fc, scIL-10:C_{H1}:C_L:Fc, and scIL-10:Fc pharmacokinetics in mice were evaluated at a single intravenous doses of 0.5 mg/kg administered into tail vein and a single subcutaneous doses of 2.5 mg/kg administered into the interscapular region. Blood samples (n=3 samples/time point/fusion protein) were collected at 0.083, 0.5, 1, 4, 6, 24, 48, 96, 168, 192 and 216 hours after administration of scIL-10:C_L:C_H1:Fc, scIL-10:C_{H1}:C_L:Fc and scIL-10:Fc. For each time point/ fusion protein/route of administration, serum was pooled and concentrations were measured using standard MSD techniques. Bioanalytical data was subjected to non-compartmental pharmacokinetic analysis using Phoenix WinNonlin 6.4 software. The pharmacokinetic parameter included standard pharmacokinetic parameters of

maximum concentration (C_{max}), time to maximum concentration (T_{max}), area under the time versus concentration curve (AUC), mean residence time (MRT), elimination half-life (t1/2), clearance (CL), distribution volume at steady state (V_{ss}), and bioavailability (%F) were determined and reported in Tables 2 and 3.

Table 2

Row ID	Compound	Dose (mg/kg)	Dose (~nMole/kg)	ROA	Cmax (nM)	Tmax (h)	Cmax/D (nM/D)	AUClast (h*nM)
1	scIL-10:Fc	0.5	3.93	IV	94.9	0.083	24.2	2080
2	scIL-10:Fc	2.5	19.63	SC	221	24	11.3	12700
3	scIL-10:C_L:C_H1:Fc	0.5	2.85	IV	140	0.083	49.2	2850
4	scIL-10:C_L:C_H1:Fc	2.5	14.25	SC	227	24	15.9	19500
5	scIL-10:C_{H1}:C_L:Fc	0.5	2.84	IV	115	0.083	40.5	1300
6	scIL-10:C_{H1}:C_L:Fc	2.5	14.2	SC	120	24	8.48	7570

Table 3

Row ID	AUCinf (h*nM)	AUCinf/D (h*nM)	MRTinf (h)	t1/2 (h)	CL (mL/hr/kg)	Vss (mL/kg)	%F
1	2170	552	33	21	1.811	59.57	NA
2	12700	649	46	11	NA	NA	~100
3	2850	999	30	7.8	1.001	29.56	NA
4	19500	1370	56	8.5	NA	NA	~100
5	1300	458	16	9.3	2.183	35.44	NA
6	7570	533	41	9.1	NA	NA	~100

Example 2: (scIL-10)-L1-HINGE-Fc Fusion Proteins.

Design of scIL-10 variant fusion proteins.

The scIL-10 of Formula 1 are fused to a single chain Fc linker of Formula 2 wherein L1 is CL-CH1-Fc as per Formula 3. The amino acid sequences of each full length scIL-10-L1-HINGE-Fc fusion variant protein synthesized is found in Table 4. The description column of Table 4 indicates the scIL-10 used in the construct with the fusion partner. For example wtIL-10:linker:D41F indicates that in accordance with Formula 1, the first monomer subunit is wt IL-10 of SEQ ID NO: 1 and is therefore unsubstituted linked to a linker which is in turn linked to the second monomer subunit wherein the wtIL10 of SEQ ID NO: 1 is substituted at amino acid 41 such that the isoleucine at amino acid 41 is substituted with phenylalanine (D41F).

For expression in mammalian cells, the N-terminal leader sequence of SEQ ID NO: 48 was added to each of the protein sequences found in Table 4.

Table 4

Description	Amino Acid Sequence/SEQ ID NO
scIL-10:CL:CH1:Fc	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 12)
M22A:linker:D41N (R1+R2 mutant)	SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK NQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD

	GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 44)
D41N:linker:M22A (R1+R2 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK NQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 43)
wtIL-10:linker:M22A, D41N (R1+R2 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK NQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 42)
wtIL-10:linker:D41N (R1 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK NQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 41)
M22A:linker:D41A (R1+R2 mutant)	SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK AQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR

	FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 40)
D41A:linker:M22A (R1+R2 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK AQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 39)
wtIL-10:linker:M22A, D41A (R1+R2 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK AQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 38)
wtIL-10:linker:D41A (R1 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK AQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE

	ALHNHYTQKS LSLSPGK (SEQ ID NO: 37)
M22A:linker:D41F (R1+R2 mutant)	SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 29)
D41F:linker:M22A (R1+R2 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 28)
wtIL-10:linker:M22A, D41F (R1+R2 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 27)
M22A:linker:D41F (R1+R2 mutant)	SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT

	HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 26)
M22A:linker:M22A (R2 mutant)	SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 25)
wtIL-10:linker:M22A (R2 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 24)
M22A:linker:wtIL-10 (R2 mutant)	SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 23)

D41F:linker:D41F (R1 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 22)
wtIL-10:linkerD41F (R1 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 21)
D41F:linker:wtIL-10 (R1 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 20)
wtIL-10:linker:I87A (vIL10 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDAKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP

	SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 19)
I87A:linker:wtIL-10 (vIL10 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDAKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 18)
I87A:linker:I87A (vIL10 mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDAKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDAKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 17)
M22A, D41F:linker:M22A, D41F (R1+R2 pan mutant)	SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 30)
M22A, D41F:linker:M22A (R1+R2 triple mutant)	SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK

	LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO:31)
M22A, D41F:linker:D41F (R1+R2 triple mutant)	SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 32)
M22A:linker:M22A, D41F (R1+R2 triple mutant)	SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO:33)
D41F:linker:M22A, D41F (R1+R2 triple mutant)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NALRDLRDAF SRVKTFFQMK FQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGGSGGGGS RTVAAPSVFI FPPSDEQLKS GTASVVCLLN NFYPREAKVQ WKVDNALQSG NSQESVTEQD SKDSTYSLSS TLTLSKADYE KHKVYACEVT HQGLSSPVTK SFNRGECGGG GSGGGGSGGG GSGGGGSAST KGPSVFPLAP SSKSTSGGTA ALGCLVKDYF PEPVTVSWNS GALTSGVHTF PAVLQSSGLY SLSSVVTVPS SSLGTQTYIC NVNHKPSNTK VDKRVEPKSC DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD

	GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 34)

Expression of sc-IL-10 variant fusion proteins

The genes were synthetically synthesized and supplied in pcDNA3.1 expression vector (GeneArt), and transiently expressed in HEK293 cells using the Expi293 expression system (Life Technologies). Proteins were purified using Protein A (GE Healthcare) with low pH elution and dialyzed against 2L 1X PBS 2 times.

The molecules were analyzed by SDS PAGE gel under reducing and non-reducing conditions. For reducing and non-reducing conditions, 2.5ug of protein was loaded onto an Any kD gel (Invitrogen) with a Precision Plus Protein Kaleidoscope standard (Invitrogen) (MW range 10kD – 250 kD). The molecule was characterized by analytical gel filtration on an XBridge Protein BEH SEC column, 200Å, 3.5 µm, 7.8 mm X 150 mm (Waters). The column was equilibrated and run at 0.9 ml/min with 100mM sodium phosphate pH 7.0 as a running buffer for all analyses. Purified samples (0.5mg/ml) were injected (15ul) and eluted with a run time of 15 min.

Mouse PBMC Cytokine Release Assay

In vitro bioactivity was assessed by evaluating the ability of our scIL-10 constructs to inhibit the production of TNFα in LPS stimulated C57BL/6 mouse PBMCs (Bioreclamation). For the assay, PBMCs cells were plated at 50,000 cells/well in RPMI media containing 10% heat inactivated fetal bovine serum. Cells were incubated for 18 hours at 37°C, 5% CO_2 with 100 ng/mL LPS and varying concentrations of the scIL-10 constructs (R&D Systems). After 18 hours, TNFα production was measured using V-Plex mouse TNFa MSD (Mesoscale Discovery). See Tables 5 and 6 below for IC50 values.

MC/9 Assay

In vitro bioactivity was assessed by evaluating the ability of our scIL-10 constructs to stimulate proliferation of the mouse mast cell line MC/9 (ATCC CRL-8306). For the assay, MC/9 cells were plated at 10,000 cells/well in DMEM media containing 10% heat inactivated fetal bovine serum, 2 mM glutamine and 0.05 mM 2-mercaptoethanol. Cells were incubated for 72 hours at 37°C, 5% CO_2 with varying concentrations of human IL-10 (R&D Systems), RDB3515, RDB3516 or RDB3509. After 72 hours, the cells were stained with CellTiter-Blue

(Promega) for 4 hours at 37°C, 5% CO_2 according to the manufacturer's protocol. Fluorescent measurements were taken at 560/590 nm. See Tables 5 and 6 below for EC50 values.

Table 5

SEQ ID NO	DESCRIPTION	PBMC + LPS (pM)	MC/9 (pM)	RATIO
	wtIL-10	**0.45**	**5**	**11.1**
12	scIL-10:CL:CH1:Fc	0.06	60	1000
13	scIL-10:Fc	0.08	409	5112.5
14	(scIL-10:Fc), hinge truncation mutant 1	0.06	494	8233.3
15	(scIL-10:Fc), hinge truncation mutant 2	0.1	864	8640
16	(scIL-10:Fc), hinge truncation mutant 3	1.4	1007	719.3
17	I87A:linker:I87A (vIL10 mutant)	0.38	1775	4671.1
18	I87A:linker:wtIL-10 (vIL10 mutant)	0.03	107	3566.7
19	wtIL-10:linker:I87A (vIL10 mutant)	0.18	346	1922.2
20	D41F:linker:wtIL-10 (R1 mutant)	0.12	264	2200
21	wtIL-10:linkerD41F (R1 mutant)	0.18	1368	7600
22	D41F:linker:D41F (R1 mutant)	ND	ND	No activity
23	M22A:linker:wtIL-10 (R2 mutant)	0.077	47	610.4
24	wtIL-10:linker:M22A (R2 mutant)	0.045	38	844.4
25	M22A:linker:M22A (R2 mutant)	0.73	541	741.1
26	M22A:linker:D41F (R1+R2 mutant)	2.1	987	470
27	wtIL-10:linker:M22A, D41F (R1+R2 mutant)	2.6	6590	2534.6
28	D41F:linker:M22A (R1+R2 mutant)	8.9	ND	>> 10000
29	M22A:linker:D41F (R1+R2 mutant)	4.4	ND	>> 10000
30	M22A, D41F:linker:M22A, D41F (R1+R2 pan mutant)	ND	ND	No activity
31	M22A, D41F:linker:M22A (R1+R2 triple mutant)	65	ND	>> 10000
32	M22A, D41F:linker:D41F (R1+R2 triple mutant)	ND	ND	No activity
33	M22A:linker:M22A, D41F (R1+R2 triple mutant)	502	ND	>> 10000
34	D41F:linker:M22A, D41F (R1+R2 triple mutant)	ND	ND	No activity
35	scIL-10:CL:CH1:Fc (scIL10 5aa linker)	0.007	0.6	85.7
36	scIL-10:CL:CH1:Fc (scIL10 3aa linker)	0.03	1.5	50

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Table 6

SEQ ID NO	DESCRIPTION	PBMC + LPS	MC/9	Ratio
12	scIL-10:CL:CH1:Fc	0.5	133	266
37	wtIL-10:linker:D41A (R1 mutant)	0.6	1430	2383.333
38	wtIL-10:linker:M22A, D41A (R1+R2 mutant)	2.4	3602	3602
39	D41A:linker:M22A (R1+R2 mutant)	11.1	ND	ND
40	M22A:linker:D41A (R1+R2 mutant)	6	ND	ND
41	wtIL-10:linker:D41N (R1 mutant)	1.4	747	533.5714
42	wtIL-10:linker:M22A, D41N (R1+R2 mutant)	0.8	791	988.75
43	D41N:linker:M22A (R1+R2 mutant)	4.6	2780	604.3478
44	M22A:linker:D41N (R1+R2 mutant)	2.1	ND	ND

As shown in Table 5, the ratio for WT IL-10 was ~11. The ratio for SEQ ID NO: 12 was 1000, showing that just by building the scIL-10 sequence on the CL:CH1:Fc scaffold, the anti-inflammatory window is increased. The following experiments were conducted with various configurations of scIL-10 molecules of Formula 1 including unsubstituted scIL-10, scIL-10 variants and LINKER lengths of various sizes on the CL:CH1:Fc scaffold.

Experiments were conducted using the constructs of Tables 5 and 6 to explore the effects unsubstituted scIL-10 and scIL-10 variants that disrupt the scIL-10 interfaces with different combinations of the two IL-10R1 and two IL-10R2 receptor chains from the scIL-10 heteropentameric signaling complex. Mutations that disrupt either one of the two IL-10R1 interfaces SEQ ID NOS: 20, 21, 37 and 41 as illustrated in FIG. 7, slightly weaken the anti-inflammatory potency, while significantly weakening the immunostimulatory potency, resulting in an increase in the anti-inflammatory window size.

Introducing a double mutation that simultaneously disrupts both IL-10R1 interfaces (SEQ ID NO: 22) results in a construct with no measurable anti-inflammatory or immunostimulatory activities. This demonstrates that in order for scIL-10 to signal via the IL-10 receptor, it must be able to recruit at least 1 IL-10R1 receptor chain. Since the IL-10R1 receptor chain is known to be the "high affinity" receptor chain (binding more tightly to IL-10 than IL-10R2 does), it is likely that mutations that simultaneously disrupt both IL-10R1 binding interfaces would eliminate or significatly weaken the ability of scIL-10 to bind to the IL-10 receptor, resulting in no signal transduction at all.

Mutations that disrupt either one of the two IL-10R2 interfaces (SEQ ID NOS: 23 and 24), as illustrated in FIG. 8, demonstrate no change in the anti-inflammatory potency,

while showing a slight increase in the immunostimulatory potency, resulting in slightly decreased anti-inflammatory window sizes. Introducing a double-mutant that simultaneously disrupts both IL-10R2 interfaces (SEQ ID NOS; 29) leads to a loss in potency for both anti-inflammatory and immunostimulatory activities, resulting in a construct with an anti-inflammatory window size similar to that of the IL-10R2 interface single mutants, which is slightly reduced relative to the native scIL-10 construct. This result demonstrates that mutations that disrupt the IL-10R2 interface do not alone have the potential to expand the anti-inflammatory window of scIL-10.

Mutations that simultaneously disrupt one of the IL-10R1 and one of the IL-10R2 interfaces were explored as illustrated in FIG. 8. Mutations located in the IL-10R1 and IL-10R2 sites from the same side of the scIL-10 fused dimer (SEQ ID NOS: 26 AND 27) demonstrate weakened potency for both anti-inflammatory and immunostimulatory activities; one of those combinations (SEQ ID NO: 27) displays a significantly increased anti-inflammatory window size. Mutations located in an IL-10R1 interface and an IL-10R2 interface from opposite sides of the scIL-10 fused dimer (SEQ ID NOS: 28 and 29) display weakened anti-inflammatory potency, and no measurable immunostimulatory activities at the concentrations tested. Therefore, they display extremely large anti-inflammatory windows. Since IL-10 receptor signal transduction requires IL-10R1 and IL-10R2 to be clustered following IL-10 binding, these data indicate that the optimal strategy for attenuating immunstimulatory activity (and thereby increasing the anti-inflammatory window) is to target both of the pairs of IL-10R1/IL-10R2 receptor chains. Since the IL-10R1 interface scan revealed that signaling requires that at least one IL-10R1 interface be competent for binding, it is necessary to target the IL-10R2 interface on the opposite side of the scIL-10 fused dimer, to effectively disrupt both pairs of IL-10R1/IL-10R2 receptor chains that cluster upon scIL-10 binding. This pattern of mutations more dramatically modulates scIL-10 bioactivity on cells that mediate immunostimulation, while the cells that mediate the anti-inflammatory effects remain quite sensitive to scIL-10 signaling.

Example 3 Varying linker length of scIL-10

The scIL-10 of Formula 1 wherein LINKER length is varied are fused to a single chain Fc linker of Formula 2 wherein L1 is CL-CH1-Fc as per Formula 3. The amino acid sequences of each full length scIL-10-L1-HINGE-Fc fusion variant protein synthesized is found in Table 7.

For expression in mammalian cells, the N-terminal leader sequence of SEQ ID NO: 48 was added to each of the protein sequences found in Table 7.

The amino acid sequences of each fusion protein are found in Table 7. Expression of peptides are as described in Example 2. Bioactivity of was tested in a mouse PBMC cytokine release assay and an MC/9 assay as described in Example 2. The results are found in Table 5 of Example 2. The results show that decreasing the size of the linker reduces the size of the anti-inflammatory window, implying that the linker length affects the strength of the IL-10R1 and IL-10R2 interfaces in ways that reduce the selectivity for anti-inflammatory potency over immunostimulatory potency.

Table 7

Description	Amino Acid Sequence
Unsubstituted scIL-10 (5aa linker):CL:CH1:Fc)	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGSPGQG TQSENSCTHF PGNLPNMLRD LRDAFSRVKT FFQMKDQLDN LLLKESLLED FKGYLGCQAL SEMIQFYLEE VMPQAENQDP DIKAHVNSLG ENLKTLRLRL RRCHRFLPCE NKSKAVEQVK NAFNKLQEKG IYKAMSEFDI FINYIEAYMT MKIRNGGGGS GGGGSRTVAA PSVFIFPPSD EQLKSGTASV VCLLNNFYPR EAKVQWKVDN ALQSGNSQES VTEQDSKDST YSLSSTLTLS KADYEKHKVY ACEVTHQGLS SPVTKSFNRG ECGGGGSGGG GSGGGGSGGG GSASTKGPSV FPLAPSSKST SGGTAALGCL VKDYFPEPVT VSWNSGALTS GVHTFPAVLQ SSGLYSLSSV VTVPSSSLGT QTYICNVNHK PSNTKVDKRV EPKSCDKTHT CPPCPAPELL GGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ YNSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR EEMTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP PVLDSDGSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP GK (SEQ ID NO: 39)
scIL-10 of Formula 1 wherein LINKER is 3 amino acid linker	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGGSPGQGTQ SENSCTHFPG NLPNMLRDLR DAFSRVKTFF QMKDQLDNLL LKESLLEDFK GYLGCQALSE MIQFYLEEVM PQAENQDPDI KAHVNSLGEN LKTLRLRLRR CHRFLPCENK SKAVEQVKNA FNKLQEKGIY KAMSEFDIFI NYIEAYMTMK IRNGGGGSGG GGSRTVAAPS VFIFPPSDEQ LKSGTASVVC LLNNFYPREA KVQWKVDNAL QSGNSQESVT EQDSKDSTYS LSSTLTLSKA DYEKHKVYAC EVTHQGLSSP VTKSFNRGEC GGGGSGGGGS GGGGSGGGGS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKRVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP

	ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK (SEQ ID NO: 36)

Example 4: Modulating the anti-inflammatory window of scIL-10 via steric crowding.

The amino acid sequences of each scIL-10 fusion protein used in this experiment are found in Table 8. Expression of peptides is as described in Example 2. Bioactivity of peptides was tested in a mouse PBMC cytokine release assay and an MC/9 assay as described in Example 2. The results are found in Table 5 of Example 2. The results show that as the hinge is shortened, the anti-inflammatory window increases in size. Without being limited to any particular theory, this implies that hinge truncation increases steric crowding between two scIL-10 moieties, resulting in modulation of the IL-10R1 and IL-10R2 interfaces, which translates to altered anti-inflammatory and immunostimulatory potencies.

Table 8

Description:	Amino Acid Sequence
scIL-10:Fc	MYRMQLLSCI ALSLALVTNS SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGSPGQG TQSENSCTHF PGNLPNMLRD LRDAFSRVKT FFQMKDQLDN LLLKESLLED FKGYLGCQAL SEMIQFYLEE VMPQAENQDP DIKAHVNSLG ENLKTLRLRL RRCHRFLPCE NKSKAVEQVK NAFNKLQEKG IYKAMSEFDI FINYIEAYMT MKIRNEPKSS DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPGK (SEQ ID NO: 13)
(scIL-10:Fc), 4AA hinge truncation	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGSPGQG TQSENSCTHF PGNLPNMLRD LRDAFSRVKT FFQMKDQLDN LLLKESLLED FKGYLGCQAL SEMIQFYLEE VMPQAENQDP DIKAHVNSLG ENLKTLRLRL RRCHRFLPCE NKSKAVEQVK NAFNKLQEKG IYKAMSEFDI FINYIEAYMT MKIRNSDKTH TCPPCPAPEL LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV DVSHEDPEVK FNWYVDGVEV HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKALPAPIEK TISKAKGQPR EPQVYTLPPS REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT PPVLDSDGSF FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS PGK (SEQ ID NO: 14)
(scIL-10:Fc), 7 aa hinge truncation	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGSPGQG TQSENSCTHF PGNLPNMLRD LRDAFSRVKT FFQMKDQLDN LLLKESLLED FKGYLGCQAL SEMIQFYLEE VMPQAENQDP DIKAHVNSLG ENLKTLRLRL RRCHRFLPCE NKSKAVEQVK NAFNKLQEKG IYKAMSEFDI FINYIEAYMT MKIRNTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA

	LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK (SEQ ID NO: 15)
(scIL-10:Fc), 10aa hinge truncation.	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGSPGQG TQSENSCTHF PGNLPNMLRD LRDAFSRVKT FFQMKDQLDN LLLKESLLED FKGYLGCQAL SEMIQFYLEE VMPQAENQDP DIKAHVNSLG ENLKTLRLRL RRCHRFLPCE NKSKAVEQVK NAFNKLQEKG IYKAMSEFDI FINYIEAYMT MKIRNTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK (SEQ ID NO: 16)

Example 5- scIL-10

Experiments were conducted with scIL-10 of Formula 1 wherein LINKER was of varying lengths. The amino acid sequences synthesized for these experiments are shown in Table 9. Expression of SEQ ID NOS: 45 and 46 is as described in Example 2. Bioactivity of SEQ ID NOS 45 was tested in an MC/9 assay as described in Example 2. The data showed that the value for SEQ ID NO: 45 in the MC/9 was 5.6 pM.

Bioactivity of SEQ ID NOS 45 and 46 will be further tested in a mouse PBMC cytokine release assay and an MC/9 assay as described in Example 2. The results will show that the scIL-10 moiety, absent any fusion partner, demonstrates highly potent bioactivity, consistent with the trends observed for scIL-10 Fc fusion proteins.

Table 9

Description	Amino Acid Sequence
scIL-10 with 5 amino acid linker	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGSPGQG TQSENSCTHF PGNLPNMLRD LRDAFSRVKT FFQMKDQLDN LLLKESLLED FKGYLGCQAL SEMIQFYLEE VMPQAENQDP DIKAHVNSLG ENLKTLRLRL RRCHRFLPCE NKSKAVEQVK NAFNKLQEKG IYKAMSEFDI FINYIEAYMT MKIRN (SEQ ID NO: 45)
scIL-10 with 10 amino acid linker	SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGGGSGG SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN (SEQ ID NO: 46)

EXAMPLE 6

The scIL-10 of Formula 1was fused to a mucin domain linker comprising a tandem repeat of MUC 20 which in turn was fused to an Fc domain. The amino acid sequence of the (scIL-10 (5aa linker))-(mucin linker)-Fc is found in Table 10. For expression in mammalian cells, the N-terminal leader sequence of SEQ ID NO: 48 was added to the protein found in Table 11.

The amino acid sequences of each fusion protein are found in Table 10. Expression of peptides are as described in Example 2. Bioactivity of was tested in a mouse PBMC cytokine release assay and an MC/9 assay as described in Example 2. The results are found in Table 11. The results show that the bioactivities of scIL-10 Fc fusion proteins are consistent regardless of the composition of the linker domain connecting the scIL-10 and Fc domains.

TABLE 10

Description	Amino Acid Sequence
(scIL-10(5aa linker))-(mucin linker)-Fc	MYRMQLLSCI ALSLALVTNS SPGQGTQSEN SCTHFPGNLP NMLRDLRDAF SRVKTFFQMK DQLDNLLLKE SLLEDFKGYL GCQALSEMIQ FYLEEVMPQA ENQDPDIKAH VNSLGENLKT LRLRLRRCHR FLPCENKSKA VEQVKNAFNK LQEKGIYKAM SEFDIFINYI EAYMTMKIRN GGSGGSPGQG TQSENSCTHF PGNLPNMLRD LRDAFSRVKT FFQMKDQLDN LLLKESLLED FKGYLGCQAL SEMIQFYLEE VMPQAENQDP DIKAHVNSLG ENLKTLRLRL RRCHRFLPCE NKSKAVEQVK NAFNKLQEKG IYKAMSEFDI FINYIEAYMT MKIRNSGSGG ASSESSASSD GPHPVITESR ASSESSASSD GPHPVITESR EPKSSDKTHT CPPCPAPELL GGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ YNSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR EEMTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP PVLDSDGSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP GK (SEQ ID NO: 52)

Table 11

SEQ ID NO	PMBC	MC/9	Ratio
52	0.11 pM	21 pM	190.9

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

While this invention has been particularly shown and described with references to preferred features thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims. It should also be understood that the various features of the invention described herein are not mutually exclusive and that features may be combined in whole or in part in accordance with the invention.

CLAIMS

1. A method of modulating the immunostimulatory and anti-inflammatory properties of IL-10 in a patient in need of IL-10 therapy comprising administering scIL-10 comprising an amino acid sequence arrangement from N-terminus to C-terminus in accordance with Formula 1:

(first monomer subunit)-LINKER-(second monomer subunit)

Formula 1

wherein the first monomer subunit, the second monomer subunit or both the first and second monomer subunits may be independently selected from: an unsubstituted IL-10 monomer subunit; or a substituted IL-10 monomer subunit comprising at least one amino acid substitution;

wherein scIL-10 modulates the immunostimulatory or anti-inflammatory properties as compared to wtIL-10,

wherein LINKER is an amino acid linker of between about 1 and about 100 amino acids in length; and

wherein scIL-10 is optionally covalently attached to a fusion partner.

2. The method of claim 1 wherein LINKER is 5-15 amino acids in length.

3. The method of claim 1 wherein the amino acid substitutions comprise the substitution of amino acids of scIL-10 that interface with IL-10R1, IL-10R2 or amino acids that interface with both IL-10R1 and IL-10R2.

4. The method of claim 1 wherein the amino acid substitutions comprise substitutions of amino acids selected from: methionine at position 22 and aspartic acid at position 41 or any combination thereof.

5. The method of claim 4 wherein aspartic acid at position 41 is substituted on the first monomer subunit or on the second monomer subunit but not both monomer subunits.

6. The method of claim 5 wherein methionine at position 22 is substituted on only one monomer subunit that is not the same monomer subunit comprising the substitution of aspartic acid at position 41.

7. The method of claim 1 wherein the amino acid substitution comprises isoleucine at position 87.

8. The method of claim 3 wherein the amino acid substitutions are selected from: methionine at position 22 to alanine (M22A); aspartic acid at position 41 to asparagine (D41N); aspartic acid at position 41 to alanine (D41A); aspartic acid at position 41 to phenylalanine (D41F).

9. The method of claim 1 comprising a fusion partner wherein scIL-10 is fused to the hinge region IgG1.

10. The method of claim 1 comprising a fusion partner wherein scIL-10 is fused to a modified hinge region if IgG1 wherein the modification to the hinge region is the deletion of between 1 and 10 amino acids from the hinge region of IgG1.

11. The method of claim 1 comprising a fusion partner wherein scIL-10 is fused to the hinge region of IgG1 via a mucin linker.

12. The method of claim 11 wherein the mucin linker comprises an amino acid sequence that is a tandem repeat of MUC20.

13. The method of claim 1 comprising a fusion partner wherein scIL-10 is fused to a single chain Fc linker wherein the fusion protein has the sequence of Formula 2

(scIL-10)-L1-HINGE-Fc

Formula 2

wherein,

L1 is a linker having the following arrangement from amino-terminus to carboxy-terminus:

L2-CL-L3-CH1-L4 or L2-CH1-L3-CL-L4

wherein,

L2 and L4 are independently polypeptide linkers or are independently absent;
L3 is a polypeptide linker;
CL is a constant region polypeptide of an immunoglobulin light chain; and
CH1 is a constant region polypeptide from a CH1 domain of an immunoglobulin heavy chain;
HINGE is a hinge sequence of an immunoglobulin or is absent with the proviso that if HINGE is absent, L4 is present; and
Fc is the carboxy-terminus of an immunoglobulin or any active fragment or derivative thereof.

14. An scIL-10 polypeptide comprising an amino acid sequence arrangement from N-terminus to C-terminus in accordance with Formula 1:

(first monomer subunit)-LINKER-(second monomer subunit)

Formula 1

wherein the first monomer subunit or the second monomer subunit may be independently selected from: an unsubstituted IL-10 monomer subunit; or a substituted IL-10 monomer subunit with the proviso that at least one of the first monomer subunit or the second monomer subunit comprises at least one amino acid substitution;
wherein LINKER is an amino acid linker of between about 1 and about 100 amino acids in length; and
wherein scIL-10 is optionally covalently attached to a fusion partner.

15. The polypeptide of claim 14 wherein LINKER is 5-15 amino acids in length.

16. The polypeptide of claim 14 wherein the amino acid substitutions comprise the substitution of amino acids of scIL-10 that interface with IL-10R1, IL-10R2 or amino acids that interface with both IL-10R1 and IL-10R2.

17. The polypeptide of claim 14 wherein the amino acid substitutions comprise substitutions of amino acids selected from: methionine at position 22 and aspartic acid at position 41 or any combination thereof.

18. The polypeptide of claim 17 wherein aspartic acid at position 41 is substituted on the first monomer subunit or on the second monomer subunit but not both monomer subunits.

19. The polypeptide of claim 18 wherein methionine at position 22 is substituted on only one monomer subunit that is not the same monomer subunit comprising the substitution of aspartic acid at position 41.

20. The polypeptide of claim 14 wherein the amino acid substitution comprises isoleucine at position 87.

21. The polypeptide of claim 16 wherein the amino acid substitutions are selected from: methionine at position 22 to alanine (M22A); aspartic acid at position 41 to asparagine (D41N); aspartic acid at position 41 to alanine (D41A); aspartic acid at position 41 to phenylalanine (D41F).

22. The polypeptide of claim 1 comprising a fusion partner wherein scIL-10 is fused to the hinge region IgG1.

23. The polypeptide of claim 14 comprising a fusion partner wherein scIL-10 is fused to a modified hinge region if IgG1 wherein the modification to the hinge region is the deletion of between 1 and 10 amino acids from the hinge region of IgG1.

24. The polypeptide of claim 14 comprising a fusion partner wherein scIL-10 is fused to the hinge region of IgG1 via a mucin linker.

25. The polypeptide of claim 24 wherein the mucin linker comprises an amino acid sequence that is a tandem repeat of MUC20.

26. An scIL-10 polypeptide of claim 14 comprising a fusion partner wherein scIL-10 is fused to a single chain Fc linker wherein the fusion protein comprises an amino acid sequence of Formula 2

(scIL-10)-L1-HINGE-Fc

Formula 2

wherein,

L1 is a linker having the following arrangement from amino-terminus to carboxy-terminus:

L2-CL-L3-CH1-L4 or L2-CH1-L3-CL-L4

wherein,

L2 and L4 are independently polypeptide linkers or are independently absent;

L3 is a polypeptide linker;

CL is a constant region polypeptide of an immunoglobulin light chain; and

CH1 is a constant region polypeptide from a CH1 domain of an immunoglobulin heavy chain;

HINGE is a hinge sequence of an immunoglobulin or is absent with the proviso that if HINGE is absent, L4 is present; and

Fc is the carboxy-terminus of an immunoglobulin or any active fragment or derivative thereof.

27. The polypeptide of claim 26, wherein CL, CH1, HINGE and Fc are at least 90% identical to the CL, CH1, hinge and Fc regions respectively of human IgG1.

28. The polypeptide of claim 26, wherein L3 is a polypeptide linker having the amino acid sequence $(GGGGS)_n$ wherein n is 1-5.

29. The polypeptide of claim 26, wherein L2 is present and is a polypeptide linker having the amino acid sequence $(GGGGS)_n$ wherein n is 1-5.

30. The polypeptide of claim 26, wherein L4 is present and is a polypeptide linker having the amino acid sequence $(GGGGS)_n$ wherein n is 1-5.

31. The polypeptide of claim 26, wherein HINGE and L2 are present and L4 is absent.

32. The polypeptide of claim 26, wherein HINGE, L2 and L4 are present.

33. The polypeptide of claim 26, wherein HINGE is absent and L4 is present.

34. The polypeptide of claim 26, wherein HINGE is absent and L2 and L4 are present.

35. A dimerized complex comprising the polypeptide of claim 26 wherein L1 is a linker having the following arrangement from amino-terminus to carboxy-terminus: L2-CL-L3-CH1-L4.

36. A polypeptide of claim 26 selected from the group consisting of: SEQ ID NOs: 20-21 and SEQ ID NOS: 37-44

37. A polypeptide of claim 26 selected from the group consisting of: SEQ ID NOS: 17, 18 and 19.

38. An scIL-10 polypeptide comprising an amino acid sequence arrangement from N-terminus to C-terminus in accordance with Formula 1:

(first monomer subunit)-LINKER-(second monomer subunit)

Formula 1

wherein the first monomer subunit, the second monomer subunit or both the first and second monomer subunits may be independently selected from: an unsubstituted IL-10 monomer subunit; or a substituted IL-10 monomer subunit;
wherein LINKER is an amino acid linker of between about 1 and about 100 amino acids in length with the proviso that the linker is not GGSGGGGSGG (SEQ ID NO: 3); and
wherein scIL-10 is covalently attached to a fusion partner.

39. A polypeptide of claim 38 comprising a fusion partner wherein scIL-10 is fused to a single chain Fc linker wherein the fusion protein comprises an amino acid sequence of Formula 2

(scIL-10)-L1-HINGE-Fc

Formula 2

wherein,
L1 is a linker having the following arrangement from amino-terminus to carboxy-terminus:

L2-CL-L3-CH1-L4 or L2-CH1-L3-CL-L4

wherein,

L2 and L4 are independently polypeptide linkers or are independently absent;

L3 is a polypeptide linker;

CL is a constant region polypeptide of an immunoglobulin light chain; and

CH1 is a constant region polypeptide from a CH1 domain of an immunoglobulin heavy chain;

HINGE is a hinge sequence of an immunoglobulin or is absent with the proviso that if HINGE is absent, L4 is present; and

Fc is the carboxy-terminus of an immunoglobulin or any active fragment or derivative thereof.

40. A polypeptide of claim 39 selected from SEQ ID NO 35 and SEQ ID NO: 36.

41. An scIL-10 polypeptide comprising an amino acid sequence arrangement from N-terminus to C-terminus in accordance with Formula 1:

(first monomer subunit)-LINKER-(second monomer subunit)

Formula 1

wherein the first monomer subunit, the second monomer subunit or both the first and second monomer subunits may be independently selected from: an unsubstituted IL-10 monomer subunit; or a substituted IL-10 monomer subunit comprising at least one amino acid substitution;

wherein LINKER is an amino acid linker of between about 1 and about 100 amino acids in length; and

wherein scIL-10 is covalently attached to a fusion partner that comprises a mucin domain polypeptide.

42. The polypeptide of claim 41 comprising SEQ ID NO: 52.

43. The method of claim 1 wherein the polypeptide comprises SEQ ID NOS: 12-21, 23-29, 31, 33, and 35-45 and 52.

44. The polypeptide of claim 38 wherein the fusion partner comprises an IgG1 Fc region including a hinge region.

45. The polypeptide of claim 44 comprising SEQ ID NO: 13.

46. The polypeptide of claim 44 wherein 1- 10 amino acids have been deleted from the hinge region.

5

47. The polypeptide of claim 46 selected from SEQ ID NOS: 14-16.

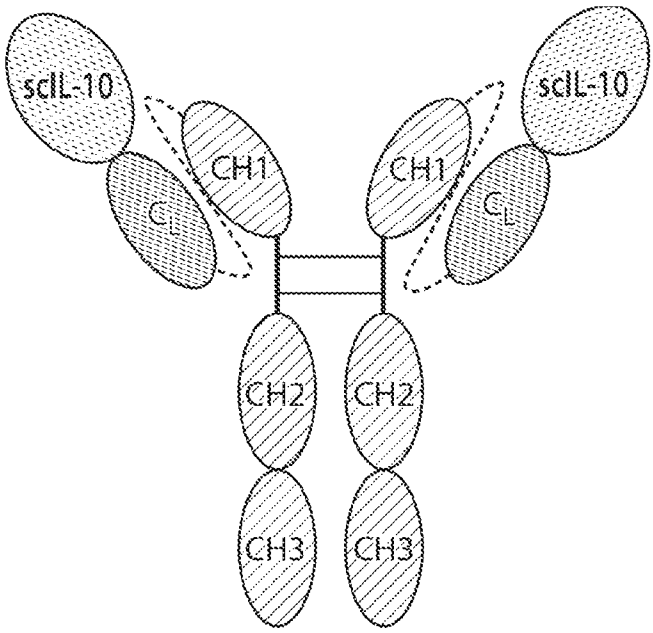
scIL-10
scIL-10
CH1
CH1
C_L
C_L
CH2
CH2
CH3
CH3

FIG. 1

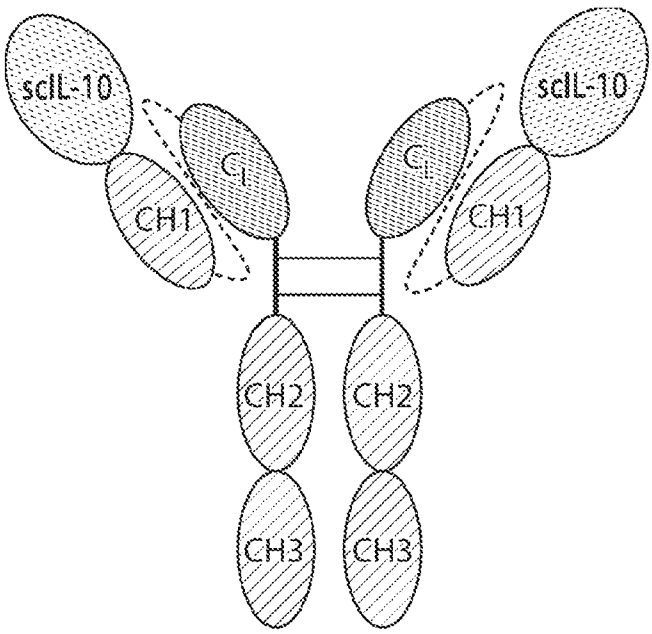
scIL-10
scIL-10
C_L
C_L
CH1
CH1
CH2
CH2
CH3
CH3

FIG. 2

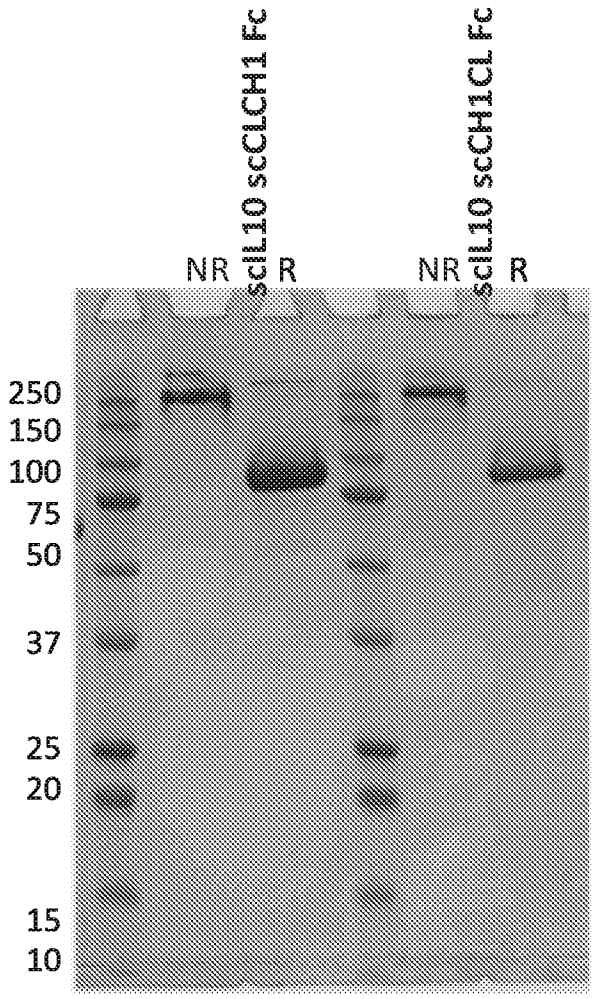
scIL10 scCLCH1 Fc
scIL10 scCH1CL Fc
NR
R
NR
R
250
150
100
75
50
37
25
20
15
10

FIG. 3

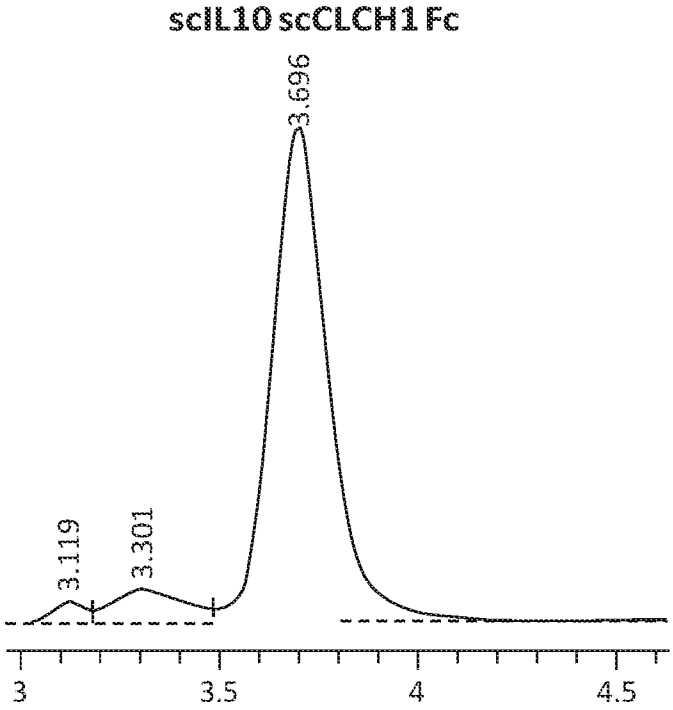
scIL10 scCLCH1 Fc
3.696
3.119
3.301
3
3.5
4
4.5

FIG. 4

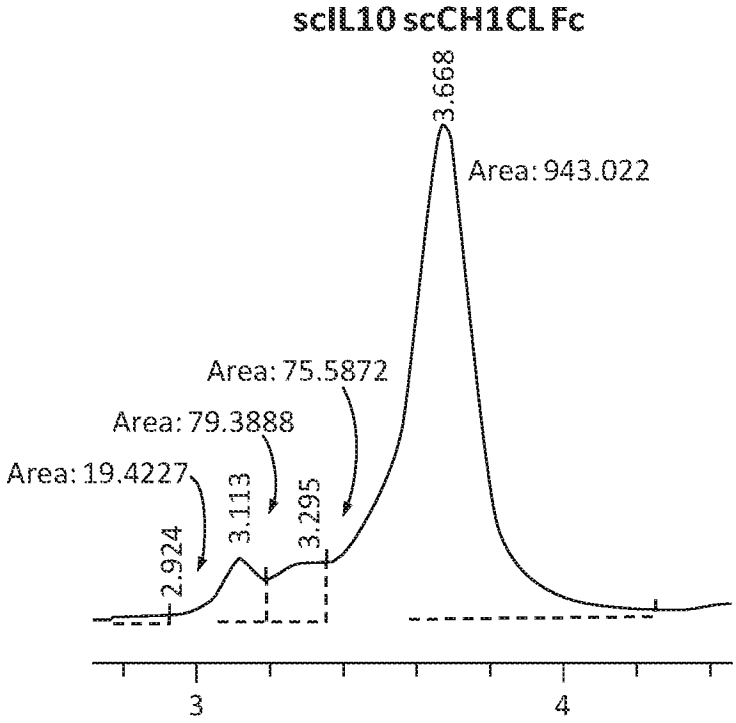
scIL10 scCH1CL Fc
3.668
Area: 943.022
Area: 75.5872
Area: 79.3888
Area: 19.4227
3.113
3.295
2.924
3
4

FIG. 5

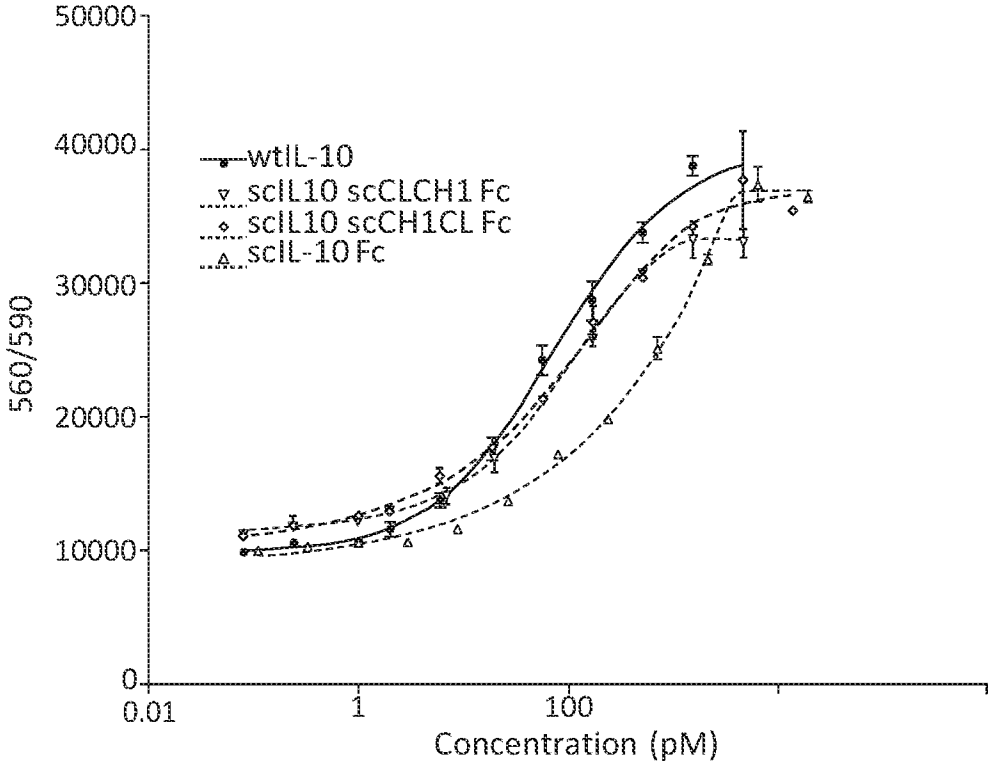
50000
40000
30000
20000
10000
0
560/590
wtIL-10
scIL10 scCLCH1 Fc
scIL10 scCH1CL Fc
scIL-10 Fc
0.01
1
100
Concentration (pM)

FIG. 6

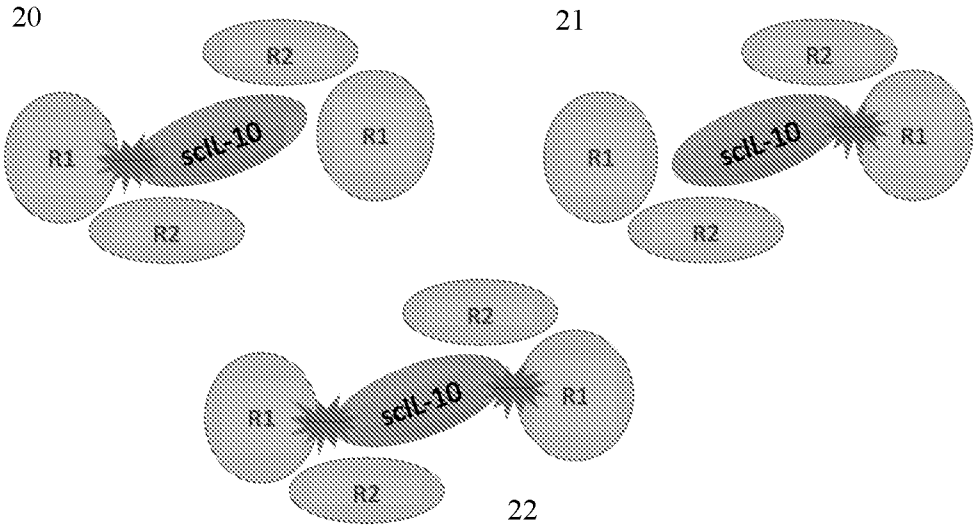

FIG. 7

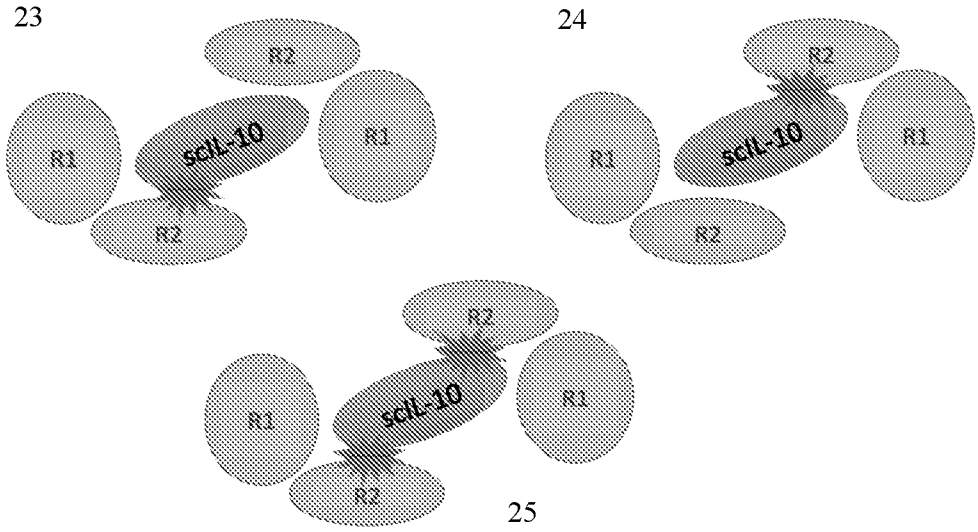

FIG. 8

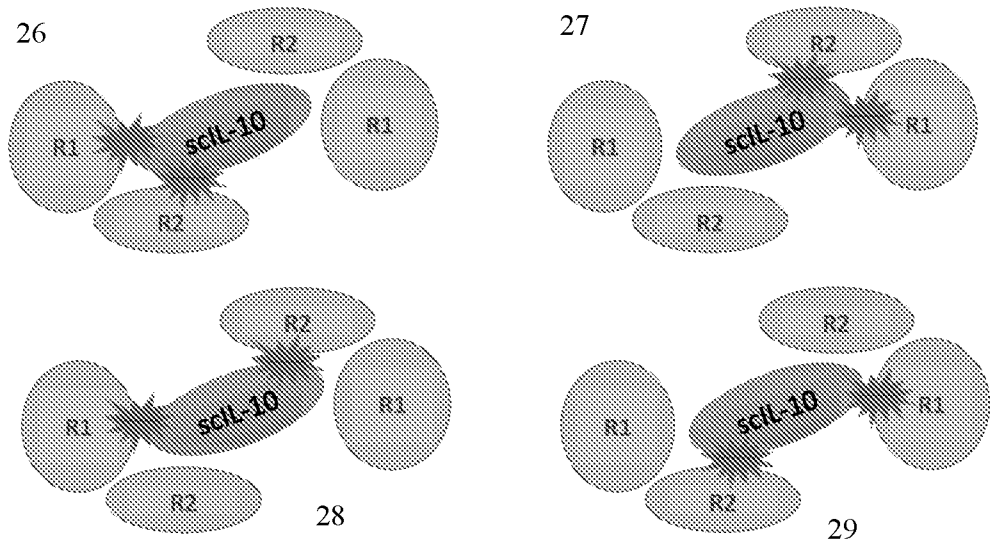
26
R2
R1
scIL-10
R1
R2
27
R2
R1
scIL-10
R1
R2
R2
R1
scIL-10
R1
R2
28
R2
R1
scIL-10
R1
R2
29

FIG. 9