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(72) Inventeurs/Inventors:
ALVARAZ, JUAN, US;
MOUSTAKAS, DEMETRI T., US;
BRODKIN, HEATHER R., US;
MCSWEENEY, LESLIE A., US

(73) Propriétaire/Owner:
ALKERMES, INC., US

(74) Agent: GOWLING WLG (CANADA) LLP

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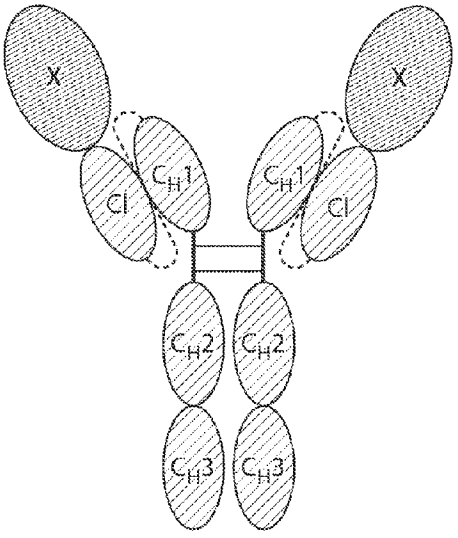


(57) Abrégé/Abstract:
The present invention provides novel, single chain Fc fusion proteins having improved properties. The invention provides single chain fusions of soluble proteins fused to the Fc region of an immunoglobulin via a novel linker comprising a constant region of an immunoglobulin light chain linked to a CHI constant region of an immunoglobulin heavy chain. This novel linker confers favorable properties on the Fc fusion proteins of the invention such as improved bioactivity and increased half-life as compared to non-Fc fusion counterparts or as compared to prior art Fc fusion proteins. The novel Fc fusion protein scaffold as described herein may be designed to include soluble proteins of interest capable of binding or interacting with any target of interest. Preferably, the Fc fusion protein of the invention is a dimer. The dimer preferably forms via a disulfide bond between free cysteine residues in the hinge region of two monomeric Fc fusion proteins of the invention.

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(71) Applicant: **ALKERMES, INC.** [US/US]; 852 Winter Street, Waltham, MA 02451 (US).

(72) Inventors: **ALVARAZ, Juan**; 6 Wyndbrook Lane, Chelmsford, MA 01824 (US). **MOUSTAKAS, Demetri, T.**; 367 Pleasant Street, Belmont, MA 02478 (US). **BRODKIN, Heather, R.**; 143 Namquid Dr., Warwick, RI 02888 (US). **MCSWEENEY, Leslie, A.**; 41 Whitewood Road, Milford, MA 01757 (US).

(74) Agents: **VANSTONE, Darlene, A.** et al.; Elmore Patent Law Group, 484 Groton Road, Westford, MA 01886 (US).

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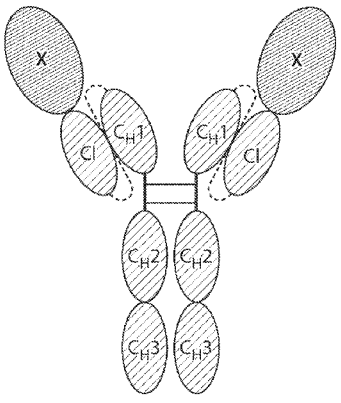


FIG. 1A

(57) Abstract: The present invention provides novel, single chain Fc fusion proteins having improved properties. The invention provides single chain fusions of soluble proteins fused to the Fc region of an immunoglobulin via a novel linker comprising a constant region of an immunoglobulin light chain linked to a CHI constant region of an immunoglobulin heavy chain. This novel linker confers favorable properties on the Fc fusion proteins of the invention such as improved bioactivity and increased half-life as compared to non-Fc fusion counterparts or as compared to prior art Fc fusion proteins. The novel Fc fusion protein scaffold as described herein may be designed to include soluble proteins of interest capable of binding or interacting with any target of interest. Preferably, the Fc fusion protein of the invention is a dimer. The dimer preferably forms via a disulfide bond between free cysteine residues in the hinge region of two monomeric Fc fusion proteins of the invention.

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# Single Chain Fc Fusion Proteins

## RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 62/094,242, filed on December 19, 2014. The entire teachings of the above application are incorporated herein by reference.

## BACKGROUND OF THE INVENTION

One strategy for increasing serum half-life of a therapeutic protein 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. However, many prior art approaches to Fc fusion protein engineering have limitations including, but not limited to, immunogenicity and poor pharmacokinetic properties.

The present invention provides monomers and dimers of Fc fusion proteins comprising novel linkers having single chain constant light (CL) and constant heavy (CH1) immunoglobulin domains. Such novel linkers are also referred to herein as scCLCH1 linkers.

Without limitation to a particular theory, the novel linkers of the invention reduce steric hindrance between the protein "payloads" on each of the single chain Fc fusion protein molecules when such molecules form dimers. Steric hindrance can result in losses in bioactivity, inefficient dimerization or reduction in the half-life of the dimer molecule for example, due to reduced binding to the FcRn. Thus incorporation of the novel linkers of the invention may result in improvement in bioactivity, increased dimer formation, in increased half-life, and the ability to incorporate larger protein payloads than those possible on prior Fc fusion proteins. Additionally, in some Fc proteins of the invention are able to form dimers that provide a more native antibody structure around the Fc domain that may improve binding of the dimer molecules to the FcRn receptor and therefore increase the circulating half-life of the novel Fc fusion proteins of the invention as compared to prior art fusion proteins.

## SUMMARY OF THE INVENTION

The present invention provides novel, single chain Fc fusion proteins having improved properties. The invention provides single chain fusions of soluble proteins fused to the Fc region of an immunoglobulin via a novel linker comprising a constant region of an immunoglobulin light chain (CL) linked to a CH1 constant region of an immunoglobulin heavy chain (scCLCH1 or scCH1CL linkers). This novel linker confers favorable properties on the Fc fusion proteins of the invention such as improved bioactivity and increased half-life as compared to non-Fc fusion counterparts or as compared to prior art Fc fusion proteins. The novel Fc fusion proteins as described herein may be designed to include soluble proteins of interest capable of binding or interacting with any target of interest with high specificity and affinity.

Preferably, an Fc fusion protein of the invention is a dimer. The dimers may be formed via covalent (e.g. disulfide linkages) or non-covalent interactions between single chain fusion proteins of the invention resulting in a homodimeric or heterodimeric protein complex retaining the advantageous properties of an antibody molecule for use as a therapeutic molecule.

In another aspect, the invention provides nucleic acids encoding the Fc fusion proteins provided herein. Also provided are vectors, including expression vectors, comprise a nucleic acid encoding any of the Fc fusion proteins described herein. Also provided are host cells containing such expression vectors and methods for producing the Fc fusion proteins described herein in the host cells.

## 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. 1A is a diagram of an Fc fusion protein homodimer comprising X fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker in accordance with the invention.

FIG. 1B is a diagram of an Fc fusion protein heterodimer of two polypeptide chains, where the first comprises X fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker, and the second comprises Y, where Y is different from X, fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker in accordance with the invention.

FIG. 1C is a diagram of an Fc fusion protein homodimer comprising X fused to the Fc region of an IgG1 antibody via the novel scCH1CL linker in accordance with the invention.

FIG. 1D is a diagram of an Fc fusion protein heterodimer of two polypeptide chains, where the first comprises X fused to the Fc region of an IgG1 antibody via the novel scCH1CL linker, and the second comprises Y, where Y is different from X, fused to the Fc region of an IgG1 antibody via the novel scCH1CL linker in accordance with the invention.

FIG. 2 is an SDS-PAGE showing expression of an Fc fusion protein comprising Factor IX fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker in accordance with the invention.

FIG. 3 is graph showing the clotting activity of an Fc fusion protein comprising Factor IX fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker in accordance with the invention.

FIG. 4 is a graph showing the *in vivo* half-life in rats when intravenously administered an Fc fusion protein comprising Factor IX fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker.

FIG. 5A is an SDS-PAGE showing expression of an Fc fusion protein comprising TNFR2 fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker under reducing conditions.

FIG. 5B is an SDS-PAGE showing expression of an Fc fusion protein comprising TNFR2 fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker under non-reducing conditions.

FIG. 6 is a graph showing the inhibition of the activation of a reporter gene by the TNFR2 fusion protein of the invention as compared to a standard TNF direct fusion protein.

FIG. 7 is a graph showing the *in vivo* half-life in rats when intravenously administered an Fc fusion protein comprising TNFR2 fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker as compared to a standard TNF direct fusion protein.

FIG. 8A is an SDS-PAGE showing expression of an Fc fusion protein comprising IL1Ra fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker under reducing conditions.

FIG. 8B is an SDS-PAGE showing expression of an Fc fusion protein comprising IL1Ra fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker under non-reducing conditions.

FIG. 9 is a graph showing the inhibition of the activation of a reporter gene by the IL1Ra fusion protein of the invention.

FIG. 10 is a graph showing the *in vivo* half-life in rats when intravenously administered an Fc fusion protein comprising IL1Ra fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker.

FIG. 11 is a graph showing the *in vivo* half-life in rats when intraocularly administered an Fc fusion protein comprising IL1Ra fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker.

FIG. 12A is a diagram of an Fc fusion protein homodimer of two polypeptide chains, wherein in each polypeptide chain comprises as X, a fusion of IL-2/IL-2Rα which is then fused to the Fc region of an IgG1 antibody via the novel scCLCH1linker.

FIG. 12B is a diagram of an Fc fusion protein homodimer of two polypeptide chains, wherein in each polypeptide chain comprises as X, a fusion of IL-2/IL-2Rα which is then fused to the Fc region of an IgG1 antibody via the novel scCH1CLlinker.

FIG. 13 is an SDS-PAGE showing expression of an Fc fusion protein comprising IL-2/IL-2Rα 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. 14A is a chromatogram showing the characterization of the IL-2/IL-2Rα fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker by analytical gel filtration.

FIG. 14B is a chromatogram showing the characterization of the IL-2/IL-2Rα fused to the Fc region of an IgG1 antibody via the novel scCH1CL linker by analytical gel filtration.

FIG. 15 is a graph showing activation of pSTAT5 by the IL-2/IL-2Rα single chain fusion proteins of the invention as compared to rhIL-2.

FIG. 16 is a graph showing the *in vivo* half-life in rats when intravenously and subcutaneously administered the IL-2/IL-2Rα single chain fusion proteins of the invention.

FIG. 17 is a diagram of an Fc fusion protein homodimer of two polypeptide chains, wherein in each polypeptide chain comprises as X, IFNβ which is then fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker of the invention.

FIG. 18 is an SDS-PAGE showing expression of an Fc fusion protein comprising IFNβ fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker under reducing and non-reducing conditions.

FIG. 19 is a chromatogram showing the characterization of IFNβ fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker by analytical gel filtration.

FIG. 20 is graph showing the activation of a reporter gene by the IFNβ fusion protein of the invention.

FIG. 21 is graph showing the mean concentration-time profile after IV (1.4 nMole/Kg and SC (3.6nMole/kg) administration of the IFNβ fusion protein of the invention.

FIG. 22A is a diagram of an Fc fusion protein homodimer of two polypeptide chains, wherein in each polypeptide chain comprises as X, IL-10 which is then fused to the Fc region of an IgG1 antibody via the novel scCLCH1 linker.

FIG. 22B is a diagram of an Fc fusion protein homodimer of two polypeptide chains, wherein in each polypeptide chain comprises as X, IL-10 which is then fused to the Fc region of an IgG1 antibody via the novel scCH1CL linker.

FIG. 23 is an SDS-PAGE showing expression of an Fc fusion protein comprising IL-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. 24A 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. 24B 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. 25 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.

## 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 chain X-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 X-L1-HINGE-Fc polypeptides forming a dimer complex, wherein said two single chain polypeptides have different amino acid sequences, in particular, X represents different soluble proteins or different portions of the same soluble protein. 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 share 100% identity, or at least 95% or at least 99% identity, the amino acid differences consisting of amino acid substitution, addition or deletion which does not affect the functional and physical properties of the polypeptide compared to the other one of the homodimer, 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 X-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.

Single Chain Fc Fusion Proteins

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

X-L1-HINGE-Fc

wherein, X is a soluble protein;

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 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, a soluble protein of interest 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".

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 the protein of interest (X) 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 the protein of interest (X) 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: 21))n or (Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 22))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: 22)) 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: 21))n or (Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 22))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: 22))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: 1).

An exemplary CH1 region corresponding to the CH1 region of hIgG1 includes:
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRV (SEQ ID NO: 2).

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: 3). 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: 4).

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: 5). 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: 6).

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.

The X-L1-HINGE-Fc fusion proteins described herein contain an X portion that may be any soluble protein of interest or any active fragment thereof or any active derivative thereof. Soluble proteins of interest (X) that may be fused to a single chain L1-HINGE-Fc scaffold in accordance with the invention include, but are not limited to: proteins or portions or fragments thereof that that can bind to, or interact with, a target molecule, cell, complex and/or tissue, such targets including enzyme substrates, proteins, nucleic acids, carbohydrates, lipids, low molecular weight compounds, and fragments thereof.

Soluble binding proteins of interest (X) include, but are not limited to, the following list of proteins, as well as active derivatives, active fragments, subunits, domains, motifs and epitopes belonging to the following list of proteins: renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VII, factor VIIIC, factor IX, tissue factor (TF), and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors such as, for example, EGFR, VEGFR; interferons such as alpha interferon (α-IFN), beta interferon (β-IFN) and gamma interferon (γ-IFN);

protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor; platelet-derived growth factor (PDGF); fibroblast growth factor such as AFGF and PFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-1, TGF-2, TGF-3, TGF-4, or TGF-5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des (1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD2, CD3, CD4, CD8, CD11a, CD14, CD18, CD19, CD20, CD22, CD23, CD25, CD33, CD34, CD40, CD40L, CD52, CD63, CD64, CD80 and CD147; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), such as M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-1 1. IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21 , IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31 , IL-32, IL-33, IL-34, IL-35; interleukin receptor antagonists such as IL1Ra; TNFα, superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope, e.g., gp120; transport proteins; homing receptors; addressins; regulatory proteins; cell adhesion molecules such as LFA-1, Mac 1, p150.95, VLA-4, ICAM-1, ICAM-3 and VCAM, a4/p7 integrin, and Xv/p3 integrin including either a or subunits thereof, integrin alpha subunits such as CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, alpha7, alpha8, alpha9, alphaD, CD11a, CD11b, CD51, CD11c, CD41, alphaIIb, alphaIELb; integrin beta subunits such as, CD29, CD 18, CD61, CD104, beta5, beta6, beta7 and beta8; Integrin subunit combinations including but not limited to, αVβ3, αVβ5 and α4β7; a member of an apoptosis pathway; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C; an Eph receptor such as EphA2, EphA4, EphB2, etc.; a Human Leukocyte Antigen (HLA) such as HLA-DR; complement proteins such as complement receptor CR1, C1Rq and other complement factors such as C3, and C5; a glycoprotein receptor such as GpIbα, GPIIb/IIIa and CD200; soluble receptors such as TNFR2.

Preferably, the soluble protein of interest (X) is Factor IX, TNFR2 (also known as TNFRSF1B) or IL1Ra. Preferably the soluble protein of interest (X) is IL-10, IL-2, IL-2Rα

or fusions thereof, or IFNβ. Preferably the soluble protein of interest (X is IL-10, IL-2, IL-2Rα (or fusions thereof), IFNβ, Factor IX, TNFR2 (also known as TNFRSF1B) or IL1Ra.

Preferably, the fusion protein has the structure of the homodimer shown in FIG. 1A where X is Factor IX, TNF-R2, or IL-1Ra or any active fragment or derivative thereof of any of the foregoing proteins. Preferably the fusion protein has the structure of the homodimers shown in FIG. 1A where X is IL-10, IL-2, IL-2Rα (or fusions thereof), or IFNβ or any active fragment or derivative of any of the foregoing proteins. Preferably, the fusion protein has the structure of the heterodimer shown in FIG. 1B where X is Factor IX, TNF-R2, or IL-1Ra and Y is different from X and is Factor IX, TNF-R2, or IL-1Ra. Preferably, the fusion protein has the structure of the heterodimer shown in FIG. 1B where X is IL-10, Factor IX, TNFR, Il-2, IL-2Rα (or fusions thereof), IFNβ or IL-1Ra and Y is different from X and is IL-10, Factor IX, TNF-R2, Il-2, IL-2Rα (or fusions thereof), IFNβ or IL-1Ra. Preferably, the fusion protein has the structure of the homodimer shown in FIG. 1C where X is Factor IX, TNF-R2, or IL-1Ra. Preferably, the fusion protein has the structure of the homodimer shown in FIG. 1C where X is IL-10, IL-2, IL-2Rα (or fusions thereof), or IFNβ. Preferably, the fusion protein has the structure of the heterodimer shown in FIG. 1D where X is Factor IX, TNF-R2, or IL-1Ra and Y is different from X and is Factor IX, TNF-R2, or IL-1Ra. Preferably, the fusion protein has the structure of the heterodimer shown in FIG. 1D where X is IL-10, Factor IX, TNF-R2, Il-2, IL-2Rα (or fusions thereof), IFNβ or IL-1Ra and Y is different from X and is IL-10, Factor IX, TNF-R2, Il-2, IL-2Rα (or fusions thereof), IFNβ or IL-1Ra.

Preferably, the soluble protein X of the formula X-L1-HINGE-Fc is factor IX and a single chain fusion protein of the invention having the formula X-L1-HINGE-Fc comprising an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to:

TVFLDHENANKILNRPKRYNSGKLEEFVQGNLERECMEEKCSFEEAREVFENTERTT
EFWKQYVDGDQCESNPCLNGGSCKDDINSYECWCPFGFEGKNCELDVTCNIKNGRC
EQFCKNSADNKVVCSCTEGYRLAENQKSCEPAVPFPCGRVSVSQTSKLTRAETVFPD
VDYVNSTEAETILDNITQSTQSFNDFTRVVGGEDAKPGQFPWQVVLNGKVDAFCGG
SIVNEKWIVTAAHCVETGVKITVVAGEHNIEETEHTEQKRNVIRIIPHHNYNAAINKY
NHDIALLELDEPLVLNSYVTPICIADKEYTNIFLKFGSGYVSGWGRVFHKGRSALVLQ
YLRVPLVDRATCLRSTKFTIYNNMFCAGFHEGGRDSCQGDSGGPHVTEVEGTSFLTG

IISWGEECAMKGKYGIYTKVSRYVNWIKEKTKLTGGGGSGGGGSRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGESGGGGSGGGGSGGGGSGGG
GSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSSDKTHTCPPCP
APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNA
KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQP
REPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:
7).

Preferably, the soluble protein X of the formula X-L1-HINGE-Fc is TNFR2 and a single chain fusion protein of the invention having the formula X-L1-HINGE-Fc comprising an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to:

LPAQVAFTPYAPEPGSTCRLREYYDQTAQMCCSKCSPGQHAKVFCTKTSDTVCDSCE
DSTYTQLWNWVPECLSCGSRCSSDQVETQACTREQNRICTCRPGWYCALSKQEGCR
LCAPLRKCRPGFGVARPGTETSDVVCKPCAPGTFSNTTSSTDICRPHQICNVVAIPGN
ASMDAVCTSTSPTRSMAPGAVHLPQPVSTRSQHTQPTPEPSTAPSTSFLLPMGPSPPA
EGSTGDGGGGSGGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQW
KVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPV
TKSFNRGESGGGGSGGGGSGGGGSGGGGSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN
HKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPQVKFNWYVDGVQVHNAKTKPREQQYNSTYRVVSVLTVLHQNWLD
GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGF
YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM
HEALHNHYTQKSLSLSPGK (SEQ ID NO: 8).

Preferably, the soluble protein X of the formula X-L1-HINGE-Fc is IL1Ra and a single fusion protein of the invention having the formula X-L1-HINGE-Fc comprises an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to:

RPSGRKSSKMQAFRIWDVNQKTFYLRNNQLVAGYLQGPNVNLEEKIDVVPIEPHALF
LGIHGGKMCLSCVKSGDETRLQLEAVNITDLSENRKQDKRFAFIRSDSGPTTSFESAA
CPGWFLCTAMEADQPVSLTNMPDEGVMVTKFYFQEDEGGGGSGGGGSRTVAAPSV
FIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST
YSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGESGGGSGGGGSGGGGS
GGGGSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHT
FPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSSDKTHTCP
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV
LDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID
NO: 9).

Preferably, the soluble protein X of the formula X-L1-HINGE-Fc is IFNβ and a single fusion protein of the invention having the formula X-L1-HINGE-Fc comprising an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to:

MSYNLLGFLQRSSNFQSQKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKEDAA
LTIYEMLQNIFAIFRQDSSSTGWNETIVENLLANVYHQINHLKTVLEEKLEKEDFTRG
KLMSSLHLKRYYGRILHYLKAKEYSHCAWTIVRVEILRNFYFINRLTGYLRNGGGGS
GGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNS
QESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGG
GGSGGGGSGGGGSGGGGSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV
SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK
RVEPKSCDKTH TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
KALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQK
SLSLSPGK. (SEQ ID NO: 18).

Preferably, the soluble protein X of the formula X-L1-HINGE-Fc comprises a protein that has been modified by circular permutation as is described in International Publication Number WO 2013/184942. Circular permutation involves the linking of the native amino

and carboxy ends of a protein, generally with a linker, and creating new amino and carboxy termini by cleaving at a new site within the protein sequence, generally a loop; such that the primary sequence of the resulting protein is reordered, while the secondary structure (and activity) is retained. Thus, creation of the new termini may provide better locations for attachment of a fusion partner relative to the native termini. Circular permutation of a protein ligand provides a means by which a protein may be altered to produce new carboxyl and amino termini without diminishing the specificity and binding affinity of the altered protein ligand for its target relative to its native form. Additionally, the new termini can be preferentially moved to a location preferential for incorporating the circularly permuted ligand into a fusion polypeptide, and demonstrate better activity compared with a fusion polypeptide containing the native (non-circularly permuted) ligand.

Preferably, the soluble protein X of formula X-L1-HINGE-Fc comprises a fusion of two different proteins designated as Q-R and wherein Q and R may be fused via an optional linker L5. Preferably Q is a soluble ligand which can form a signaling complex with a membrane associated receptor and R is the extracellular domain of one receptor chain from the membrane associated receptor. Preferably, Q-L5-R is IL-2 or circularly permuted IL-2 fused to the extracellular domain of IL-2Rα via an optional linker.

Preferably, the soluble protein X of the formula X-L1-HINGE-Fc is a fusion of IL-2/IL-2Rα wherein the single chain protein comprises an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to:

SKNFHLRPRDLISNINVIVELKGSETTFMCEYADETATIVEFLNRWITFSQSIISTLTGGS
SSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEE
ELKPLEEVLNLAQGSGGGSELCDDDPPEIPHATFKAMAYKEGTMLNCECKRGFRRIK
SGSLYMLCTGNSSHSSWDNQCQCTSSATRNTTKQVTPQPEEQKE RKTTEMQSPMQP
VDQASLPGHCREPPPWENEATERIYHFVVGQMVYYQCVQGYRALHRGPAESVCKM
THGKTRWTQPQLICTGGGGGSGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNF
YPREAKVQWKVDNALSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEV
THQGLSSPVTKSFNRGECGGGGSGGGGSGGGGSGGGGSASTKGPSVFPLAPSSKSTS
GGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
GTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT

VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVS
LTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 19).

Preferably, the soluble protein X of the formula X-L1-HINGE-Fc is a fusion of IL-2/IL-2Rα wherein the single chain protein comprises an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to:

SKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFSQSIISTLTG
GSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCL
EEELKPLEEVLNLAQGSGGGSELCDDDPPEIPHATFKAMAYKEGTMLNCECKRGFRR
IKSGSLYMLCTGNSSHSSWDNQCQCTSSATRNTTKQVTPQPEEQKERKTTEMQSPM
QPVDQASLPGHCREPPPWENEATERIYHFVVGQMVYYQCVQGYRALHRGPAESVC
KMTHGKTRWTQPQLICTGGGGGSGGGGSASTKGPSVFPLAPSSKSTSGGTAALGCLV
KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH
KPSNTKVDKRVGGGGSGGGGSGGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNN
FYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYAC
EVTHQGLSSPVTKSFNRGECGGSGGEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPK
DTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQYNSTYRVV
SVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK. (SEQ ID NO: 20).

Preferably, the soluble protein X of the formula X-L1-HINGE-Fc is IL-10 wherein the single chain protein comprises an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to:

MYRMQLLSCIALSLALVTNSSPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFF
QMKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNS
LGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIE
AYMTMKIRNGGSGGGGSGGSPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFF
QMKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNS
LGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIE
AYMTMKIRNGGGGSGGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAK
VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL

SSPVTKSFNRGECGGGGSGGGGSGGGGSGGGGSASTKGPSVFPLAPSSKSTSGGTAA
LGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYI
CNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTP
EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD
WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLV
KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCS
VMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 23).

Preferably, the soluble protein X of the formula X-L1-HINGE-Fc is IL-10 wherein the single chain protein comprises an amino acid sequence that is 50%, 60%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to:

MYRMQLLSCIALSLALVTNSSPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFF
QMKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNS
LGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIE
AYMTMKIRNGGSGGGGSGGSPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFF
QMKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNS
LGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIE
AYMTMKIRNGGGGSGGGGSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT
VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVD
KRVGGGGSGGGGSGGGGSGGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYP
REAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVT
HQGLSSPVTKSFNRGECGGSGGEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVS
LTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 24).

Preferably, the X-L1-HINGE-Fc fusion proteins of the invention are dimer complexes comprising two monomeric single chain X-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. the protein of interest (X) 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. 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 X-L1-HINGE-Fc dimer complex.

Another improved property associated with X-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.

Recombinant Production of X-L1-HINGE-Fc Fusion Proteins

The invention also provides nucleic acids encoding any of the various Fc 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 .

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 Fc 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: 10), 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: 16) region where N may be any nucleotide. At the 3′ end of most eukaryotic genes is an AATAAA (SEQ ID NO: 17) 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 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 Fc fusion proteins described herein, as well as methods for producing the Fc 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 Fc 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 Fc 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 Fc fusion proteins can also be produced by chemical synthesis.

The Fc 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 Fc 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 Fc fusion protein is sufficiently pure for use as a pharmaceutical product.

Uses of X-L1-HINGE-Fc Fusion Proteins

In one aspect, the invention provides Fc fusion proteins that are useful as diagnostic or therapeutic agents. In one aspect, the invention provides Fc fusion proteins useful in the treatment of disorders. The diseases or disorders that may be treated will be dictated by the identity of the protein (X) fused to the Fc domain via the novel L1 linker of the invention and include, but are not limited to: cancer, inflammatory diseases, arthritis, osteoporosis, infections in particular hepatitis, bacterial infections, viral infections, genetic diseases, pulmonary diseases, diabetes, hormone-related disease, Alzheimer's disease, cardiac diseases, myocardial infarction, deep vein thrombosis, diseases of the circulatory system, hypertension, hypotension, allergies, pain relief, dwarfism and other growth disorders, intoxications, blot clotting diseases, diseases of the innate immune system, embolism, wound healing, healing of burns, Crohn's disease, asthma, ulcer, sepsis, glaucoma, cerebrovascular ischemia, respiratory distress syndrome, corneal ulcers, renal disease, diabetic foot ulcer, anemia, factor IX deficiency, factor VIII deficiency, factor VII deficiency, mucositis, dysphagia, thrombocyte disorder, lung embolism, infertility, hypogonadism, leucopenia, neutropenia, endometriosis, Gaucher disease, obesity, lysosome storage disease, AIDS, premenstrual syndrome, Turners syndrome, cachexia, muscular dystrophy, Huntington's disease, colitis, SARS, Kaposi sarcoma, liver tumor, breast tumor, glioma, Non-Hodgkin lymphoma, Chronic myelocytic leukemia; Hairy cell leukemia; Renal cell carcinoma; Liver tumor; Lymphoma; Melanoma, multiple sclerosis, Kaposis sarcoma, papilloma virus, emphysema, bronchitis, periodontal disease, dementia, parturition, non-small cell lung cancer, pancreas tumor, prostate tumor, acromegaly, psoriasis, ovary tumor, Fabry disease, lysosome storage disease.

Exemplary therapeutic soluble proteins (X) that may be bound to an Fc domain include, for example, factor IX, IL1Ra, and TNFR. Exemplary therapeutic soluble proteins (X) that may be bound to an Fc domain include, for example, IL-10, IL-2, IL-2Rα or fusions thereof, or IFNβ. Exemplary therapeutic soluble proteins (X) that may be bound to an Fc domain include, for example, IL-10, IL-2, IL-2Rα or fusions thereof, IFNβ, factor IX, IL1Ra, and TNFR2.

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 a fusion protein. 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, the invention provides a fusion protein X-L1-HINGE-Fc wherein X is factor IX. Preferably, the invention provides a dimer complex of X-L1-HINGE-Fc wherein X is factor IX. Preferably the dimer complex is a homodimer complex. Factor IX fusion proteins in accordance with the invention may be used to treat patients who are deficient in factor IX and suffer from hemophilia B for e.g., control and prevention of bleeding episodes, routine prophylaxis to prevent or reduce the frequency of bleeding episodes, and perioperative management (surgical prophylaxis).

A patient in need of control or prevention of bleeding or bleeding episodes is preferably a human patient. The patient can be bleeding at the time of administration or be expected to be bleeding, or can be susceptible to bleeding in minor hemorrhage, hemarthroses, superficial muscle hemorrhage, soft tissue hemorrhage, moderate hemorrhage, intramuscle or soft tissue hemorrhage with dissection, mucous membrane hemorrhage, hematuria, major hemorrhage, hemorrhage of the pharynx, hemorrhage of the retropharynx, hemorrhage of the retroperitonium, hemorrhage of the central nervous system, bruises, cuts, scrapes, joint hemorrhage, nose bleed, mouth bleed, gum bleed, intracranial bleeding, intraperitoneal bleeding, minor spontaneous hemorrhage, bleeding after major trauma, moderate skin bruising, or spontaneous hemorrhage into joints, muscles, internal organs or the brain. Such patients also include those in need of perioperative management, such as management of bleeding associated with surgery or dental extraction. The patient is preferably in need of prophylaxis of one or more bleeding episodes. The patient is preferably in need of individualized interval prophylaxis. The patient is preferably in need of on-demand treatment of one or more bleeding episodes. The patient is preferably in need of perioperative management of one or more bleeding episodes.

When treating hemophilia with a fusion protein of the invention comprising factor IX, an "effective dose" reduces or decreases frequency of bleeding or bleeding disorder. An "effective dose" preferably stops on-going, uncontrollable bleeding or bleeding episodes. Preferably an "effective dose" prevents spontaneous bleeding or bleeding episodes in a

subject susceptible to such spontaneous bleeding or bleeding episodes. A "therapeutic dose" need not cure hemophilia.

Preferably, the invention provides a fusion protein X-L1-HINGE-Fc wherein X is IL-10. Preferably, the invention provides a dimer complex of X-L1-HINGE-Fc wherein X is IL-10. Preferably the dimer complex is a homodimer complex. An IL-10 fusion protein and/or a dimerized complex thereof in accordance with the invention may be 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 which may be treated by the IL-10 fusion proteins of the invention 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 IL-10 fusion proteins 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 which may be treated by the IL-10 fusion proteins of the invention 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 which may be treated by the IL-10 fusion proteins of the invention 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 which may be treated by the IL-10 fusion proteins of the invention 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 which may be treated by the IL-10 fusion proteins of the invention 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 which may be treated by the IL-10 fusion proteins of the invention 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 which may be treated by the IL-10 fusion proteins of the invention include, but are not limited to: conductive hearing loss, sensorineural hearing loss (SNHL), mixed hearing loss.

Examples of psychiatric disorders which may be treated by the IL-10 fusion proteins of the invention include, but are not limited to: major depressive disorder, treatment-refractory depression, treatment-resistant depression.

Examples of trauma and injury which may be treated by the IL-10 fusion proteins of the invention 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 IL-10 dimerized complex 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; 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, an IL-10 dimerized complex 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 an IL-10 fusion protein or dimerized complex thereof in accordance with 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, an IL-10 fusion protein or dimerized complex thereof in accordance with 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, an IL-10 fusion protein or dimerized complex thereof in accordance with 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, an IL-10 fusion protein or dimerized complex 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).

The invention also provides Fc fusion proteins of the inventions for use as a medicament. Preferably, the invention provides a fusion protein X-L1-HINGE-Fc wherein X is a soluble protein of interest as described earlier for use as a medicament. Preferably X is factor IX, IL1Ra, or TNFR. Preferably X is IL-10, IL-2, IL-2Rα (or fusions thereof), or IFNβ

for use as a medicament. Preferably the invention provides a dimer complex of X-L1-HINGE-Fc wherein X is a soluble protein of interest as described earlier for use as a medicament.

The invention also provides Fc fusion proteins of the inventions for use as a medicament to treat disease. Preferably, the invention provides a fusion protein X-L1-HINGE-Fc wherein X is a soluble protein of interest as described earlier for use as a medicament to treat diseases as described earlier. Preferably X is factor IX, for use as a medicament to treat bleeding. Preferably X is IL-10 for treatment of Crohn's disease (CD), rheumatoid arthritis (RA), psoriasis, viral infections such as chronic hepatitis C and human immunodeficiency virus (HIV).

Preferably the invention provides a dimer complex of X-L1-HINGE-Fc wherein X is a soluble protein of interest as described earlier for use as a medicament to treat disease. Preferably X is factor IX, IL1Ra, or TNFR, for use as a medicament to treat cancer, autoimmune disease and bleeding disorders. Preferably X is IL-10, IL-2, IL-2Rα or fusions thereof, or IFNβ for use in treating, for example, auditory disorders, renal cell carcinoma, melanoma, psoriasis, fibrosis, depression, and inflammatory bowel disease (IBD).

A factor IX dimerized fusion protein complex in accordance with the invention may also be used in the manufacture of a medicament to treat patients who are deficient in factor IX and suffer from hemophilia B for e.g., control and prevention of bleeding episodes, routine prophylaxis to prevent or reduce the frequency of bleeding episodes, and perioperative management (surgical prophylaxis).

An IL-10 fusion protein or dimerized complex 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 the Fc fusion proteins described herein. Therapeutic formulations comprising Fc fusion 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 Fc fusion proteins 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 Fc fusion protein will be dependent on the identity of the soluble protein (X), 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. A fusion protein of 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, the Fc fusion proteins 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 Fc fusion proteins, 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, the Fc fusion protein 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 Fc fusion proteins will depend on the type of disease to be treated, the severity and course of the disease, whether the Fc fusion proteins are administered for preventive or therapeutic purposes, the course of previous therapy, the patient's clinical history and response to the Fc fusion protein, and the discretion of the attending physician. The Fc fusion protein is suitably administered to the patient at one time or over a series of treatments.

## EXAMPLES

***Example 1: Factor IX***

***Design of Factor IX scC$_L$C$_H$1-Fc***

The single chain factor IX molecule contains the factor IX sequence followed by a 10 residue linker having the amino acid sequence: GGGGSGGGGS (SEQ ID NO: 11), the CL domain of IgG1 followed by a 20 residue linker having the amino acid sequence:

GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 12) followed by the CH1, hinge and Fc portions of human IgG1.

***Expression and characterization of Factor IX scC$_L$C$_H$1-Fc***

The gene, having the following DNA sequence:

ATGTACCGGATGCAGCTGCTGAGCTGTATCGCCCTGTCTCTGGCCCTCGTGACCA
ACAGCACCGTGTTTCTGGACCACGAGAACGCCAACAAGATCCTGAACCGGCCCA
AGCGGTACAACAGCGGCAAGCTGGAAGAGTTCGTGCAGGGCAACCTGGAACGCG
AGTGCATGGAAGAGAAGTGCAGCTTCGAAGAGGCCAGAGAGGTGTTCGAGAAC
ACCGAGCGGACCACCGAGTTCTGGAAGCAGTACGTGGACGGCGACCAGTGCGAG
AGCAACCCCTGTCTGAATGGCGGCAGCTGCAAGGACGACATCAACAGCTACGAG
TGCTGGTGCCCCTTCGGCTTCGAGGGCAAGAACTGCGAGCTGGACGTGACCTGCA
ACATCAAGAACGGCAGATGCGAGCAGTTCTGCAAGAACAGCGCCGACAACAAG
GTCGTGTGCTCCTGCACCGAGGGCTACAGACTGGCCGAGAACCAGAAGTCCTGC
GAGCCCGCCGTGCCTTTCCCATGTGGAAGAGTGTCCGTGTCCCAGACCAGCAAGC
TGACCAGAGCCGAGACAGTGTTCCCCGACGTGGACTACGTGAACTCCACCGAGG
CCGAGACAATCCTGGACAACATCACCCAGAGCACCCAGTCCTTCAACGACTTCAC
CAGAGTCGTGGGCGGCGAGGATGCCAAGCCTGGACAGTTCCCGTGGCAGGTGGT
GCTGAACGGAAAGGTGGACGCCTTTTGCGGCGGCAGCATCGTGAACGAGAAGTG
GATCGTGACAGCCGCCCACTGCGTGGAAACCGGCGTGAAGATTACAGTGGTGGC
CGGCGAGCACAACATCGAGGAAACCGAGCACACAGAGCAGAAACGGAACGTGA
TCAGAATCATCCCCCACCACAACTACAACGCCGCCATCAACAAGTACAACCACG
ACATTGCCCTGCTGGAACTGGACGAGCCCCTGGTGCTGAATAGCTACGTGACCCC
CATCTGCATTGCCGACAAAGAGTACACCAACATCTTTCTGAAGTTCGGCAGCGGC
TACGTGTCCGGCTGGGGCAGAGTGTTTCACAAGGGCAGATCCGCTCTGGTGCTGC
AGTACCTGAGAGTGCCTCTGGTGGACCGGGCCACCTGTCTGAGAAGCACCAAGT
TCACCATCTACAACAACATGTTCTGCGCCGGCTTCCATGAGGGCGGCAGAGATAG
CTGTCAGGGCGATTCTGGCGGCCCTCACGTGACAGAAGTGGAAGGCACCAGCTT
TCTGACCGGCATCATCAGCTGGGGCGAGGAATGCGCCATGAAGGGGAAGTACGG
CATCTACACCAAGGTGTCCAGATATGTGAACTGGATCAAAGAAAAGACCAAGCT
GACAGGCGGCGGAGGCTCTGGCGGAGGCGGATCTAGAACAGTGGCCGCTCCCAG

CGTGTTCATCTTCCCACCTAGCGACGAGCAGCTGAAGTCCGGCACAGCCTCTGTC
GTGTGCCTGCTGAACAACTTCTACCCCCGCGAGGCCAAGGTGCAGTGGAAGGTG
GACAATGCCCTGCAGAGCGGCAACAGCCAGGAAAGCGTGACCGAGCAGGACAG
CAAGGACTCCACCTACAGCCTGAGCAGCACCCTGACCCTGAGCAAGGCCGACTA
CGAGAAGCACAAGGTGTACGCCTGCGAAGTGACCCACCAGGGCCTGTCTAGCCC
AGTGACCAAGAGCTTCAACCGGGGCGAATCTGGGGGCGGAGGATCAGGCGGGG
GAGGAAGTGGGGGAGGGGGAAGCGGAGGGGGAGGATCTGCCTCTACAAAGGGC
CCTAGCGTGTTCCCCCTGGCCCCTAGCAGCAAGTCTACAAGCGGAGGCACAGCTG
CCCTGGGCTGCCTCGTGAAGGACTACTTCCCTGAGCCCGTGACCGTGTCCTGGAA
CAGCGGAGCACTGACAAGCGGCGTGCACACCTTTCCAGCCGTGCTGCAGAGCAG
CGGCCTGTACTCTCTGAGCAGCGTCGTGACAGTGCCCAGCAGCTCTCTGGGCACC
CAGACCTACATCTGCAACGTGAACCACAAGCCCAGCAATACCAAAGTGGACAAG
CGGGTGGAACCCAAGAGCAGCGACAAGACCCACACCTGTCCCCCTTGTCCTGCC
CCCGAACTGCTGGGAGGCCCTTCCGTGTTCCTGTTCCCCCCAAAGCCCAAGGACA
CCCTGATGATCAGCCGGACCCCTGAAGTGACCTGCGTGGTGGTGGATGTGTCCCA
CGAGGACCCAGAAGTGAAGTTCAATTGGTATGTGGACGGGGTGGAAGTGCACAA
CGCCAAGACCAAACCCAGAGAGGAACAGTACAATAGCACCTACCGGGTGGTGTC
CGTGCTGACAGTGCTGCACCAGGACTGGCTGAATGGCAAAGAGTATAAGTGCAA
AGTGTCCAACAAGGCCCTGCCTGCCCCCATCGAGAAAACCATCAGCAAGGCCAA
GGGCCAGCCCCGCGAACCCCAGGTGTACACACTGCCCCCAAGCCGGGAAGAGAT
GACCAAGAACCAGGTGTCCCTGACCTGTCTCGTGAAAGGCTTCTACCCTTCCGAT
ATCGCCGTGGAATGGGAGAGCAACGGCCAGCCCGAGAACAATTACAAGACCACC
CCCCCTGTGCTGGACTCCGACGGCTCATTCTTCCTGTACAGCAAACTGACCGTGG
ACAAGAGCCGGTGGCAGCAGGGAAACGTGTTCAGCTGCAGCGTGATGCACGAGG
CCCTGCACAACCACTACACCCAGAAAAGCCTGAGCCTGTCCCCTGGCAAG (SEQ
ID NO: 13); was synthesized (Genewiz), cloned into pcDNA/UCOE and transiently
expressed in HEK293 cells using the Expi293 expression system (Life Technologies).
Proteins were purified first using Protein A (GE Healthcare) with low pH elution and
dialyzed against 2L 25mM TRIS pH 7.5, 150mM NaCl 3 times. Following dialysis, the
protein was loaded onto a Q sepharose FF column and eluted with step gradients of $CaCl_2$ in

25mM TRIS pH 7.5, 150mM NaCl. The most active fractions were pooled and dialyzed against 1X PBS for further analysis.

The molecule was analyzed by SDS PAGE gel under reducing and non-reducing conditions (FIG. 2). For non-reducing conditions, 5ug of purified protein was loaded onto a NuPAGE® NOVEX® 3-8% TRIS-Acetate gel (Invitrogen) with a HIMARK™ pre-stained protein standard (Invitrogen) (MW range 31kD – 460kD). For reducing conditions, 5ug of protein was loaded onto an any kD™ gel (Invitrogen) with a PRECISION PLUS PROTEIN™ Kaleidoscope standard (Invitrogen) (MW range 10kD – 250 kD).

***Bioactivity of Factor IX scC$_L$C$_H$1-Fc (APTT assay)***

An automated Factor IX activity assay was performed using the KC-1 Delta™ instrument (Tcoag, Wicklow, IRE) to quantify the ability of the FIX component of the Factor IX scC$_L$C$_H$1-Fc protein to restore the clotting activity of FIX-deficient plasma. Test samples were mixed with equal volumes of human FIX-deficient plasma (George King Bio-Medical Inc, Overland Park, KS) and cephalin-containing ellagic acid activator (aPTT-soluble activator, Helena Laboratories, cat. #5389), and after 4 min incubation, 5 mM calcium chloride (25 mM stock, VWR) was added and the time to clot measured. Activity was calculated based on a calibration curve of clotting times versus activity unit concentration (IU/mL) of serial dilutions of rHuman Factor IX (FIX) (Haematologic Technologies Inc. Essex Junction, VT) standard for purified proteins. Factors of intrinsic coagulation systems are activated by incubating the plasma with the optimal amount of phospholipids and a surface activator at 37°C. The addition of calcium ions triggers the coagulation process, and the clotting time is them measured. The APTT is the time taken for a fibrin clot to form (FIG. 3).

***Rat PK of Factor IX scC$_L$C$_H$1-Fc***

Single intravenous doses of 51 IU/kg factor IX scC$_L$C$_H$1-Fc were administered into the lateral tail vein of 3 rats. Blood samples were collected at 0.25, 4, 8, 24, 48, 72, 96, and 168 hours after administration of factor IX scC$_L$C$_H$1-Fc, and citrated plasma (0.32% final) prepared. Concentrations were measured using standard MSD techniques with Goat anti-Human factor IX Affinity purified IgG (Enzyme Research Laboratories, South Bend, IN) as

the capture antibody and Goat anti-Human IgG Fc cross-adsorbed antibody biotinylated (Bethyl Laboratories, Montgomery, TX) as the detection antibody. Pharmacokinetic analysis was performed using non-compartmental modeling with WINNONLIN® software (Pharsight Corporation, Mountain View, CA). The pharmacokinetic parameter estimates derived from MSD data included maximum concentration (Cmax), area under the time versus concentration curve (AUC), and elimination half-life ($t_{1/2}$) (FIG. 4 and Table 1).

Table 1

| Test Article | Testing Animal | Dosing Route | Dose (mg/kg) | Dose (IU/kg) | $C_{max}$ (ug/mL) | $AUC_{0-\infty}$ (ug-h/mL) | $t_{1/2}$ (h) |
|---|---|---|---|---|---|---|---|
| FIXscLCLCH1Fc | rat | IV | 7.9 | 51 | 17.0 ± 11.4 | 69.6 ± 7.78 | 53.7 ± 12.5 |
| Mono* FIXFc | rat | IV | | 200 | | | 34.8 ± 5.3 |
| rhFIX** | rat | IV | | 50 | 2.6 | 8.2 | 5.0 |

* Peters, R. T. et al. Prolonged activity of factor IX as a monomeric Fc fusion protein. Blood. (2013).

**Keith, J.C. et al. Evaluation of Recombinant Human Factor IX: Pharmacokinetic Studies in the Rat and the Dog. *Thrombosis and Haemostasis* 73(1):101-105 (1994).

***Example 2: TNF-R2***

***Design of TNF-R2 scC$_L$C$_H$1-Fc***

The single chain TNFR2 molecule contains the TNFR2 sequence followed by a 10 residue linker, GGGGSGGGGS (SEQ ID NO: 11), the CL domain of IgG1 followed by a 20 residue linker GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 12) followed by the CH1, hinge and Fc portions of human IgG1.

***Expression of TNF-R2 scC$_L$C$_H$1-Fc***

The gene, having the following DNA sequence:

ATGTATAGGATGCAGCTCCTCAGCTGCATCGCTCTGTCCCTCGCCCTGGTGACCA
ACAGCCTCCCTGCCCAGGTGGCCTTTACACCCTACGCTCCTGAGCCCGGAAGCAC
CTGCAGGCTCAGGGAGTACTACGATCAGACCGCCCAAATGTGTTGCAGCAAGTG
CTCCCCTGGCCAGCACGCCAAGGTGTTCTGCACCAAGACAAGCGATACCGTGTGC
GATAGCTGTGAGGACAGCACCTACACCCAGCTGTGGAATTGGGTGCCCGAGTGC
CTGAGCTGTGGCAGCAGGTGCAGCAGCGATCAGGTGGAGACACAGGCCTGCACC
AGAGAGCAGAACAGGATTTGTACCTGCAGGCCCGGCTGGTATTGCGCCCTGAGC
AAGCAGGAGGGATGTAGGCTGTGCGCCCCTCTGAGGAAATGCAGACCTGGCTTT

GGAGTGGCTAGGCCCGGCACCGAGACATCCGACGTGGTGTGCAAGCCTTGTGCC
CCTGGCACCTTTTCCAACACCACCAGCTCCACCGACATCTGCAGGCCCCATCAGA
TTTGCAACGTGGTGGCCATCCCCGGAAACGCTAGCATGGATGCCGTGTGCACCTC
CACCTCCCCTACCAGGAGCATGGCCCCTGGAGCCGTGCATCTGCCTCAACCCGTC
AGCACCAGAAGCCAGCACACACAGCCCACCCCCGAACCTAGCACCGCTCCCTCC
ACCAGCTTCCTGCTGCCTATGGGACCCTCCCCTCCTGCCGAAGGGAGCACCGGAG
ATGGAGGAGGAGGAAGCGGCGGAGGAGGCTCCAGAACAGTGGCTGCCCCTAGC
GTGTTCATTTTCCCTCCCTCCGACGAGCAGCTCAAGTCCGGAACCGCTTCCGTGG
TCTGCCTGCTGAACAACTTCTACCCCAGAGAGGCCAAGGTGCAGTGGAAAGTCG
ACAATGCTCTGCAGAGCGGAAACTCCCAGGAGTCCGTCACCGAGCAGGACAGCA
AGGACTCCACATATAGCCTGTCCTCCACCCTGACCCTGAGCAAGGCCGACTATGA
GAAACACAAGGTGTATGCCTGCGAAGTGACCCACCAGGGCCTGTCCAGCCCCGT
CACCAAGTCCTTCAATAGGGGCGAGAGCGGAGGCGGCGGGAGCGGCGGCGGCG
GGAGCGGAGGAGGAGGGAGCGGAGGAGGCGGAAGCGCTTCCACCAAGGGACCT
AGCGTGTTTCCCCTCGCCCCCAGCTCCAAGAGCACAAGCGGAGGCACAGCCGCT
CTGGGCTGTCTGGTGAAGGATTACTTCCCCGAGCCCGTCACAGTGAGCTGGAACT
CCGGAGCCCTGACCTCCGGAGTGCACACCTTTCCTGCCGTGCTGCAGAGCAGCGG
ACTGTACAGCCTGTCCAGCGTGGTCACAGTGCCCTCCAGCTCCCTGGGCACCCAG
ACCTACATCTGCAACGTGAACCACAAGCCCAGCAACACAAAGGTGGACAAGAGA
GTGGAACCTAAGTCCTGTGACAAAACCCATACCTGCCCTCCCTGCCCTGCCCCTG
AGCTGCTGGGAGGACCTAGCGTGTTTCTGTTTCCCCCCAAACCCAAGGATACCCT
GATGATCAGCAGGACCCCTGAGGTGACATGCGTGGTGGTGGACGTGTCCCACGA
GGACCCTCAGGTCAAGTTCAACTGGTACGTGGATGGCGTCCAGGTGCACAATGCT
AAGACCAAGCCCAGGGAGCAGCAATACAATTCCACCTACAGGGTGGTGTCCGTG
CTCACCGTCCTCCACCAGAACTGGCTCGACGGCAAAGAATACAAGTGCAAAGTG
AGCAACAAGGCTCTCCCCGCCCCTATCGAGAAGACCATTTCCAAAGCCAAGGGC
CAGCCCAGAGAACCTCAAGTCTACACCCTGCCCCCCAGCAGGGAGGAGATGACC
AAGAACCAGGTGAGCCTGACCTGCCTCGTCAAGGGATTCTATCCCAGCGACATC
GCCGTGGAATGGGAGTCCAATGGCCAGCCCGAGAATAACTACAAGACCACACCC
CCCGTGCTGGATTCCGATGGCAGCTTTTTCCTGTACAGCAAGCTGACAGTGGATA
AGAGCAGGTGGCAGCAGGGCAACGTGTTCAGCTGCTCCGTCATGCACGAAGCCC

TGCACAATCACTACACCCAGAAGAGCCTGTCCCTCAGCCCCGGCAAG (SEQ ID NO: 14); was synthesized (Genewiz), cloned into pcDNA/UCOE and transiently expressed in HEK293 cells using the Expi293 expression system (Life Technologies). Proteins were purified first using Protein A (GE Healthcare) with low pH elution and dialyzed against 2L 1X PBS 3 times.

The molecule was analyzed by SDS PAGE gel under reducing and non-reducing conditions (FIGs. 5A and 5B). For non-reducing conditions, 5ug of purified protein was loaded onto a NuPAGE® Novex® 3-8% TRIS-Acetate gel (Invitrogen) with a HiMark™ Pre-stained protein standard (Invitrogen) (MW range 31kD – 460kD). For reducing conditions, 5ug of protein was loaded onto Any kD™ gel (Invitrogen) with a PRECISION PLUS PROTEIN™ Kaleidoscope standard (Invitrogen) (MW range 10kD – 250 kD).

***Bioactivity of TNF-R2 scC$_L$C$_H$1-Fc***

HEK-Blue™ TNF-α cells (InvivoGen) are human embryonic kidney cells specifically designed to detect bioactive TNF-α *in vitro* by monitoring the activation of the NF-κB/AP-1 pathways. The cell line expresses an inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene under control of the IFN-β minimal promoter fused to five NFκb and five AP-1 binding sites. For the TNF-α antagonist assay, HEK-Blue TNF-α cells were plated at 50,000 cells/well in DMEM media containing 2 mM L-glutamine, 4.5 g/l glucose and 10% heat inactivated FBS (Gibco) and 235 pM TNF-α 1a (InvivoGen). Cells were incubated for 20 hours at 37°C, 5% $CO_2$ with varying concentrations of TNF-R2 direct fusion or TNF-R2 single chain fusion body (TNF-R2 scC$_L$C$_H$1-Fc). SEAP production was detected by adding QUANTI-Blue and incubating for 3 hours at 37°C, 5% $CO_2$ and read on a plate reader at 630 nm. Activation of the SEAP gene can be inhibited by the TNF-α antagonist TNF-R2 in a dose dependent manner. The TNF-R2 single chain fusion body molecule inhibited activation of the SEAP gene with an $IC_{50}$ of 51pM vs the direct fusion of TNF-R2 with an $IC_{50}$ of 112pM (FIG. 6).

***Rat PK of TNF-R2 scC$_L$C$_H$1-Fc***

Single intravenous doses of 5 mg/kg TNF-R2 scC$_L$C$_H$1-Fc were administered into the lateral tail vein of 3 rats. Blood samples were collected at 0.083, 1, 6, 24, 48 hr, 5, 7, 9, 12,

15, 21, 28 days after administration of TNF-R2 $scC_LC_H1$-Fc. Concentrations were measured using standard MSD techniques with Goat anti-Human F(ab')2 IgG Fc (Thermo Scientific, Rockford, IL) as the capture antibody and Goat anti-Human IgG Fc cross-adsorbed antibody biotinylated (Bethyl Laboratories, Montgomery, TX) as the detection antibody.

Pharmacokinetic analysis was performed using non-compartmental modeling with WinNonlin® software (Pharsight Corporation, Mountain View, CA). The pharmacokinetic parameter estimates derived from MSD data included maximum concentration (Cmax), area under the time versus concentration curve (AUC), and elimination half-life ($t_{1/2}$) (FIG. 7 and Table 2).

Table 2

| Test Article | Testing Animal | Dosing Route | Dose (mg/kg) | $C_{max}$ (nM) | $AUC_{0-\infty}$ (nM-h) | $t_{1/2}$ (h) |
|---|---|---|---|---|---|---|
| TNF-R2 direct fusion | rat | IV | 5 | 584 ± 59.2 | 3445 ± 967 | 24 ± 6.5 |
| TNF-R2 $scC_LC_H1$-Fc | rat | IV | 5 | 412 ± 109 | 3207 ±157 | 102 ± 33 |

***Example 3: IL1Ra***

***Design of IL1Ra $scC_LC_H1$-Fc***

The single chain IL1Ra molecule contains the IL1Ra sequence followed by a 10 residue linker, GGGGSGGGGS (SEQ ID NO: 11), the CL domain of IgG1 followed by a 20 residue linker, GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 12) followed by the CH1, hinge and Fc portions of human IgG1.

***Expression of IL1Ra $scC_LC_H1$-Fc***

The gene having the following DNA sequence:

ATGTACCGGATGCAGCTGCTGTCCTGTATCGCCCTGTCTCTGGCCCTGGTC
ACCAACTCCAGACCCTCTGGCCGGAAGTCCTCCAAGATGCAGGCCTTCCGGATCT
GGGACGTGAACCAGAAAACCTTCTACCTGCGGAACAACCAGCTGGTGGCCGGCT
ATCTGCAGGGCCCCAACGTGAACCTGGAAGAGAAGATCGACGTGGTGCCCATCG
AGCCCCACGCCCTGTTTCTGGGAATCCACGGCGGCAAGATGTGCCTGTCCTGCGT
GAAGTCCGGCGACGAGACACGGCTGCAGCTGGAAGCCGTGAACATCACCGACCT

GTCCGAGAACCGGAAGCAGGACAAGAGATTCGCCTTCATCAGATCCGACTCCGG
CCCTACCACCTCCTTCGAGTCTGCTGCTTGCCCCGGCTGGTTCCTGTGCACCGCCA
TGGAAGCTGACCAGCCCGTGTCCCTGACCAACATGCCTGACGAGGGCGTGATGG
TCACCAAGTTCTATTTTCAGGAAGATGAGGGCGGAGGCGGCTCTGGCGGCGGAG
GATCTAGAACAGTGGCCGCTCCCTCCGTGTTCATCTTCCCACCTTCCGACGAGCA
GCTGAAGTCTGGCACCGCCTCTGTCGTGTGCCTGCTGAACAACTTCTACCCTCGC
GAGGCCAAGGTGCAGTGGAAGGTGGACAACGCCCTGCAGTCCGGCAACTCCCAG
GAATCCGTCACCGAGCAGGACTCCAAGGACAGCACCTACTCCCTGTCCTCCACCC
TGACCCTGTCCAAGGCCGACTACGAGAAGCACAAGGTGTACGCCTGCGAAGTGA
CCCACCAGGGCCTGTCTAGCCCCGTGACCAAGTCTTTCAACCGGGGCGAAAGCG
GAGGCGGAGGTTCAGGTGGTGGTGGATCAGGTGGCGGCGGATCTGGCGGTGGTG
GCTCTGCTTCTACCAAGGGCCCTTCCGTGTTCCCTCTGGCCCCTTCCAGCAAGTCT
ACCTCTGGCGGCACAGCCGCTCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAGC
CTGTGACCGTGTCCTGGAACTCTGGCGCTCTGACATCCGGCGTGCACACCTTCCC
TGCTGTGCTGCAGTCCTCCGGCCTGTACAGCCTGTCCTCCGTCGTGACCGTGCCTT
CCAGCTCTCTGGGCACCCAGACCTACATCTGTAACGTGAACCACAAGCCCTCCAA
CACCAAAGTGGACAAGCGGGTGGAACCCAAGTCCTCCGACAAGACCCACACCTG
TCCTCCCTGCCCTGCTCCTGAACTGCTGGGCGGACCTAGCGTGTTCCTGTTCCCTC
CAAAGCCCAAGGACACCCTGATGATCTCCCGGACCCCTGAAGTGACCTGCGTGG
TGGTCGATGTGTCCCACGAGGACCCAGAAGTGAAGTTCAATTGGTACGTGGACG
GCGTGGAAGTGCACAATGCCAAGACCAAGCCCAGAGAGGAACAGTACAACTCCA
CCTACCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAGGATTGGCTGAACGGCAA
AGAGTACAAGTGCAAGGTGTCCAACAAGGCCCTGCCTGCCCCTATCGAAAAGAC
CATCTCCAAGGCCAAGGGCCAGCCCCGGGAACCTCAGGTGTACACCCTGCCTCCC
AGCCGGGAAGAGATGACCAAGAACCAGGTGTCACTGACCTGTCTGGTCAAGGGC
TTCTACCCCTCCGACATTGCCGTGGAATGGGAGTCCAACGGCCAGCCCGAGAAC
AACTACAAGACCACCCCTCCCGTGCTGGACTCCGACGGCTCATTCTTCCTGTACT
CCAAGCTGACCGTGGACAAGTCCCGGTGGCAGCAGGGCAACGTGTTCTCCTGCTC
CGTGATGCACGAGGCCCTGCACAACCACTACACCCAGAAGTCCCTGTCCCTGAGC
CCCGGCAAG (SEQ ID NO: 15); was synthesized (Genewiz, Inc.), cloned into pcDNA/UCOE and transiently expressed in HEK293 cells using the Expi293 expression

system (Life Technologies). Proteins were purified first using Protein A (GE Healthcare) with low pH elution and dialyzed against 2L 1X PBS 3 times. The molecule was analyzed by SDS PAGE gel under reducing and non-reducing conditions (FIGs. 8A and 8B). For non-reducing conditions, 5ug of purified protein was loaded onto a NuPAGE® NOVEX® 3-8% TRIS-Acetate gel (Invitrogen) with a HIMARK™ Pre-stained protein standard (Invitrogen) (MW range 31kD – 460kD). For reducing conditions, 5ug of protein was loaded onto an Any kD™ gel (Invitrogen) with a PRECISION PLUS PROTEIN™ Kaleidoscope standard (Invitrogen) (MW range 10kD – 250 kD).

***Bioactivity of IL1Ra scC$_L$C$_H$1-Fc***

HEK-Blue™ IL-1β cells (InvivoGen) are human embryonic kidney cells specifically designed to detect bioactive IL-1β *in vitro* by monitoring the IL-1β-induced activation of the NF-κB/AP-1 pathways. The cell line expresses an inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene under control of the IFN-β minimal promoter fused to five NFκb and five AP-1 binding sites. For the IL-1β antagonist assay, HEK-Blue IL-1β cells were plated at 50,000 cells/well in DMEM media containing 2 mM L-glu and 10% heat inactivated FBS (Gibco) and 57 pM IL-1β (R&D systems). Cells were incubated for 20 hours at 37°C, 5% $CO_2$ with varying concentrations of IL1RascC$_L$C$_H$1-Fc. SEAP production was detected by adding QUANTI-Blue and incubating for 3 hours at 37°C, 5% $CO_2$ and read on a plate reader at 630 nm. IL-1β activation of the SEAP gene can be inhibited by the IL-1β antagonist IL-1Ra in a dose dependent manner. The IL-1Ra single chain molecule inhibited IL-1β activation of the SEAP gene with an $IC_{50}$ of 12.5 Nm (FIG. 9).

***Rat PK of IL1Ra scC$_L$C$_H$1-Fc***

Single intravenous doses of 2 mg/kg IL1Ra scC$_L$C$_H$1-Fc were administered into a jugular vein catheter of 3 rats. Blood samples were collected at 0.083, 0.25, 1, 2, 6, 24, 48, 72, 96 and 168 hours after administration of IL1Ra scC$_L$C$_H$1-Fc. Single subcutaneous doses of 5 mg/kg IL1Ra scC$_L$C$_H$1-Fc were administered into the interscapular region of 3 rats. Blood samples were collected at 0.25, 1, 2, 4, 6, 24, 48, 72, 96 and 168 hours after administration of IL1Ra scC$_L$C$_H$1-Fc. Concentrations were measured using standard MSD techniques with Goat anti-Human F(ab')2 IgG Fc (Thermo Scientific, Rockford, IL) as the

capture antibody and Mouse anti-Human IL1Ra biotin conjugate (Invitrogen, Grand Island, NY) as the detection antibody. Pharmacokinetic analysis was performed using non-compartmental modeling with WINNONLIN® software (Pharsight Corporation, Mountain View, CA). The pharmacokinetic parameter estimates derived from MSD data included maximum concentration (Cmax), area under the time versus concentration curve (AUC), and elimination half-life ($t_{1/2}$) (FIG. 10 and Table 3).

Table 3

| Test Article | Testing Animal | Dosing Route | Dose (mg/kg) | $C_{max}$ (nM) | $AUC_{0-\infty}$ (nM-h) | $t_{1/2}$ (h) |
|---|---|---|---|---|---|---|
| IL1Ra-$scC_LC_H1$-Fc | rat | IV | 2 | 375 ± 7.6 | 1828 ± 139 | 9.8 ± 0.9 |
| IL1Ra-$scC_LC_H1$-Fc | rat | SC | 5 | 24.7 ± 34.1 | 363 ± 476 | 9.4 ± 1.6 |
| rhIL-1Ra* | rat | IV | 1 | 448.5 ± 134 | 98.5 ± 5.8 | 1.15 ± 0.5 |
| rhIL-1Ra* | rat | SC | 1 | 25.3 ± 3.5 | 74.1 ± 9.3 | 0.85± 0.08 |

*Source: FDA document BLA: 103950/0. PK parameters were converted to nM concentrations using a MW of 17257.6 g/mole for rhIL-1Ra

***Intra-Ocular PK of IL1Ra $scC_LC_H1$-Fc***

A bolus intravitreal injection of 0.5 mg IL1Ra $scC_LC_H1$-Fc was administered into each eye of 8 male rabbits. Blood samples from two animals were collected at 4, 96, 168 and 336 hours after administration of IL1Ra $scC_LC_H1$-Fc. At the time of sacrifice, both eyes from each animal were collected and flash frozen in liquid nitrogen. Concentrations were measured using standard MSD techniques. Pharmacokinetic analysis was performed using non-compartmental modeling with WINNONLIN® software (Pharsight Corporation, Mountain View, CA). The pharmacokinetic parameter estimates derived from MSD data included maximum concentration (Cmax), area under the time versus concentration curve (AUC), and elimination half-life ($t_{1/2}$) (Table 4 and FIG. 11).

Table 4

| Test Article | Testing Animal | Matrix | $C_{max}$ (ug/mL) | $AUC_{0-\infty}$ (ug/mL-h) | $t_{1/2}$ (h) |
|---|---|---|---|---|---|
| IL1Ra $scC_LC_H1$-Fc | rabbit | Aqueous | 2.53 | 369 | 83 |
| IL1Ra $scC_LC_H1$-Fc | rabbit | Vitreous | 265 | 2904 | 129 |

***Example 4: IL-2/IL2Rα***

***Design of IL-2/IL-2Rα $scC_LC_H1$-Fc and IL-2/IL-2Rα $scC_{H1}C_L$-Fc***

The IL-2/IL-2Rα single chain fusion body molecule contains a circularly permuted human IL-2 linked to the extracellular domain of IL-2Rα fusion protein linked to the CL-CH1-Fc domain (SEQ ID NO: 19) or the CH1-CL-Fc (SEQ ID NO: 20) of the IgG1 heavy chain (FIGs. 12A and 12B) referred to herein as IL-2/IL-2Rα $scC_LC_H1$-Fc and IL-2/IL-2Rα $scC_{H1}C_L$-Fc, respectively. For expression in mammalian cells, the N-terminal leader sequence of SEQ ID NO: 10 was added to the protein of SEQ ID NO: 19 and SEQ ID NO: 20).

***Expression of IL-2/IL-2Rα $scC_LC_H1$-Fc and IL-2/IL-2Rα $scC_{H1}C_L$-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. 13). For reducing and non-reducing conditions, 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 a BioSuite Ultra High Resolution SEC column, 250Å, 4 µm, 4.6 mm X 300 mm (Waters). The column was equilibrated and run at 0.3 ml/min with 150mM 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 25 min (FIGs. 14A and 14B).

***Bioactivity of IL-2/IL-2Rα $scC_LC_H1$-Fc and IL-2/IL-2Rα $scC_{H1}C_L$-Fc***

*In vitro* bioactivity was assessed by evaluating the ability of IL-2/IL-2Rα $scC_LC_H1$-Fc and IL-2/IL-2Rα $scC_{H1}C_L$-Fc to activate pSTAT5 in the human HH T-cell lymphoma cell line (ATCC CRL-2105) using the Phospho-STAT5A/B (Tyr694/Tyr699) InstantOne™ ELISA kit from eBioscience. For the assay, HH cells were plated at $2x10^5$ cells/well in RPMI1640 media containing 10% FBS. Samples were incubated with decreasing concentrations of wild-type IL-2 (wtIL-2), IL-2/IL-2Ra scClCH1 Fc or IL-2/IL-2Ra scCH2Cl Fc from approximately 50 nM, or unstimulated, for approximately 25 ± 5 minutes in a 37°C, 5% $CO_2$ incubator. Stimulation reaction was terminated by prompt addition of 25 µL of cell lysis mix (provided in kit) and incubated at room temperature for 10 minutes with constant shaking at 300 rpm on a titer plate shaker. 50 µL aliquots of resulting lysates were added to each well in the assay plate (provided in kit). After adding 50 µL of antibody cocktail to each well, the plate was covered and incubated at room temperature for 1 hour with constant shaking at 300 rpm on a titer plate shaker. Plate was subsequently washed three times with 300 µL/well of 1X wash buffer. 100 µL of detection reagent was added to each well and incubated at room temperature for 30 minutes with constant shaking at 300 rpm. Detection reaction was stopped by addition of 100 µL of stop solution and the absorbance at 450 nM was measured using a SynergyMx plate reader. IL-2/IL-2Ra scClCH1 Fc ($EC_{50}$ = 0.97 nM), or IL-2/IL-2Ra scCH2Cl Fc ($EC_{50}$ = 1.1 nM) and wtIL-2 ($EC_{50}$ = 0.80 nM) were active in a dose dependent fashion (FIG. 15).

***Rat PK of IL-2/IL-2Rα $scC_LC_H1$-Fc and IL-2/IL-2Rα $scC_{H1}C_L$-Fc***

Single intravenous doses of 1 mg/kg IL-2/IL-2Rα $scC_LC_H1$-Fc and IL-2/IL-2Rα $scC_{H1}C_L$-Fc were administered into a tail vein of 3 rats. Blood samples were collected at 0.083, 0.25, 0.5, 1, 3, 8, 24, 48, 72, 96 and 168 hrs after administration of IL-2/IL-2Rα $scC_LC_H1$-Fc and IL-2/IL-2Rα $scC_{H1}C_L$-Fc. Single subcutaneous doses of 2 mg/kg IL-2/IL-2Rα $scC_LC_H1$-Fc and IL-2/IL-2Rα $scC_{H1}C_L$-Fc were administered into the interscapular region of 3 rats. Blood samples were collected at 0.25, 0.5, 1, 2, 6, 8, 24, 48, 72, 96 and 168 hrs after administration of IL-2/IL-2Rα $scC_LC_H1$-Fc and IL-2/IL-2Rα $scC_{H1}C_L$-Fc.

Concentrations were measured using standard MSD techniques. Pharmacokinetic analysis was performed using non-compartmental modeling with WinNonlin software (Pharsight Corporation, Mountain View, CA). The pharmacokinetic parameter estimates derived from MSD data included maximum concentration (Cmax), area under the time versus concentration curve (AUC), and elimination half-life ($t_{1/2}$) (FIG. 16 and Table 5).

Table 5

| Test Article | Dosing Route | Dose (nmol/kg) | $T_{max}$ (h) | $C_{max}$ (nM) | $AUC_{0-\infty}$ (nM-h) | $t_{1/2}$ (h) | MRT (h) | CL (mL/h/kg) | Vd (mL/kg) | F (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| IL-2/IL-2Ra scC1CH1 Fc | IV | 6 | 0.083 | 122.9 ± 13.0 | 876 ± 16.2 | 33.4 ± 4.4 | 25.6 ± 4.2 | 6.78 ± 0.125 | 374 ± 33.5 | |
| IL-2/IL-2Ra scC1CH1 Fc | SC | 12 | 48 | 5.67 ± 0.35 | 483 ± 56.5 | 15.3 ± 3.6 | 55.0 ± 1.7 | | | 28 |
| IL-2/IL-2Ra scCH1C1 Fc | IV | 6 | 0.083 | 65.5 ± 7.6 | 505 ± 66.4 | 39.5 ± 4.7 | 26.3 ± 1.7 | 11.9 ± 1.6 | 313 ± 47.7 | |
| IL-2/IL-2Ra scCH1C1 Fc | SC | 12 | (24, 48) | 3.36 ± 0.84 | 293 ± 1.0 | 32.9 ± 0.1 | 70.3 ± 8.8 | | | 29 |

Note: mean ± SD for all parameters except median in $T_{max}$, max, for $T_{max}$, n=3 unless otherwise noted; F = %ratio of dose normalized $AUC_{0-\infty}$ after SC vs IV

***Example 5: IFNβ***

***Design of IFNβ scC$_L$C$_H$1-Fc***

The IFNβ single chain fusion body molecule contains IFNβ (C17S) linked to the CL-CH1-Fc domain of the IgG1 heavy chain (FIG. 17). For expression in mammalian cells, the N-terminal leader sequence of SEQ ID NO: 10 was added to the protein of SEQ ID NO: 18.

***Expression of IFNβ scC$_L$C$_H$1-Fc***

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

The molecule was analyzed by SDS PAGE gel under reducing and non-reducing conditions (FIG. 18). For reducing and non-reducing conditions, 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 a BioSuite Ultra High Resolution SEC column, 250Å, 4 μm, 4.6 mm X 300 mm (Waters). The column was equilibrated and run at 0.3 ml/min with 150mM 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 60 min (FIG. 19).

***Bioactivity of IFNβ scC$_L$C$_H$1-Fc***

HEK-Blue™ IFNα/β cells (InvivoGen) are human embryonic kidney cells specifically designed to detect bioactive Type I IFNs *in vitro* by monitoring the activation of the ISGF3 pathway. The cell line expresses an inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene under control of the IFNα/β inducible ISG54 promoter. For the IFNβ agonist assay, HEK-Blue IFNα/β cells were plated at 50,000 cells/well in DMEM media containing 2 mM L-glutamine, 4.5 g/l glucose and 10% heat inactivated FBS (Gibco). Cells were incubated for 20 hours at 37°C, 5% $CO_2$ with varying concentrations of IFNβ scC$_L$C$_H$1-Fc or wtIFNβ (Peprotech). SEAP production was detected by adding QUANTI-Blue and incubating for 3 hours at 37°C, 5% $CO_2$ and read on a plate reader at 630 nm. IFNβ scC$_L$C$_H$1-Fc ($EC_{50}$ = 0.9 pM) and wtIFNβ ($EC_{50}$ = 0.6 pM) were active in a dose dependent fashion (FIG. 20).

***Rat PK of IFNβ scC$_L$C$_H$1-Fc***

A single intravenous dose of 0.5 mg/kg IFNβ scC$_L$C$_H$1-Fc was administered into a surgically implanted jugular vein catheter of 3 rats. Blood samples were collected at 0.083, 0.25, 0.5, 1, 3, 8, 24, 48, 72, 96 and 168 hrs after administration of IFNβ scC$_L$C$_H$1-Fc. A Single subcutaneous dose of 1 mg/kg IFNβ scC$_L$C$_H$1-Fc was administered into the interscapular region of 3 rats. Blood samples were collected at 0.25, 0.5, 1, 2, 6, 8, 24, 48, 72, 96 and 168 hrs after administration of IFNβ scC$_L$C$_H$1-Fc. Concentrations were measured using standard MSD techniques. Pharmacokinetic analysis was performed using non-compartmental modeling with WinNonlin software (Pharsight Corporation, Mountain View, CA). The pharmacokinetic parameter estimates derived from MSD data included maximum concentration (Cmax), area under the time versus concentration curve (AUC), and elimination half-life ($t_{1/2}$) (FIG. 21 and Table 6).

Table 6

| Route | Dose | Animal ID | Cmax | Tmax | Cmax/D | $AUC_{\infty}$ | $AUC_{\infty}/D$ | CL | $V_{ss}$ | $t_{1/2}$ | MRT | %F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (nMole/kg) | | (nM) | (h) | (kg*nM/nmol) | (h*nM) | (h*kg*nM/nmol) | (mL/h/kg) | (mL/kg) | (h) | (h) | (%) |
| IV | 1.4 | 67363 | 61.1 | 0.083 | 42.7 | 1320 | 921 | 1.09 | 111 | 87 | 100 | |
| | | 67364 | 54.7 | 0.083 | 38.2 | 1320 | 924 | 1.08 | 129 | 110 | 120 | |
| | | 67366 | 109 | 0.083 | 76.6 | 1790 | 1250 | 0.801 | 106 | 120 | 130 | |
| | | **Mean** | **75.1** | **0.083** | **52.5** | **1480** | **1030** | **0.991** | **115** | **100** | **120** | |
| | | **SD** | **30** | **NA** | **21** | **272** | **190** | **0.164** | **12.3** | **14** | **15** | |
| SC | 3.6 | 67367 | 16.4 | 48 | 4.57 | ND | ND | NA | NA | ND | ND | |
| | | 67369 | 12.8 | 24 | 3.57 | 2150 | 600 | NA | NA | 87 | 140 | |
| | | **Mean** | **14.6** | **36** | **4.07** | **2150** | **600** | **NA** | **NA** | **87** | **140** | **58.3** |
| | | **SD** | **NA** | **NA** | **NA** | **NA** | **NA** | **NA** | **NA** | **NA** | **NA** | |

***Example 6: IL-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 domain or the CH1-CL-Fc of the IgG1 heavy chain (FIGs. 22A and 22B). The amino acid sequences of each molecule synthesized is found in Table 7.

| Protein | Sequence |
|---|---|
| scIL-10:CL:CH1:Fc | MYRMQLLSCIALSLALVTNSSPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNL LLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFL PCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRNGGSGGGGSGGSPGQGTQS ENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLE EVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKA MSEFDIFINYIEAYMTMKIRNGGGGSGGGGSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCP PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV MHEALHNHYTQKSLSLSPGK (SEQ ID NO: 23) |
| scIL-10:CH1:CL:Fc | MYRMQLLSCIALSLALVTNSSPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNL LLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFL PCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRNGGSGGGGSGGSPGQGTQS ENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLE EVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKA MSEFDIFINYIEAYMTMKIRNGGGGSGGGGSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVGGGGSG GGGSGGGGSGGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNS QESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGECGGSGGEPKSCDK THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKT KPREEQVNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRE EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 24) |
| scIL-10:Fc (Control) | MYRMQLLSCIALSLALVTNSSPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNL LLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFL PCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEAYMTMKIRNGGSGGSPGQGTQSENSCTH FPGNLPNMLRDLRDAFSRVKTFFQMKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLEEVMPQA ENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPCENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFI NYIEAYMTMKIRNEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT ISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 25) |

Table 7

***Expression of scIL-10:$C_L$:$C_{H}1$: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. 23). 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. 24A and 24B).

***Bioactivity of scIL-10:$C_L$:$C_H$1:Fc and scIL-10:$C_{H1}$:$C_L$:Fc***

*In vitro* bioactivity was assessed by evaluating the ability of scIL-10:$C_L$:$C_H$1:Fc and scIL-10:$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_H$1:Fc, scIL-10:$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_H$1:Fc ($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. 25).

***Mouse PK of scIL-10:$C_L$:$C_H$1:Fc and scIL-10:$C_{H1}$:$C_L$:Fc***

scIL-10:$C_L$:$C_H$1:Fc, scIL-10:$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_H$1:Fc, scIL-10:$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 8 and 9.

Table 8

| Row ID | Compound | Dose | Dose | ROA | Cmax | Tmax | Cmax/D | AUClast |
|---|---|---|---|---|---|---|---|---|
| | | (mg/kg) | (~nMole/kg) | | (nM) | (h) | (nM/D) | (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_H$1:Fc | 0.5 | 2.85 | IV | 140 | 0.083 | 49.2 | 2850 |
| 4 | scIL-10:$C_L$:$C_H$1:Fc | 2.5 | 14.25 | SC | 227 | 24 | 15.9 | 19500 |
| 5 | scIL-10:$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 9

| Row ID | AUCinf | AUCinf/D | MRTinf | t1/2 | CL | Vss | %F |
|---|---|---|---|---|---|---|---|
| | (h*nM) | (h*nM) | (h) | (h) | (mL/hr/kg) | (mL/kg) | |
| 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 |

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art.

## CLAIMS

1. A single chain fusion protein having the following arrangement from amino-terminus to carboxy-terminus:

X-L1-HINGE -Fc

wherein,

X is a soluble protein comprising Factor IX, tumor necrosis factor receptor 2 (TNFR2) interleukin 1 receptor antagonist (IL1Ra), interleukin-10 (IL-10), interleukin-2 (IL-2), interleukin-2 receptor alpha (IL-2Rα), interferon-beta (IFNβ), circularly permuted IL-2, or circularly permuted IL-2 fused to the extracellular domain of interleukin-2 receptor alpha (IL-2Rα);

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;

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.

2. The fusion protein of claim 1, wherein CL, CH1, HINGE and Fc are at least 99% identical to the CL, CH1, hinge and Fc regions respectively of human IgG1, wherein CL, CH1, HINGE and Fc retains a biological activity of the fusion protein of claim 1.

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

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

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

6. The fusion protein of claim 1, wherein HINGE and L2 are present and L4 is absent.

7. The fusion protein of claim 1, wherein HINGE, L2 and L4 are present.

8. The fusion protein of claim 1, wherein HINGE is absent and L4 is present.

9. The fusion protein of claim 1, wherein HINGE is absent and L2 and L4 are present.

10. A dimerized complex comprising the fusion protein of claim 1.

11. The dimerized complex of claim 10, wherein the dimerized complex is a homodimeric complex.

12. Use of the fusion protein of claim 1 or a homodimeric complex thereof wherein X comprises IL-10 for treating auditory disorders, renal cell carcinoma, melanoma, psoriasis, fibrosis, depression, or inflammatory bowel disease in a patient.

13. The fusion protein of claim 1 or dimerized complex of claim 10, for use in the manufacture of a medicament for treating auditory disorders, renal cell carcinoma, melanoma, psoriasis, fibrosis, depression or inflammatory bowel disease, wherein X comprises IL-10.

14. The fusion protein of claim 1 or a homodimeric complex thereof, for use in the treatment of cancer, autoimmune disease or bleeding disorders wherein X comprises Factor IX, TNFR2 or IL1Ra.

15. A single chain fusion protein having the following arrangement from amino-terminus to carboxy-terminus:

X-L1-HINGE -Fc

wherein,

X comprises IL-10;

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;

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.

16. The fusion protein of claim 15, wherein CL, CH1, HINGE and Fc are at least 99% identical to the CL, CH1, HINGE and Fc regions respectively of human IgG1, wherein CL, CH1, HINGE and Fc retains a biological activity of the fusion protein of claim 15.

17. The fusion protein of claim 15, wherein L3 is a polypeptide linker having the amino acid sequence $(GGGGS)_n$ wherein n is 1-5.

18. The fusion protein of claim 15, wherein L2 is present and is a polypeptide linker having the amino acid sequence $(GGGGS)_n$ wherein n is 1-5.

19. The fusion protein of claim 15, wherein L4 is present and is a polypeptide linker having the amino acid sequence $(GGGGS)_n$ wherein n is 1-5.

20. The fusion protein of claim 15, wherein HINGE and L2 are present and L4 is absent.

21. The fusion protein of claim 15, wherein HINGE, L2 and L4 are present.

22. The fusion protein of claim 15, wherein HINGE is absent and L4 is present.

23. The fusion protein of claim 15, wherein HINGE is absent and L2 and L4 are present.

24. A dimerized complex comprising the fusion protein of claim 15.

25. A single chain fusion protein comprising amino acids 21-817 of SEQ ID NO: 23.

26. A homodimeric complex of the fusion protein of claim 25.

27. A single chain fusion protein comprising amino acids 21-822 of SEQ ID NO: 24.

28. A homodimeric complex of the fusion protein of claim 27.

29. A single chain fusion protein comprising an amino acid sequence that is 99% identical to amino acids 21-817 of SEQ ID NO: 23, wherein the single chain fusion protein retains a biological activity of SEQ ID NO: 23.

30. A homodimeric complex of the fusion protein of claim 29.

31. A single chain fusion protein comprising an amino acid sequence that is 99% identical to amino acids 21-822 of SEQ ID NO: 24, wherein the single chain fusion protein retains a biological activity of SEQ ID NO: 24.

32. A homodimeric complex of the fusion protein of claim 31.

33. A nucleic acid encoding the fusion protein of any one of claims 25, 27, 29 and 31.

34. A vector comprising the nucleic acid of claim 33.

35. A host cell comprising the vector of claim 34.

36. Use of the fusion protein of any one of claims 15, 25, 27, 29 and 31 or a homodimeric complex thereof for treating auditory disorders, renal cell carcinoma, melanoma, psoriasis, fibrosis, depression, or inflammatory bowel disease in a patient.

37. The fusion protein of any one of claims 15, 25, 27, 29 and 31, for use in the manufacture of a medicament for treating auditory disorders, renal cell carcinoma, melanoma, psoriasis, fibrosis, depression or inflammatory bowel disease.

38. The fusion protein of claim 15, where X is an IL-10 homodimer fusion protein.

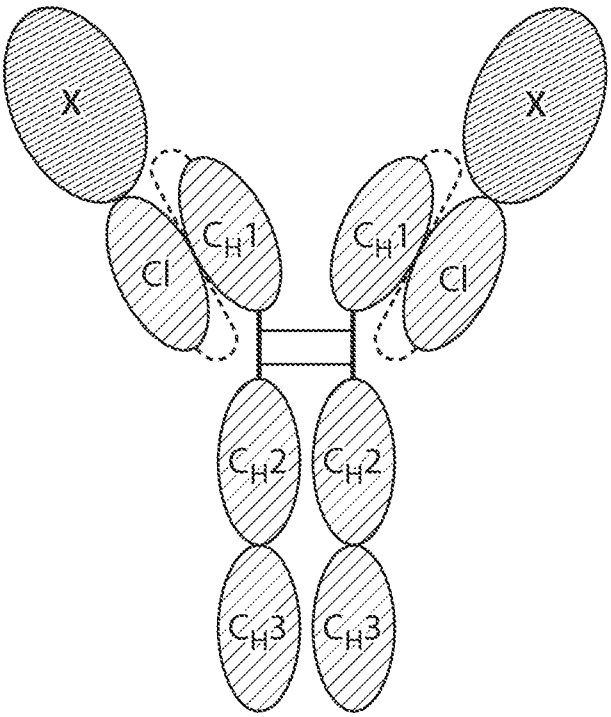



FIG. 1A

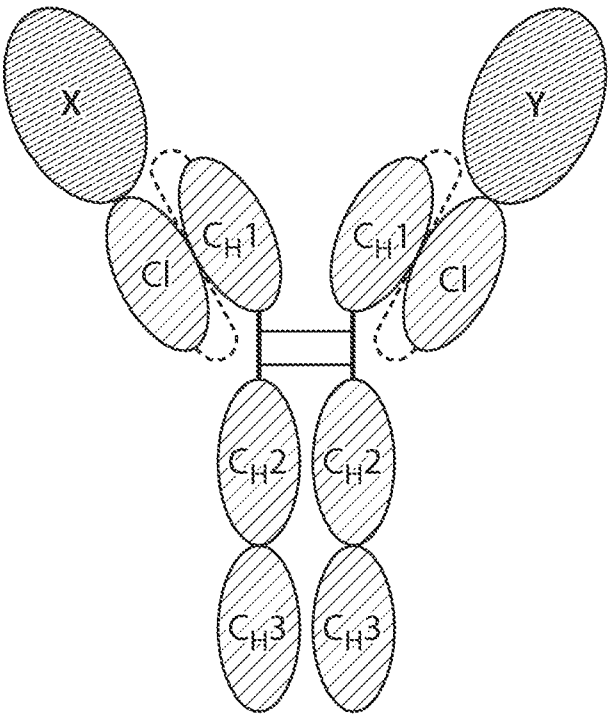



FIG. 1B

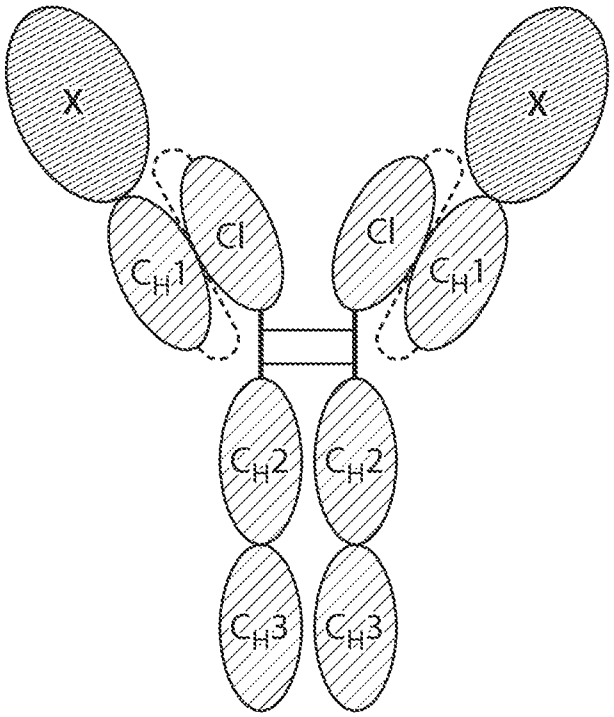



FIG. 1C

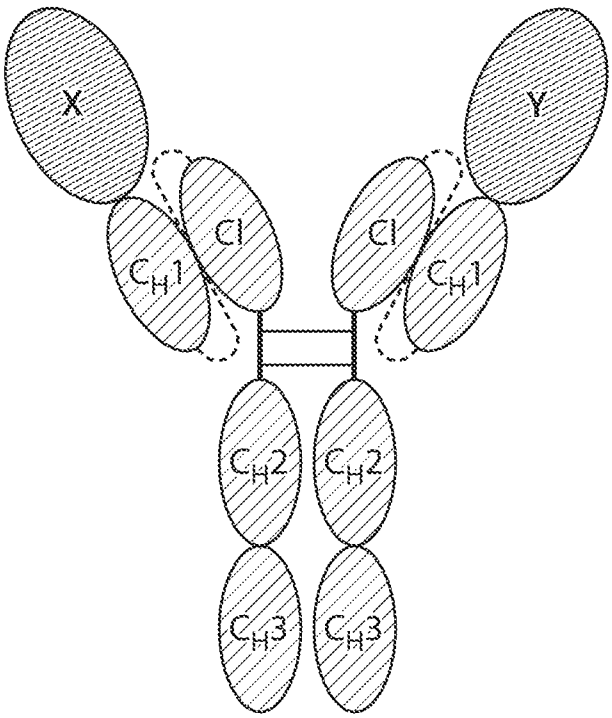



FIG. 1D

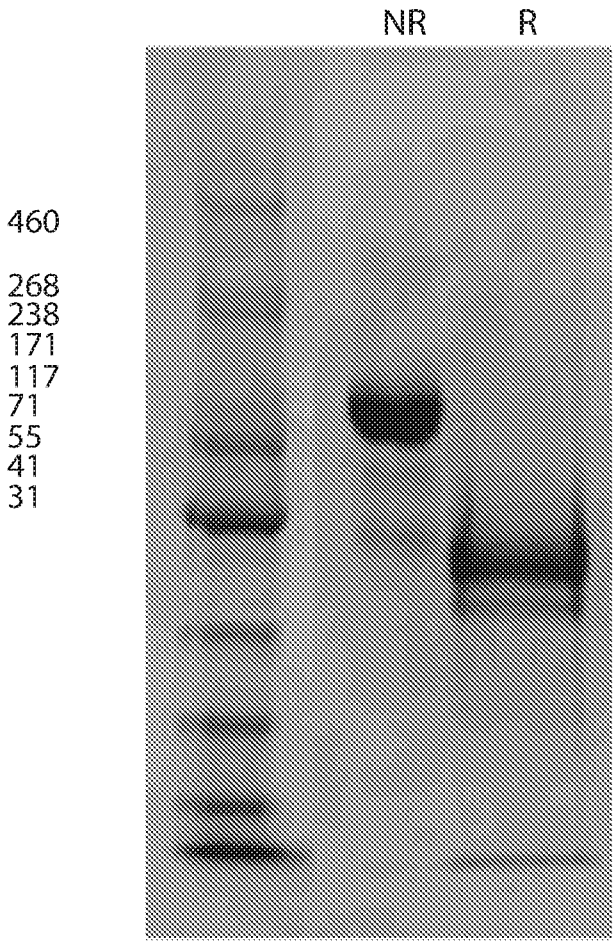



FIG. 2

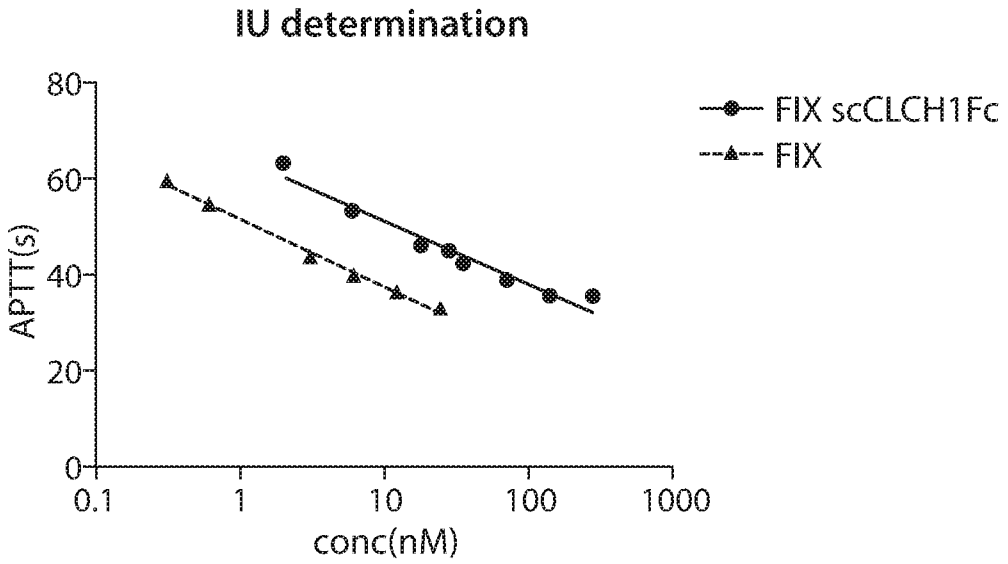

IU determination
80
60
40
20
0
APTT(s)
0.1
1
10
100
1000
conc(nM)
FIX scCLCH1Fc
FIX

FIG. 3

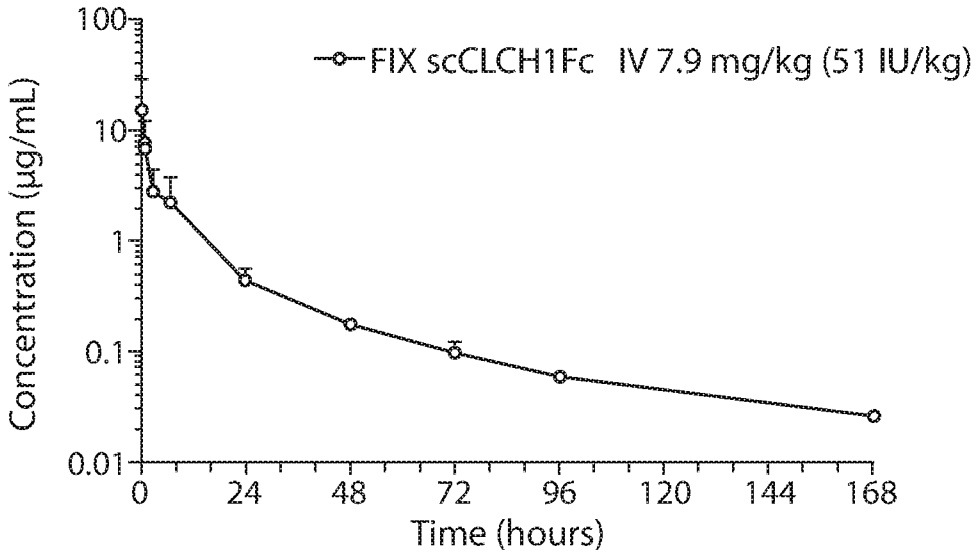

100
10
1
0.1
0.01
Concentration (µg/mL)
0
24
48
72
96
120
144
168
Time (hours)
FIX scCLCH1Fc IV 7.9 mg/kg (51 IU/kg)

FIG. 4

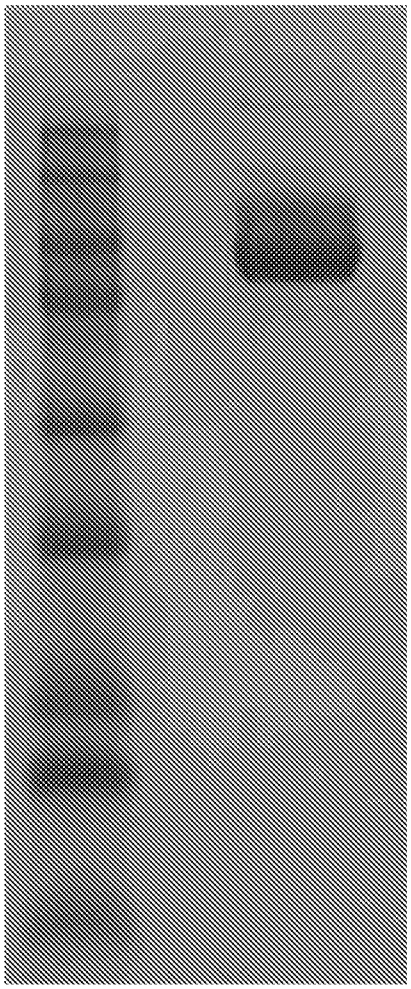



FIG. 5A

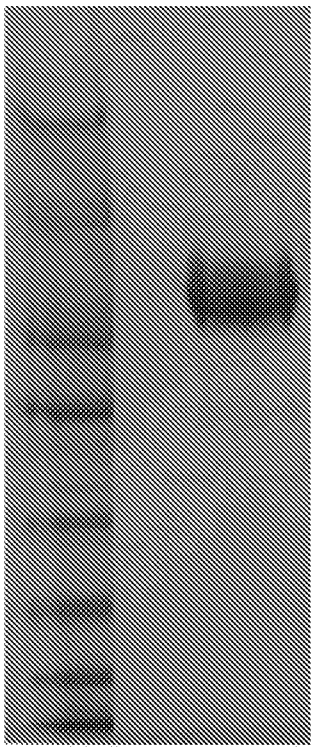
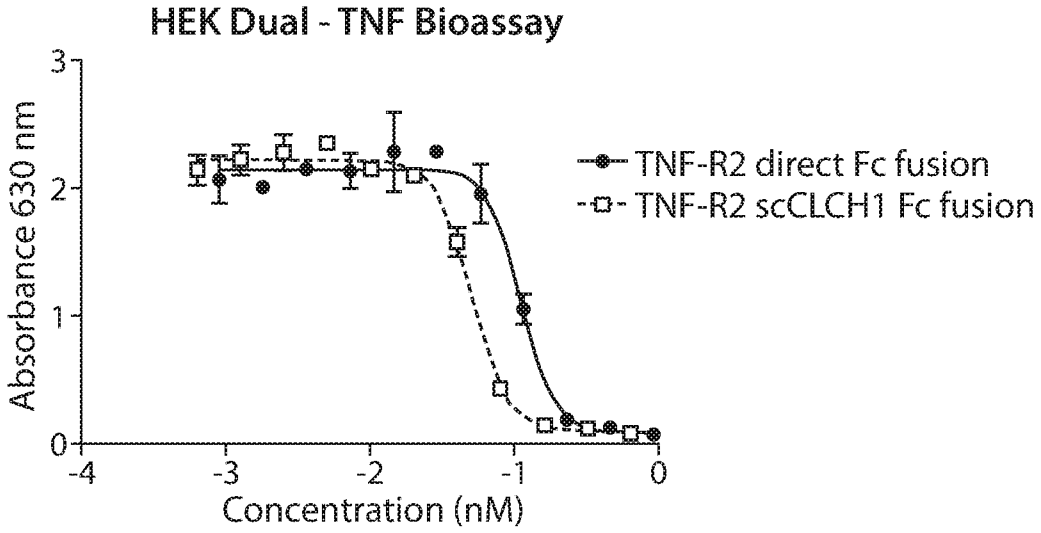



FIG. 5B




TNF-R2 direct Fc IC50 = 12 ng/mL (112 pM)
TNF-R2 scCLCH1 Fc IC50 = 7.85 ng/mL (51 pM)

FIG. 6

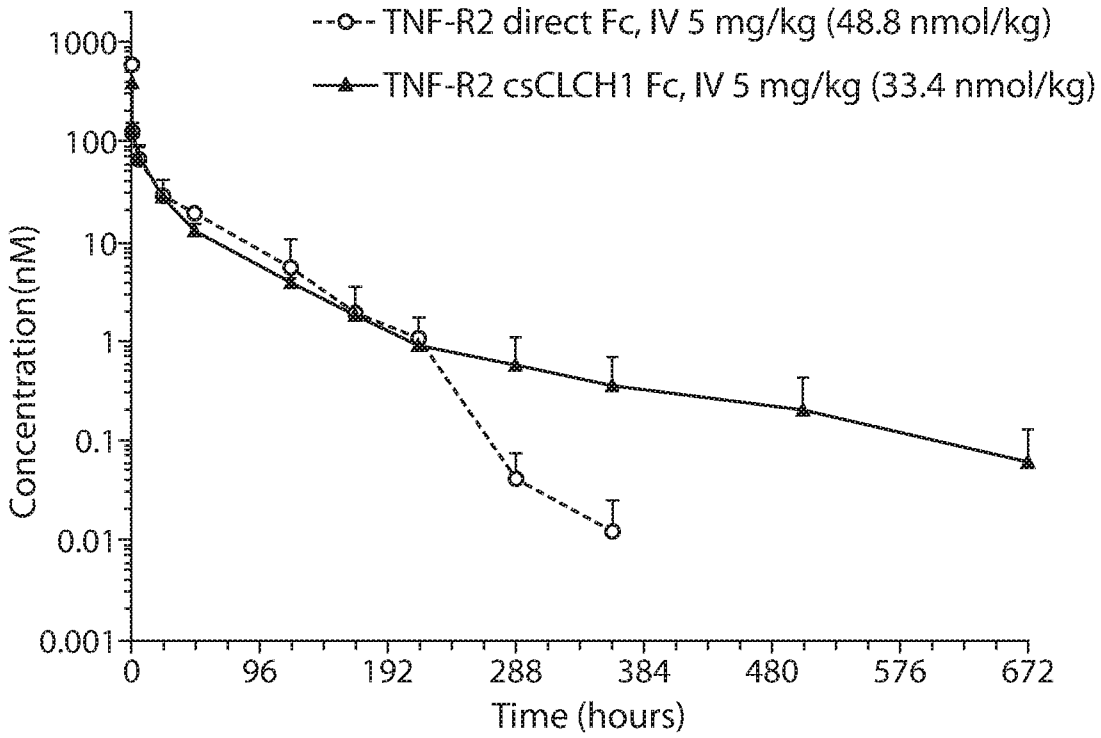



FIG. 7

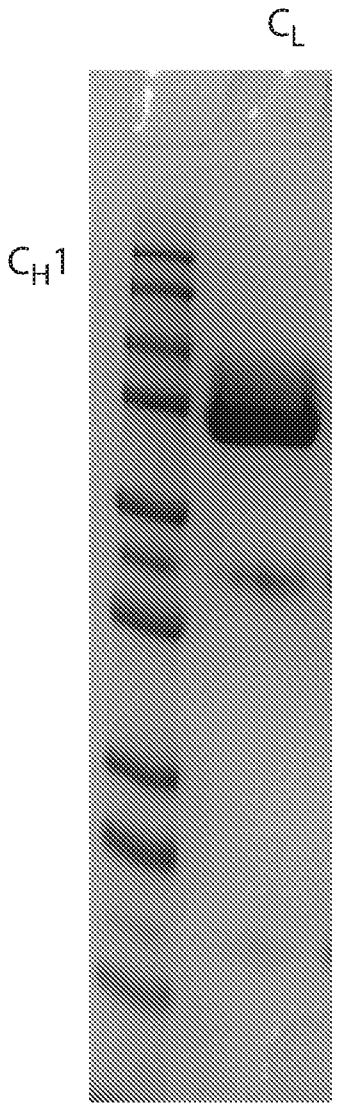



FIG. 8A

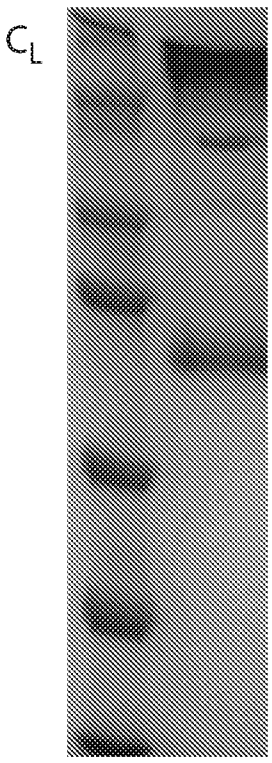



FIG. 8B

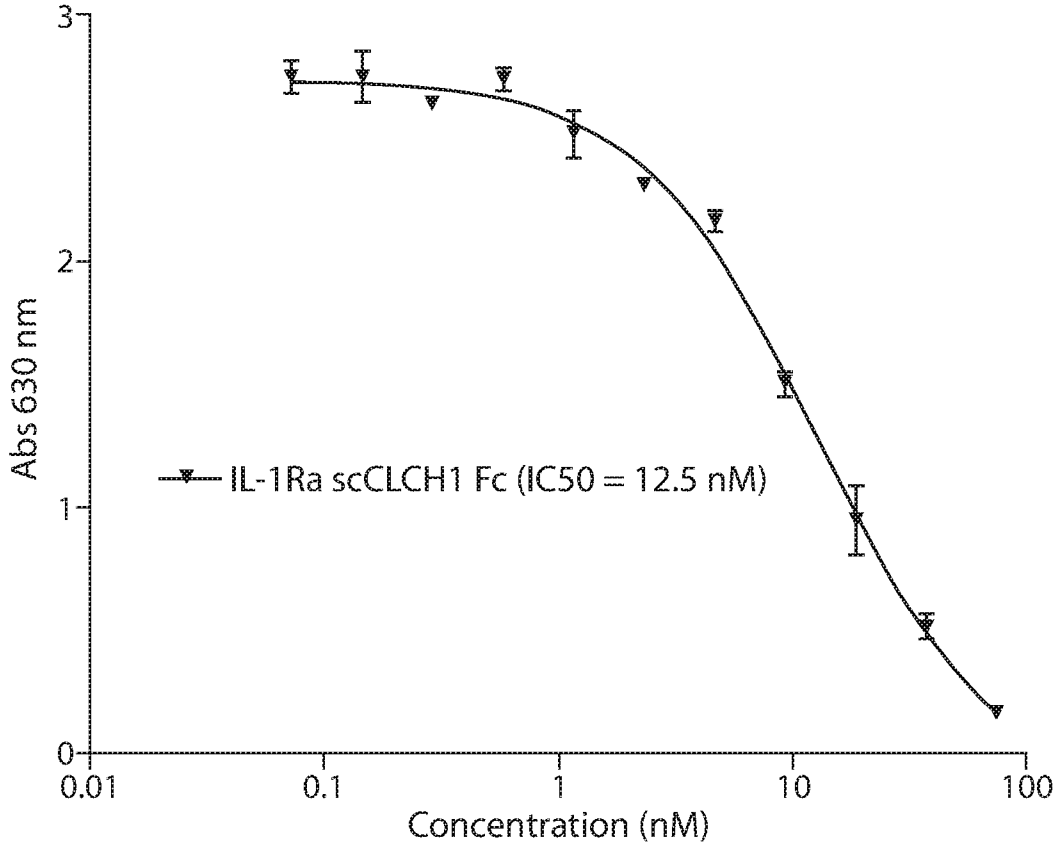

Abs 630 nm
3
2
1
0
0.01
0.1
1
10
100
Concentration (nM)
IL-1Ra scCLCH1 Fc (IC50 = 12.5 nM)

FIG. 9

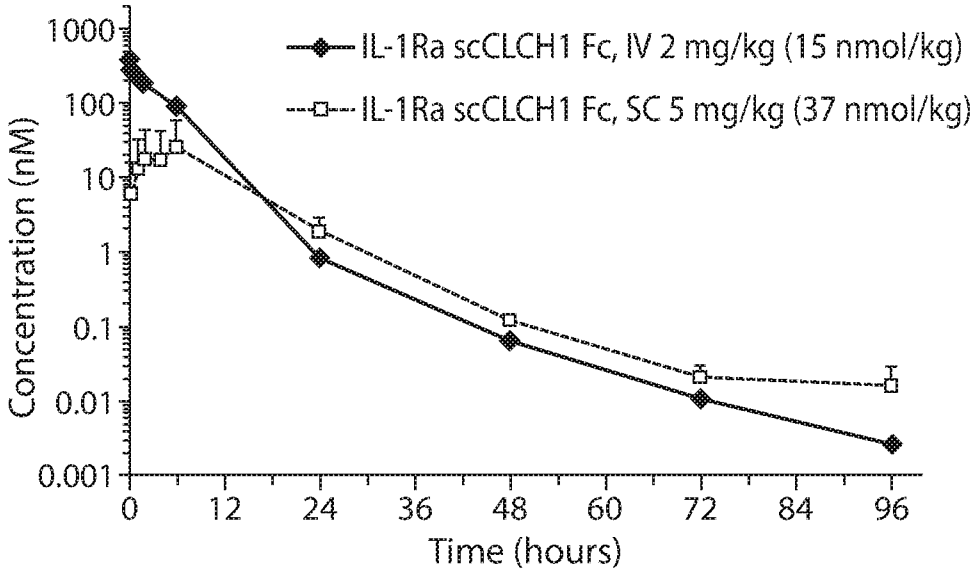

IL-1Ra scCLCH1 Fc, IV 2 mg/kg (15 nmol/kg)
IL-1Ra scCLCH1 Fc, SC 5 mg/kg (37 nmol/kg)
Concentration (nM)
1000
100
10
1
0.1
0.01
0.001
0
12
24
36
48
60
72
84
96
Time (hours)

FIG. 10

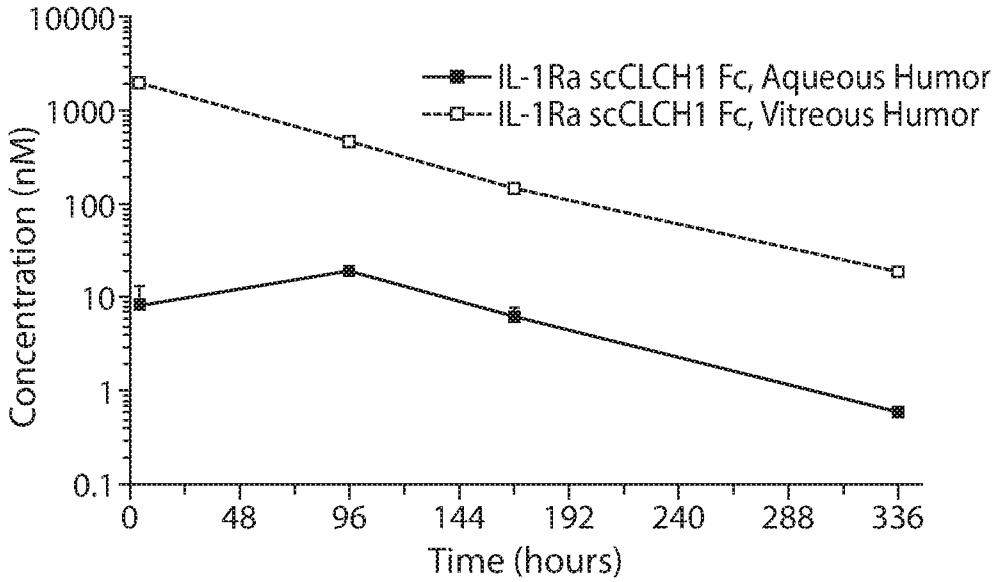

IL-1Ra scCLCH1 Fc, Aqueous Humor
IL-1Ra scCLCH1 Fc, Vitreous Humor
Concentration (nM)
10000
1000
100
10
1
0.1
0
48
96
144
192
240
288
336
Time (hours)

FIG. 11

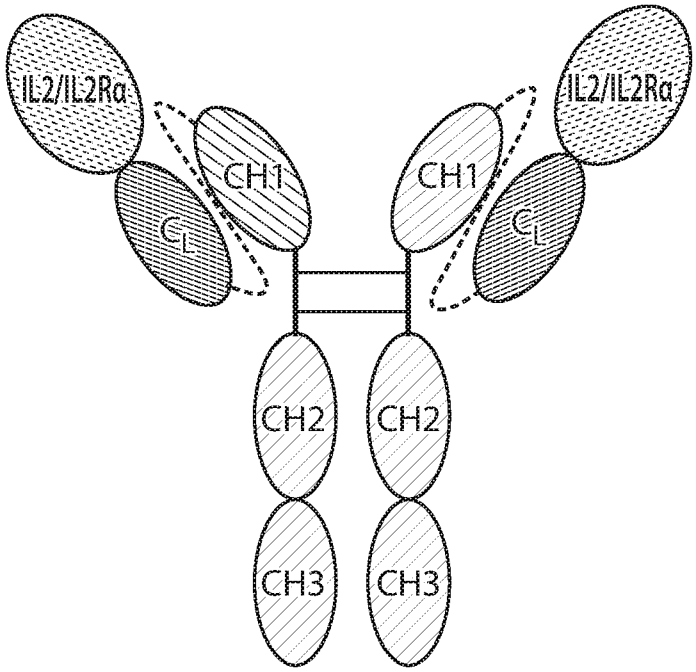



FIG. 12A

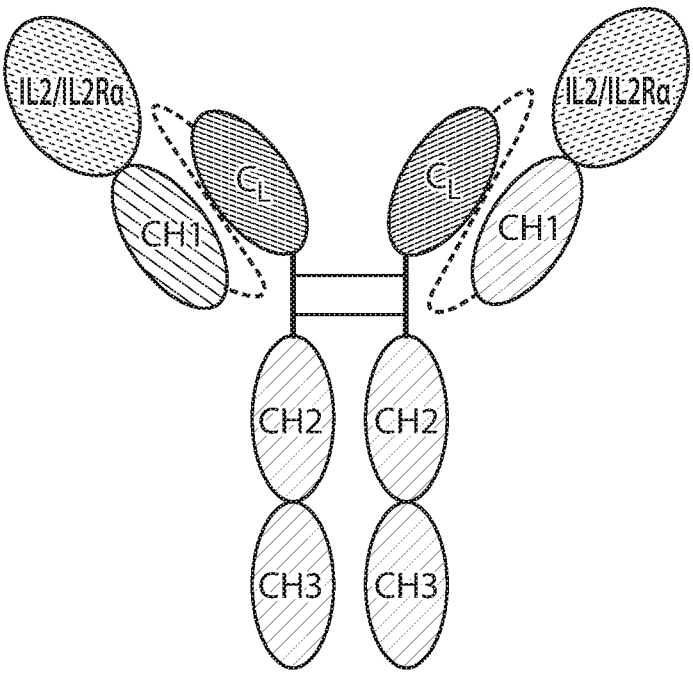



FIG. 12B

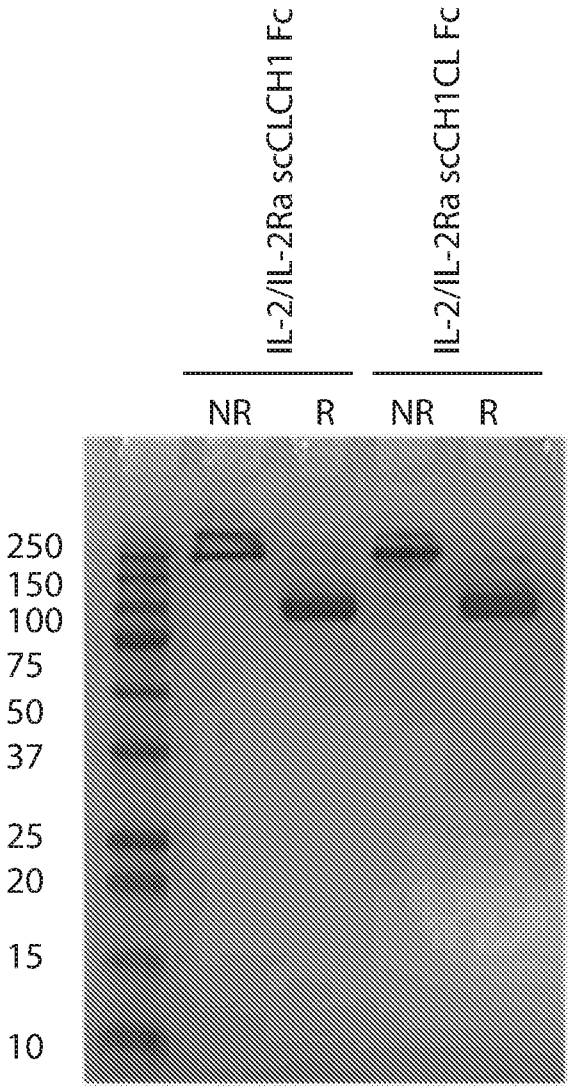

IL-2/IL-2Ra scCLCH1 Fc
IL-2/IL-2Ra scCH1CL Fc
NR
R
NR
R
250
150
100
75
50
37
25
20
15
10

FIG. 13

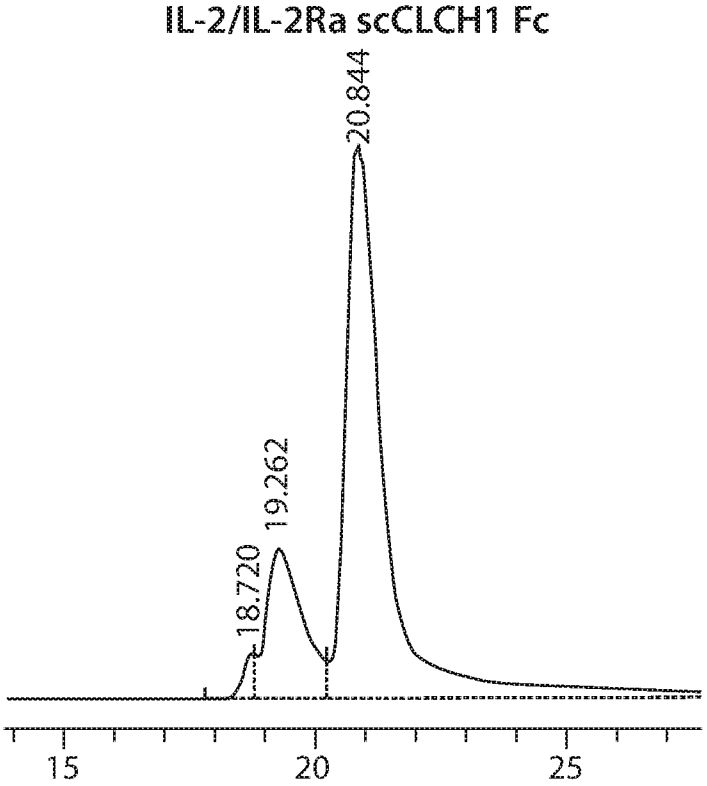



FIG. 14A

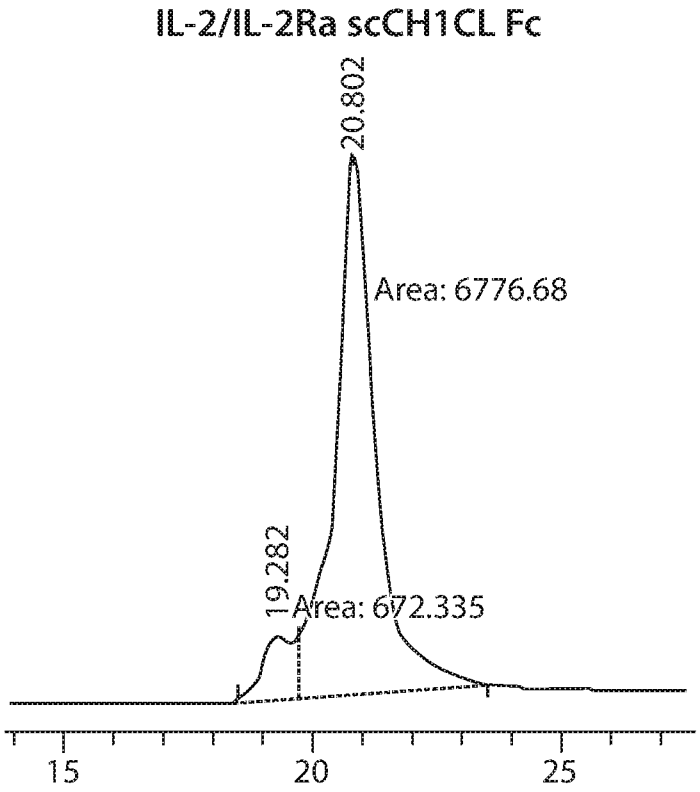



FIG. 14B

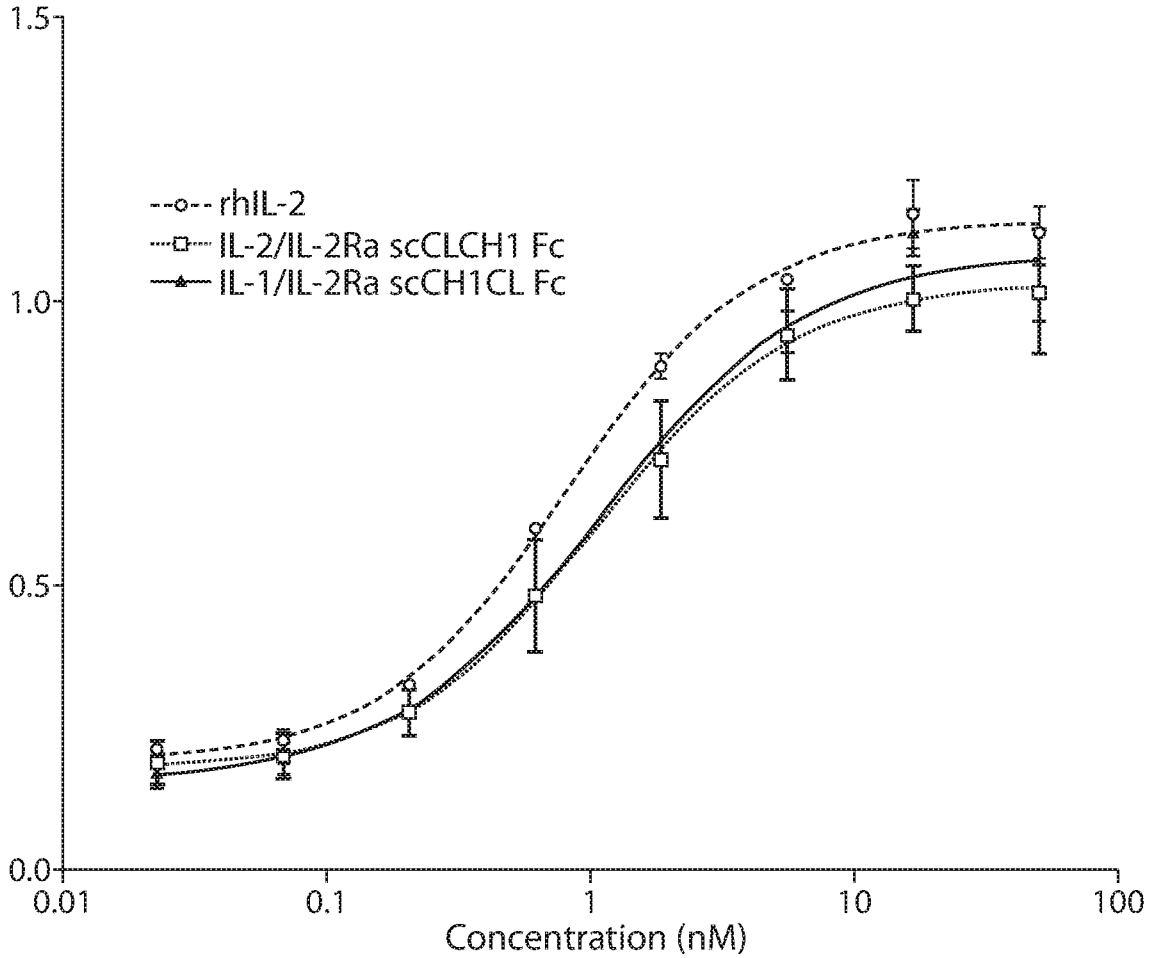

1.5
1.0
0.5
0.0
rhIL-2
IL-2/IL-2Ra scCLCH1 Fc
IL-1/IL-2Ra scCH1CL Fc
0.01
0.1
1
10
100
Concentration (nM)

FIG. 15

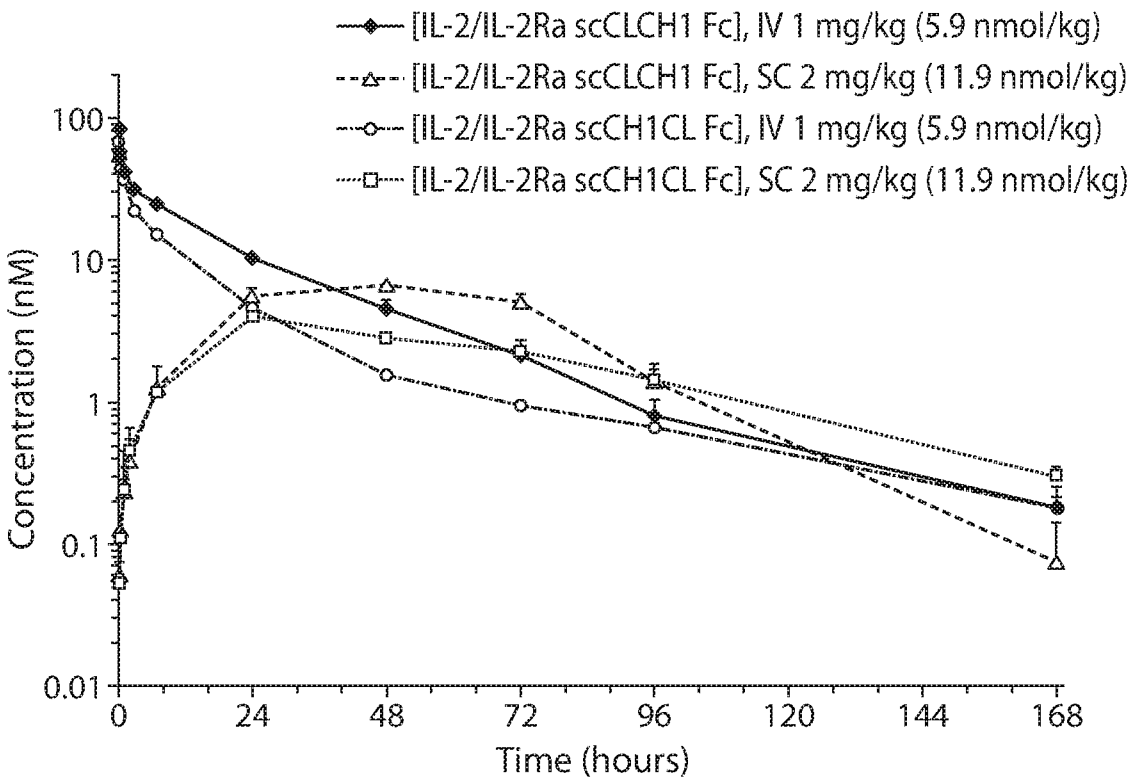

[IL-2/IL-2Ra scCLCH1 Fc], IV 1 mg/kg (5.9 nmol/kg)
[IL-2/IL-2Ra scCLCH1 Fc], SC 2 mg/kg (11.9 nmol/kg)
[IL-2/IL-2Ra scCH1CL Fc], IV 1 mg/kg (5.9 nmol/kg)
[IL-2/IL-2Ra scCH1CL Fc], SC 2 mg/kg (11.9 nmol/kg)
Concentration (nM)
100
10
1
0.1
0.01
0
24
48
72
96
120
144
168
Time (hours)

FIG. 16

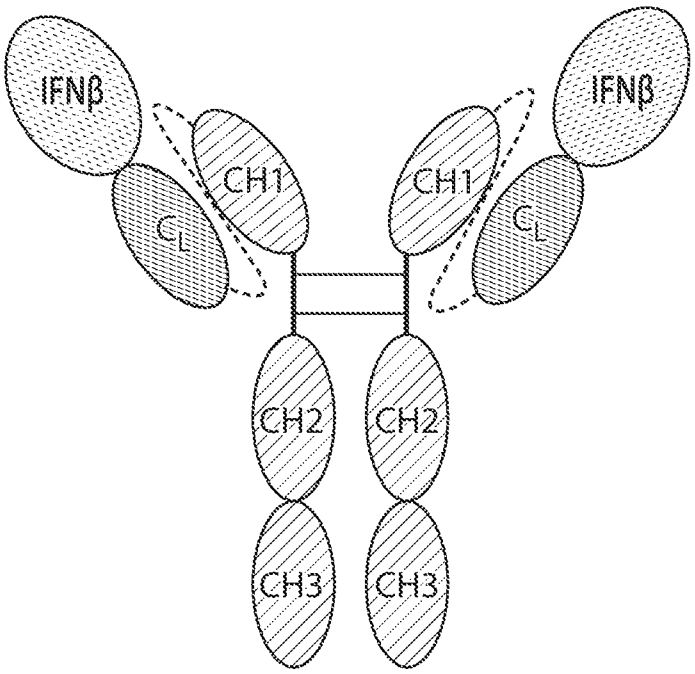

IFNβ
IFNβ
CH1
CH1
$C_L$
$C_L$
CH2
CH2
CH3
CH3

FIG. 17

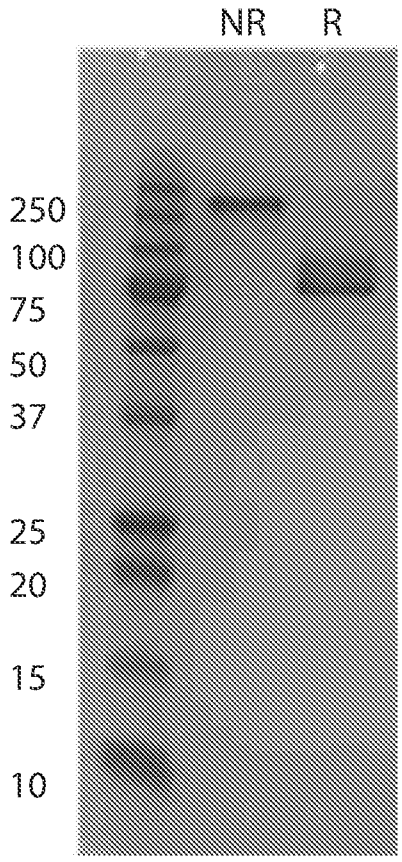

NR
R
250
100
75
50
37
25
20
15
10

FIG. 18

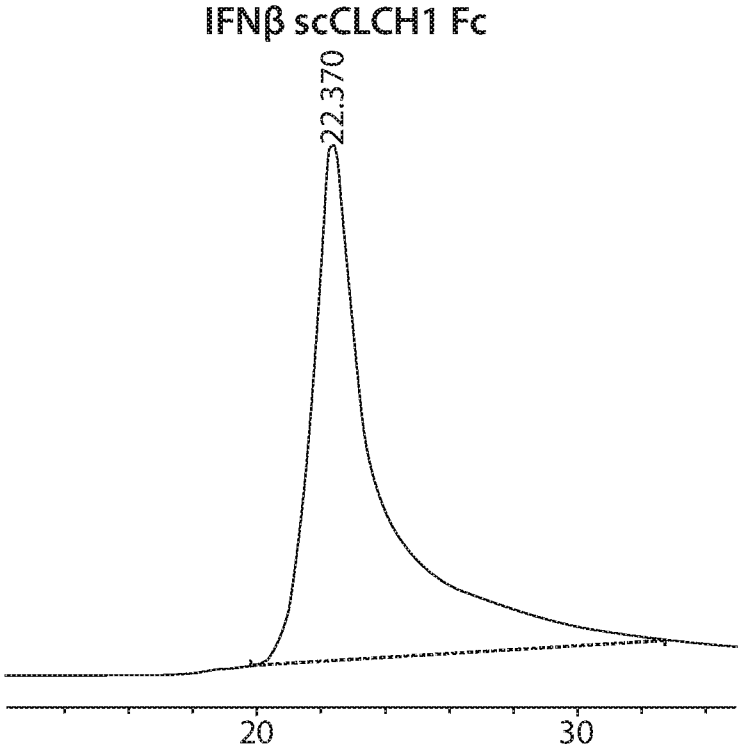

IFNβ scCLCH1 Fc
22.370
20
30

FIG. 19

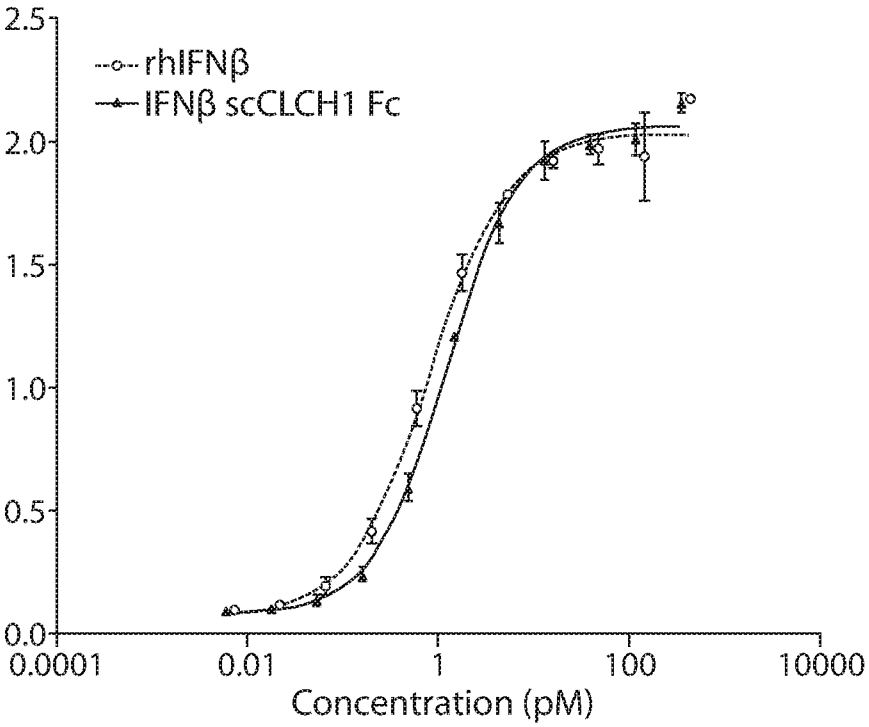

rhIFNβ
IFNβ scCLCH1 Fc
2.5
2.0
1.5
1.0
0.5
0.0
0.0001
0.01
1
100
10000
Concentration (pM)

FIG. 20

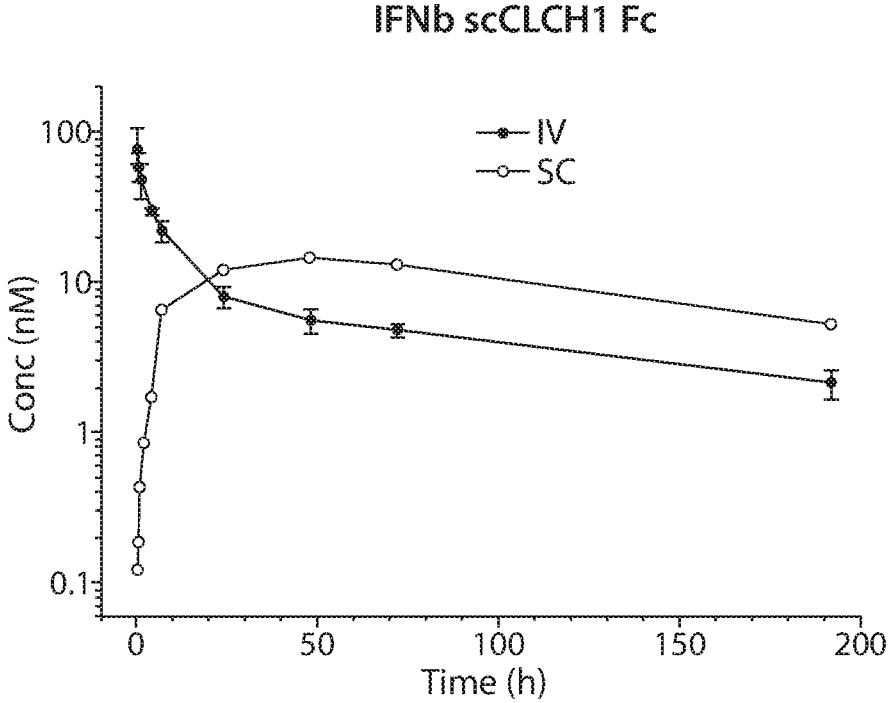



FIG. 21

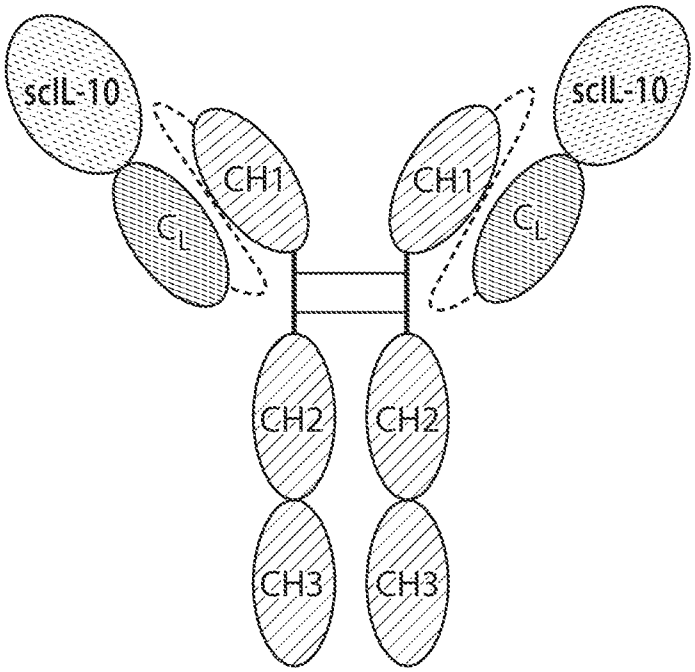



FIG. 22A

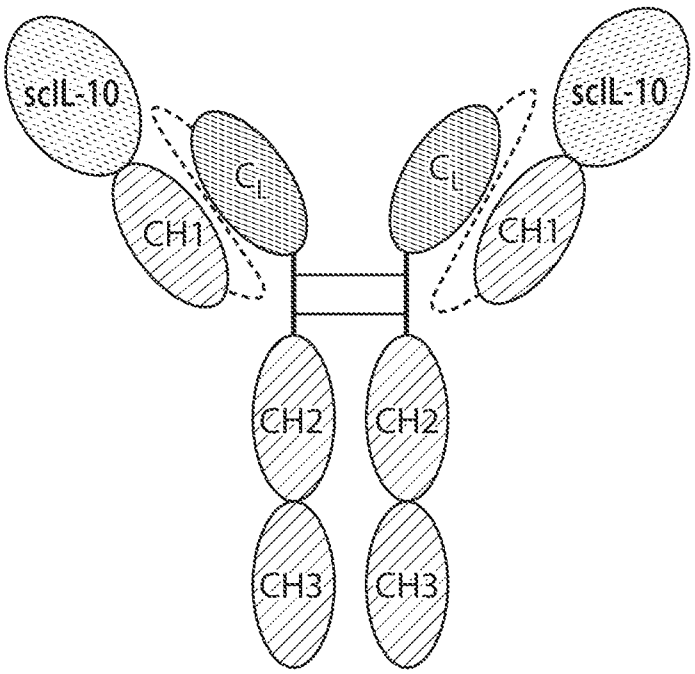



FIG. 22B

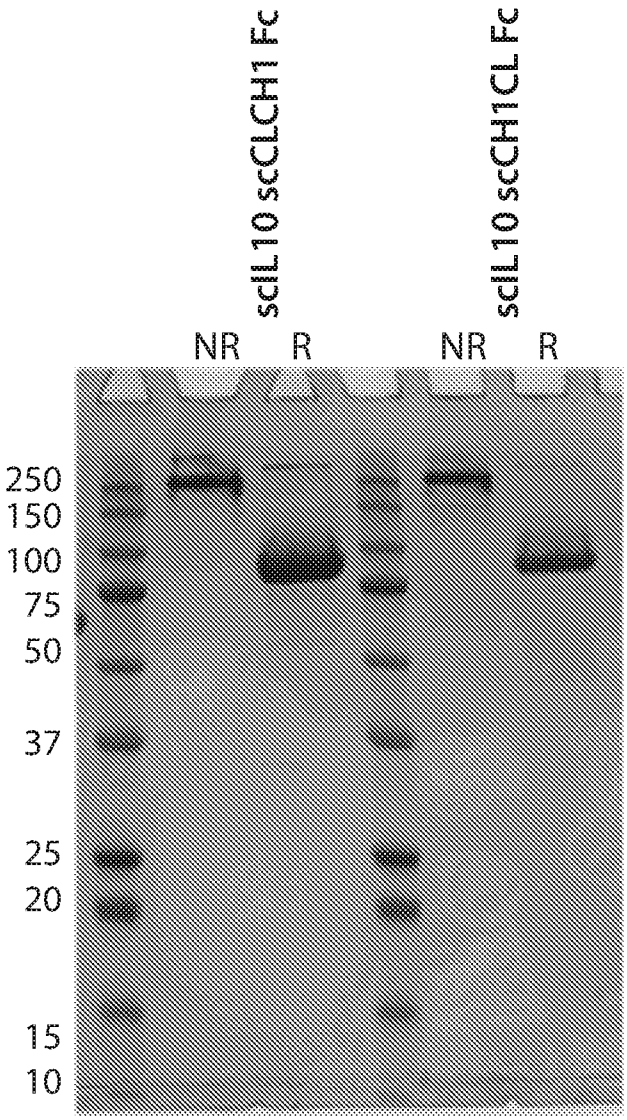

scIL10 scCLCH1 Fc
scIL10 scCH1CL Fc
NR
R
NR
R
250
150
100
75
50
37
25
20
15
10

FIG. 23

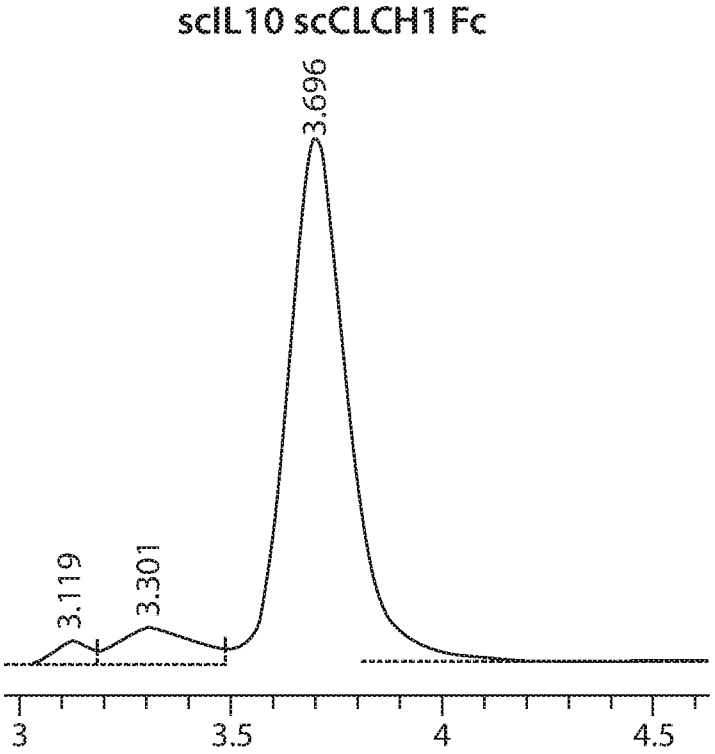



FIG. 24A

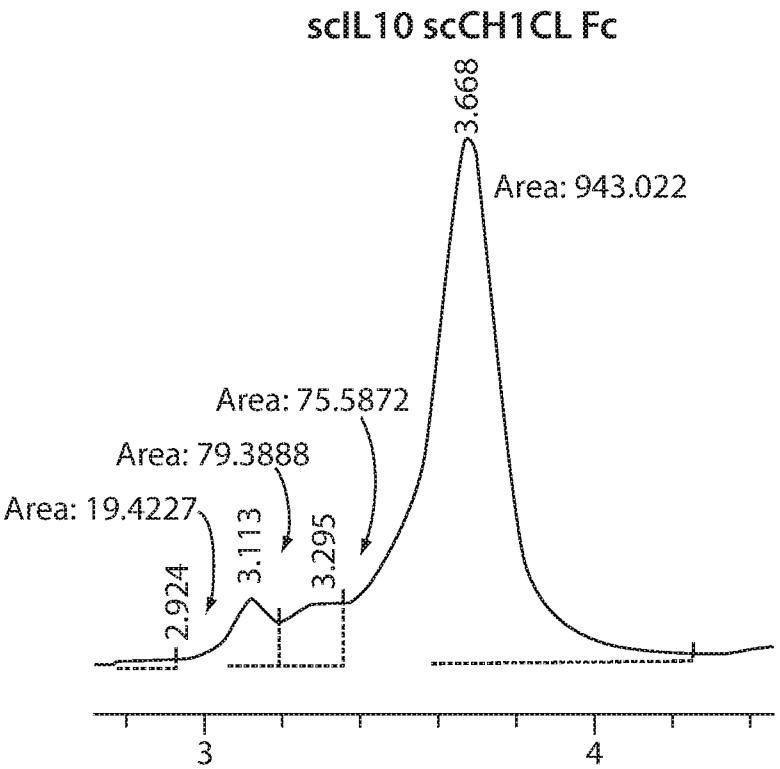



FIG. 24B

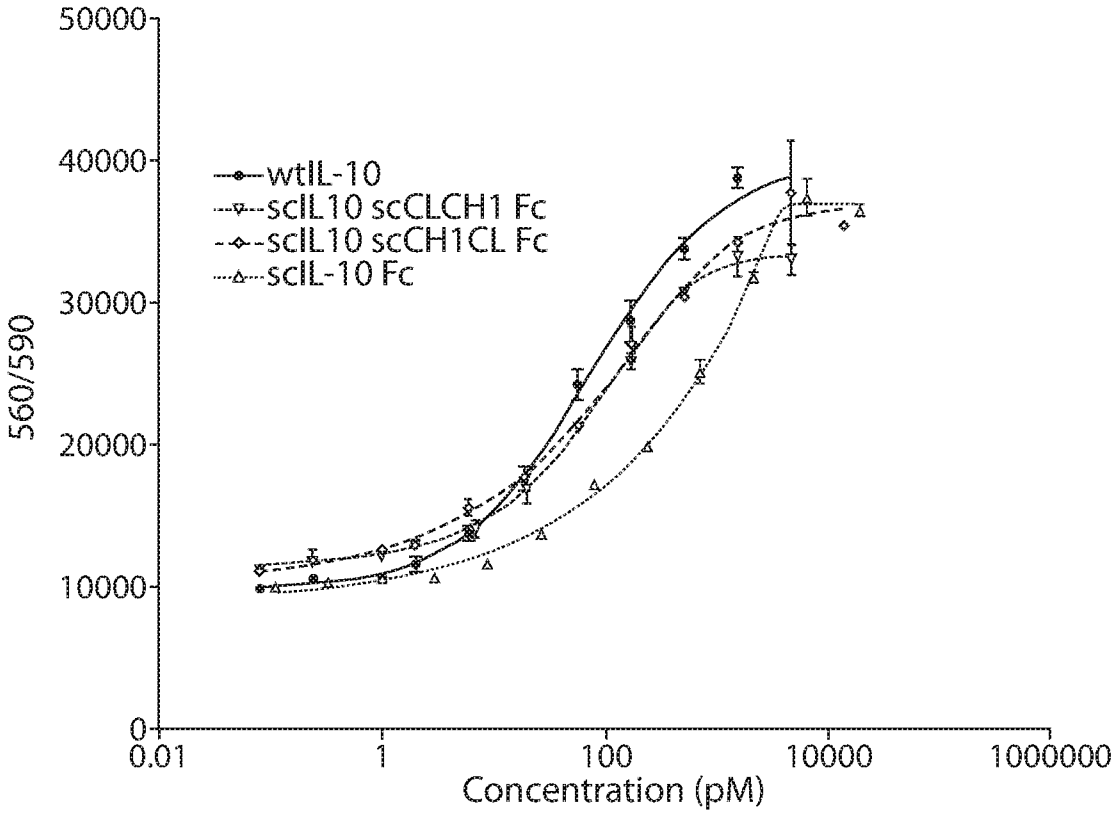
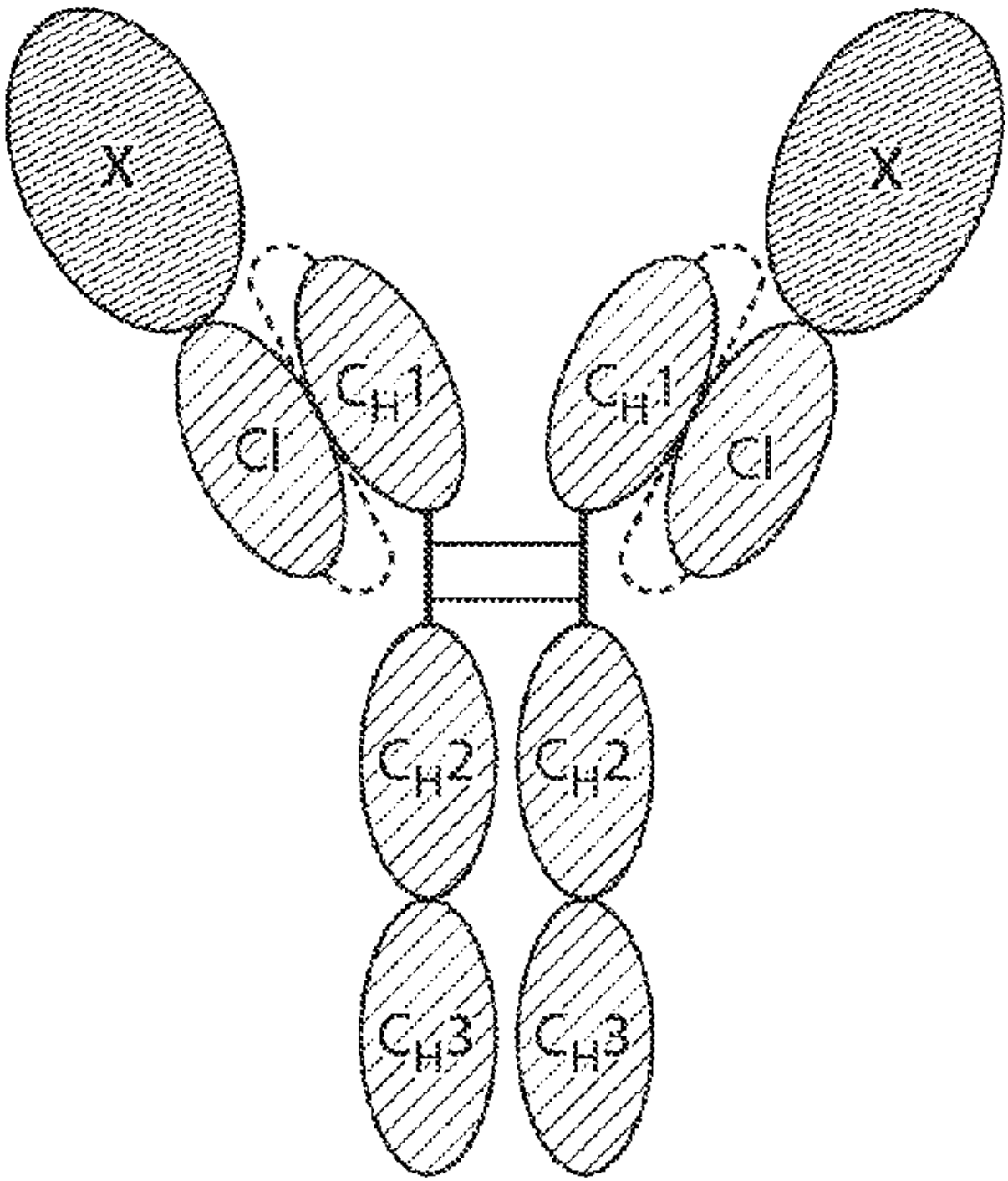

50000
40000
30000
20000
10000
0
560/590
wtIL-10
scIL10 scCLCH1 Fc
scIL10 scCH1CL Fc
scIL-10 Fc
0.01
1
100
10000
1000000
Concentration (pM)

FIG. 25


X
X
$C_{H}1$
$C_{H}1$
Cl
Cl
$C_{H}2$
$C_{H}2$
$C_{H}3$
$C_{H}3$