ANTIBODY FUSION PROTEINS WITH MODIFIED FcRn BINDING SITES

FIELD OF THE INVENTION

[0001] The invention is directed to antibody fusion proteins with a modified FcRn binding site and nucleic acid molecules encoding them. The antibody fusion protein include two polypeptide chains, wherein the first polypeptide chain includes a biologically, preferably therapeutically active molecule linked to at least a portion of an immunoglobulin constant region. The second polypeptide chain includes at least a portion of an immunoglobulin constant region. One of the polypeptide chains includes a mutation in the constant region that results in reduction or loss of FcRn binding but does not cause substantial reduction of serum half-life of said antibody fusion protein. In a preferred embodiment the invention relates to respectively constructed Fc-IFNß fusion proteins.

BACKGROUND

[0002] An antibody fusion protein linking a protein of interest to an immunoglobulin constant region possesses both the biological activity of the linked protein as well as the advantages associated with the presence of the immunoglobulin moiety. The creation of such fusion proteins helps to ensure the efficient production and secretion of proteins of interest. Furthermore, these fusion proteins often exhibit novel properties such as increased circulating half-life that are of significant therapeutic advantage.

[0003] In adult mammals, FcRn, also known as the neonatal Fc receptor, plays a key role in maintaining serum antibody levels by acting as a protective receptor that binds and salvages antibodies of the IgG isotype from degradation. IgG molecules are endocytosed by endothelial cells, and if they bind to FcRn, are recycled out into circulation. In contrast, IgG molecules that do not bind to FcRn enter the cells and are targeted to the lysosomal pathway where they are degraded. A variant IgG1 in which His435 is mutated to alanine results in the selective loss of FcRn binding and a significantly reduced serum half-life (Firan *et al.* 2001, International Immunology 13:993).

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[0004] The Fc portion of an IgG molecules includes two identical polypeptide chains, with each polypeptide chain engaging a single FcRn molecule through its FcRn binding site (Martin *et al.*, 1999, Biochemistry 38:12639). Earlier studies indicate that each Fc portion requires both FcRn binding sites for serum persistence. A heterodimeric Fc fragment containing a wild-type FcRn-binding polypeptide chain and a mutated non-FcRn-binding polypeptide chain has a significantly reduced serum half-life compared to a homodimeric wild-type Fc fragment (Kim *et al.*, 1994, Scand. J. Immunol. 40:457-465). Such a heterodimeric Fc fragment containing only one FcRn binding site is recycled less efficiently than a homodimeric wild-type Fc fragment and is preferentially trafficked to lysosomes for degradation (Tesar *et al.*, 2006, Traffic 7:1127). These observations have direct relevance to the effective application of therapeutics involving FcRn binding partners including IgG antibodies or their Fc portions. For example, U.S. Patent No 7,348,004 describes a fusion protein that specifically requires intact FcRn binding or even enhanced FcRn binding for its improved biological properties such as increased serum half-life and enhanced bioavailability.

[0005] More recent studies have identified the expression of FcRn in phagocytes and suggested a novel role of FcRn in IgG-mediated phagocytosis (Vidarsson *et al.* 2006, Blood 108:3573). IgG-opsonized pathogens are internalized into phagosomes by FcRn, providing an effective means to mark pathogens for ingestion and destruction by phagocytes. The IgG1 variant with a H435A mutation exhibits significantly reduced opsonization activity.

SUMMARY OF THE INVENTION

[0006] The present invention is based in part on the surprising discovery that an antibody fusion protein with a mutation in one of its constituent immunoglobulin constant region polypeptide chains that reduces or eliminates FcRn binding exhibits comparable biological properties such as prolonged circulating half-life as corresponding fusion proteins without the mutation. Furthermore, given the role of FcRn in IgG-mediated phagocytosis, it is contemplated that the antibody fusion protein with a mutation that reduces FcRn binding may have low opsonization activity leading to reduced immunogenicity.

[0007] The present invention provides methods and compositions for expressing soluble, biologically active antibody fusion proteins with a mutation in the FcRn binding site that reduces FcRn binding. The fusion protein includes two polypeptide chains. The first polypeptide chain includes a biologically active molecule linked to at least a portion of an immunoglobulin constant region, and the second polypeptide chain includes at least a portion of an immunoglobulin constant region. The amino acid sequence of the portion of the immunoglobulin constant region of one of the polypeptide chains differs from the amino acid sequence of the portion of the immunoglobulin constant region of the second polypeptide chain in that it contains a mutation in the FcRn binding site. The difference can be an amino acid deletion, insertion, substitution, or modification. In one embodiment, the difference is an amino acid substitution at specific positions within the constant region of the immunoglobulin such as 386, 388, 400, 415, 433, 435, 436, and 439. In a preferred embodiment, the amino acid substitution is at positions H435, preferably H435A.

[0008] The invention relates to fusion proteins linking a biologically active molecule to at least a portion of an immunoglobulin constant region. The biologically active molecule may be linked to the amino-terminus of the immunoglobulin constant region. Alternatively, the biologically active molecule may be linked to the carboxy-terminus of the immunoglobulin constant region. In a further embodiment, the fusion protein may include two biologically active molecules linked to two polypeptide chains of at least a portion of an immunoglobulin constant region.

[0009] The invention relates to fusion proteins that include at least a portion of the immunoglobulin constant region. It is contemplated that the portion of the immunoglobulin constant region can be an Fc fragment. In various embodiments, the Fc fragment is the Fc fragment of an IgG1, IgG2, IgG3, or IgG4. In another embodiment, the fusion protein includes an immunoglobulin variable region in at least one of the polypeptide chains. Preferred Fc- fusion proteins according to this invention are molecules wherein an Fc portion is fused via its C- or N-terminus of one or both CH2-CH3 chains to a therapeutically effective molecule, preferably a protein or polypeptide such as a cytokine, a growth factor or a growth hormone, another therapeutically effective polypeptide, a small chemical molecule, or a nucleic acid, wherein only one of the CH2-CH3 chains is mutated in order to eliminate or reduce

FcRn binding of the respective immunoglobulin chain and preferably of the complete fusion protein. Dependent on the nature of the fusion protein it may be more advantageous to mutate the immunoglobulin chain to which the biologically active molecule is fused, or it may be advantageous to mutate the naked immunoglobulin chain. These Fc fusion proteins of the invention may be homodimers (in case that two biologically active molecules are fused to the antibody Fc portion, one molecule to each heavy chain of the antibody portion) or they may be heterodimers if only one biologically active molecule is fused to one of the two heavy chains of the antibody or Fc portions, respectively.

[0010] The present invention contemplates the use of any biologically active molecule as the therapeutic molecule of the invention. For example, the biologically active molecule can be a human interferon, such as interferon-β (IFNß). To improve folding and to reduce aggregation, an interferon-β sequence can include an amino acid alteration of at least one of positions 17, 50, 57, 130, 131, 136, and 140 corresponding to native, mature interferon-β. The alteration can be an amino acid substitution. In one embodiment, the amino acid substitution is selected from the group consisting of C17S, C17A, C17V, C17M, F50H, L57A, L130A, H131A, K136A, H140A, and H140T. In another embodiment, the biologically active molecule is another cytokine or a growth factor such as human erythropoietin. In a preferred embodiment the IFNß molecule is fused to the N- or C-terminus of an Fc portion of an respectively FcRn mutated immunoglobulin. A specifically preferred embodiment is a (dimeric) Fc-IFNß fusion protein, wherein one optionally modified IFNß molecule is fused to the C- or optionally to the N-terminus, preferably the C-terminus, of the constant region of the first immunoglobulin CH2-CH3 chain, and the second CH2-CH3 immunoglobulin chain lacks IFNß, and the mutation that prevents or reduces FcRn binding is located in the first or the second CH2-CH3 chain, preferably in that chain which is naked, and the mutation is carried out at position 435 and is specifically H435A. Such preferred Fc fusion molecules are heterodimers. Moreover, a further preferred Fc-IFNß fusion protein possesses besides these structural characteristics an IgG1 hinge region coupled to a CH2-CH3 region of IgG2 or IgG4.

[0011] The invention also provides methods for encoding and expressing fusion proteins of the invention. For example, one aspect of the invention relates to a

nucleic acid encoding a polypeptide chain that includes a biologically active molecule and at least a portion of an immunoglobulin constant region comprising a mutation that reduces FcRn binding. In another aspect, the invention relates to a composition of one nucleic acid encoding a polypeptide chain that includes a biologically active molecule and at least a portion of an immunoglobulin constant region, and a second nucleic acid encoding a second polypeptide chain that includes at least a portion of an immunoglobulin constant region. One of the nucleic acid sequences contains a mutation in the FcRn binding site. In one embodiment, the mutation is an amino acid substitution at one or more positions in the constant region, for example positions 386, 388, 400, 415, 433, 435, 436, and 439, wherein the numbering of the residues is that of the EU index according to Kabat. In a further preferred embodiment, the amino acid substitution is at position 435, and specifically H435A. In another aspect, the nucleic acid molecule or the nucleic acid composition of the invention can be incorporated within a replicable expression vector, which can then be introduced into a host cell and be recombined with and integrated into the host cell genome. The replicable expression vector can include the aforementioned nucleic acid or the nucleic acid composition. In another embodiment, the invention encompasses the host cells containing the aforementioned nucleic acid or the nucleic acid composition.

[0012] The invention herein provides for a pharmaceutical composition comprising the aforementioned fusion proteins and a pharmaceutically acceptable carrier. Depending on the intended use or mode of administration, solid or liquid pharmaceutically acceptable carriers can be employed in the pharmaceutical composition.

[0013] A further aspect of the invention relates to methods for treating a mammal with a disease or condition alleviated by the administration of any of the aforementioned fusion proteins. In one embodiment, the disease or condition is viral infection. In another embodiment, the disease or condition is anemia, while in yet another embodiment, the disease or condition is multiple sclerosis.

[0014] . In summary, the invention relates to the following subject-matters:

- A protein comprising two polypeptide chains, wherein the first polypeptide chain comprises a biologically active molecule and at least a portion of an

immunoglobulin constant region, the second polypeptide chain comprises at least a portion of an immunoglobulin constant region, and one of the polypeptide chains comprises a mutation in the FcRn binding site.

- An antibody fusion protein comprising a first and a second heavy chain domain each domain comprising at least a portion of an immunoglobulin constant region, wherein at least one of said first or second heavy chain domain is fused to a biologically active molecule, and only one of said first or second heavy chain domain comprises a mutation of the amino acid sequence within the constant region, wherein the mutation results in a partial or complete loss of binding FcRn, and does not cause a reduction or a substantial reduction of serum half-life of the fusion protein. A substantial reduction of serum half life of the antibody fusion proteins is according to the invention a reduction of more than 10%, preferably more than 5%, and most preferably more than 3% of the serum half-life of the fusion protein without a FcRn binding site mutation at one or more positions, preferably at positions 386, 388, 400, 415, 433, 435, 436, 439 and 447. and most preferred at position 435.
- A respective protein or antibody fusion protein, wherein the biologically active molecule is linked to the amino-terminus of the immunoglobulin constant region or to the carboxy-terminus of the immunoglobulin constant region.
- A respective protein or antibody fusion protein, wherein the first and the second heavy chain domain comprises a biologically active molecule.
- An respective protein or antibody fusion protein which is an antibody Fc fragment, wherein the first and the second heavy chain domain are CH2 and CH3 domains including a hinge region and thus constitute said Fc fragment.
- Alternatively, an antibody fusion protein, wherein the first and / or the second heavy chain domain includes an antibody variable domain thus forming a full complete antibody that allows binding of an antigen target.
- A respective protein or antibody fusion protein, wherein the immunoglobulin is IgG, preferably IgG1, IgG2 or IgG4.
- A preferred antibody fusion protein, which is a Fc fragment or portion of antibody, wherein (i) the first and the second heavy chain domain are CH2

and CH3 domains of IgG including a hinge region and constitute a Fc fragment; (ii) the first heavy chain domain is fused via its C-terminal to a biologically active molecule; and (iii) the second heavy chain domain is not fused to a biologically active molecule, thus forming a Fc heterodimeric fusion protein. In this embodiment the mutation leading to loss or reduction of FcRn binding may be located in the heavy chain domain that is not fused to the biologically active molecule, or it may be located in the heavy chain domain that is fused to the biologically active molecule.

- A respective protein or antibody fusion protein, wherein the biologically active and therapeutically effective molecule is human interferon is a cytokine, such as an interferon or interleukin, a growth factor such as erythropoietin or a growth hormone such as human growth hormone. Most preferred according to the invention is a respective Fc fusion protein that contains an interferon-β (IFNß) molecule fused only to one heavy chain of the Fc molecule, preferably fused to the C-terminus of the Fc molecule..

- A respective antibody fusion protein, preferably a Fc fusion protein, wherein the amino acid sequence of human IFNß is altered, preferably substituted, at one or more positions preferably at positions 17, 50, 57, 130, 131, 136, and 140 corresponding to native mature human IFNß. Preferably the Fc-IFNß is substituted at one or more positions within the IFNß sequence as follows: C17S, C17A, C17V, C17M, F50H, L57A, L130A, H131A, K136A, H140A, and H140T.

- A respective Fc fragment comprising the FcRn mutation in that heavy chain which is not fused to native or modified IFNß, or vice versa, wherein the FcRn mutation is carried out in that chain, which is fused to native or modified IFNß, wherein in both alternatives the hinge region of the Fc fusion protein derives from a different IgG as the IgG heavy chains, for example, a respective Fc fusion molecule, wherein the hinge region derives from IgG1 and the CH2-CH3 heavy chains derive from IgG2 or IgG4.

- A nucleic acid composition suitable for preparing an antibody fusion protein as described above and below comprising (i) a nucleic acid encoding a first heavy

chain domain comprising a biologically active molecule fused to at least a portion of an IgG constant region, and (ii) a nucleic acid encoding a second heavy chain domain comprising at least a portion of an IgG constant region that is not fused to a biologically active molecule, wherein only one of said IgG constant regions or portions thereof comprises a mutation that results in a partial or complete loss of binding FcRn.

- A pharmaceutical composition comprising the respective protein or antibody fusion protein, preferably the respective Fc fusion protein, most preferably the Fc-IFNß fusion protein as described and a pharmaceutically acceptable carrier, excipient or diluent.
- A respective protein or antibody fusion protein, preferably the respective Fc fusion protein, most preferably the Fc-IFNß fusion protein as described or said pharmaceutical composition for use in the treatment of a diseases such as viral infection, anemia, cancer or multiple sclerosis.

DESCRIPTION OF DRAWINGS

[0015] FIGURES **1A-1D** schematically illustrate how a biologically active molecule (circle) may be linked to the Fc subunits of an antibody, shown here as a hinge (small rectangle), a CH2 domain and a CH3 domain (large rectangle). One of the Fc subunits includes a mutation in the FcRn binding site (diamond). The biologically active molecule may be linked to the N-terminus (FIGURES 1A and 1B) or the C-terminus (FIGURES 1C and 1D) of the Fc subunit.

[0016] FIGURES **2A-2F** illustrate a subset of ways in which two biologically active molecules may be linked to the Fc subunits of an antibody. One of the Fc subunits includes a mutation in the FcRn binding site. The two biologically active molecules may be same or different (black or white circles).

[0017] FIGURES **3A-3H** show how a biologically active molecule may be linked to an antibody that include a constant region (rectangles) and a variable region (stippled rectangle) of the heavy chain. The antibody fusion protein may include a single variable region (FIGURES 3A-3D), or the antibody fusion protein may include two

variable regions (FIGURES 3E-3H). One of the heavy chains includes a mutation in the FcRn binding site.

[0018] FIGURES **4A-4F** show how two biologically active molecules may be linked to an antibody that include a constant region (rectangles) and a variable region (stippled rectangle) of the heavy chain. The two biologically active molecules may be same or different (black or white circles). One of the heavy chains includes a mutation in the FcRn binding site.

[0019] FIGURES **5A-5D** show a subset of ways in which a biologically active molecule may be linked to an intact immunoglobulin such as an IgG. The constant regions of the heavy and light chains are shown as rectangles and the variable regions are shown as stippled rectangles. One of the heavy chains includes a mutation in the FcRn binding site. The biologically active molecule may be linked to the heavy chain (FIGURES 5A and 5B) or the light chain (FIGURES 5C and 5D).

[0020] FIGURES **6A-6F** illustrate how two biologically active molecules can be linked to an intact immunoglobulin such as an IgG. One of the heavy chains includes a mutation in the FcRn binding site. The biologically active molecules may be linked to the heavy chains (FIGURES 6A and 6B) or the light chains (FIGURES 6C and 6D). Alternatively, one of the biologically active molecule may be linked to the heavy chain and the other biologically active molecule is linked to the light chain (FIGURES 6E and 6F).

[0021] FIGURES **7A-7F** illustrate how two different biologically active molecules can be linked to an intact immunoglobulin such as an IgG. One of the heavy chains includes a mutation in the FcRn binding site.

[0022] FIGURE **8** shows an SDS-PAGE analysis of huFcγ4h-mono-L-DI-IFNß produced by transient coexpression of the human Fcγ4h chain and the human Fcγ4h-Linker-DI-IFNß chain in 293T cells under reducing (left panel) and non-reducing conditions (middle panel). In the left panel, lane 2 shows that both the Fcγ4h chain and the Fcγ4h-Linker-DI-IFNß chain were expressed, while lane 1 is a control showing the expression of Fcγ4h alone. In the middle panel, the transient coexpression of the human Fcγ4h chain and the human Fcγ4h-Linker-DI-IFNß chain

was analyzed under non-reducing conditions. Duplicate samples of the Fcγ4h chain (the two lanes labeled 1a and 1 b) or the coexpression of the Fcγ4h chain and the Fcγ4h-Linker-DI-IFNß chain (the two lanes labeled 2a and 2b) were analyzed. The right panel shows that coexpression of Fc and Fc-IL-2 chains from two stably transfected clones (the two lanes labeled 3 and 4) gave the normal and expected ratio of naked Fc homodimer : Fc/Fc-IL2 heterodimer : Fc-IL2 homodimer on SDS-PAGE under non-reducing conditions.

[0023] FIGURE **9** shows an SDS-PAGE analysis of conditioned media from a number of stable NS0 clones coexpressing the Fcγ4h chain and the Fcγ4h-Linker-DI-IFNß chain under non-reducing conditions. All the clones produced the Fcγ4h homodimer and Fcγ4h/Fcγ4h- IFNß heterodimer, but little or no Fcγ4h-IFNßhomodimer.

[0024] FIGURE **10** shows the biological activities of purified Fcγ4h-mono-L-DI-IFNß (squares) and Fcγ4h-mono-L-DI-IFNß variant containing the H435A substitution (triangles) in a cytopathic effect (CPE) inhibition assay. The human epithelial lung carcinoma line A549 was used as a host for the encephalomyocarditis virus (EMCV). Rebif (circles) was used as the positive control.

[0025] FIGURE **11** compares the pharmacokinetic profiles of intravenously administered huFcγ4h-mono-L-DI-IFNß (diamonds) and huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution in the form of the huFcγ4h(H435A)/huFcγ4h-L-DI-IFNß heterodimer (squares). Mice (n=3) were injected intravenously with 25 µg of total protein/mouse. Serum concentrations of the injected protein at different time points were determined by anti-hu Fc ELISA.

[0026] FIGURE **12** compares the pharmacokinetic profiles of intravenously administered huFcγ4h-mono-L-DI-IFNß (diamonds) and huFc4h-mono-L-DI-IFNß variant containing the H435A substitution in the form of the huFcγ4h(H435A)/huFcγ4h-L-DI-IFNß heterodimer (squares). Mice (n=3) were injected intravenously with 25 µg of total protein/mouse. Serum concentrations of the injected protein at different time points were determined by an ELISA consisting of anti-hu (H&L) capture and anti-hu IFNß detection.

[0027] FIGURE 13 compares the pharmacokinetic profiles of subcutaneously administered huFc4γh-mono-L-DI-IFNß (diamonds) and huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution in the form of the huFcγ4h(H435A)/huFcγ4h-L-DI-IFNß heterodimer (squares). Mice (n=3) were injected subcutaneously with 50 µg of total protein/mouse. Serum concentrations of the injected protein at different time points were determined by anti-hu Fc ELISA.

[0028] FIGURE 14 compares the pharmacokinetic profiles of subcutaneously administered huFcγ4h-mono-L-DI-IFNß (diamonds) and huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution in the form of the huFcγ4h(H435A)/huFcγ4h-L-DI-IFNß heterodimer (squares). Mice (n=3) were injected subcutaneously with 50 µg of total protein/mouse. Serum concentrations of the injected protein at different time points were determined by an ELISA consisting of anti-hu (H&L) capture and anti-hu IFNß detection.

[0029] FIGURE 15 compares the antibody titers of mice that were immunized with huFcγ4h-mono-L-DI-IFNß (circles) or huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution (diamonds) following boost injections. Mouse antibody titers were determined by ELISA.

[0030] FIGURE 16 is a Western blot analysis of the mouse serum samples from the intravenous pharmacokinetics studies under reducing conditions. Top left: huFcγ4h-mono-L-DI-IFNß probed with anti-hu Fc; bottom left: huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution probed with anti-hu Fc; top right: huFcγ4h-mono-L-DI-IFNß probed with anti-hu IFNß; bottom right: huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution probed with anti-hu IFNß.

[0031] FIGURE 17 is a Western blot analysis of the mouse serum samples from the subcutaneous pharmacokinetics studies under reducing conditions. Top left: huFcγ4h-mono-L-DI-IFNß probed with anti-hu Fc; bottom left: huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution probed with anti-hu Fc; top right: huFcγ4h-mono-L-DI-IFNß probed with anti-hu IFNß; bottom right: huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution probed with anti-hu IFNß.

DETAILED DESCRIPTION

Definitions

[0032] An "effective amount" or "pharmaceutically effective amount" of a therapeutic or composition contemplated herein is an amount sufficient to produce a desired effect, e.g., reducing the severity of disease symptoms. The pharmaceutically effective amount will vary depending upon the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like. For example, certain compositions of the present invention may be administered in a sufficient amount to produce a reasonable benefit/risk ratio applicable to such treatment.

[0033] The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA and RNA. DNA may be in the form of, e.g., plasmid DNA, pre-condensed DNA, a PCR product, vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chromosomal DNA, or derivatives and combinations of these groups. RNA may be in the form of mRNA, tRNA, rRNA, tRNA, vRNA, and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide nucleic acids (PNAs). "Nucleotides" contain a deoxyribose (DNA) or ribose (RNA), a sugar, a nitrogenous base, and a phosphate group or analog thereof. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

[0034] The term "small molecule" refers to a molecule with a molecular weight of less than 1 kDa. The small molecule can be any of a variety of molecules, naturally

occurring or synthetic. The biological active molecule can be a small organic molecule, a small inorganic molecule, a sugar molecule, a lipid molecule, or the like. The small molecule can be a drug.

[0035] The term "pharmaceutically acceptable excipient" refers to a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent, used in formulating pharmaceutical products. Each excipient must be "acceptable" in the sense of being compatible with the subject composition and its components and not injurious to the patient. Some examples of materials which may serve as pharmaceutically acceptable excipients include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

Antibody Fusion Proteins

[0036] Antibody fusion proteins linking a protein of interest to an immunoglobulin constant region, for example, an immunoglobulin Fc region, possess both the biological activity of the linked protein as well as the advantages associated with the presence of the immunoglobulin moiety. Such fusion proteins exhibit enhanced stability and increased bioavailability compared to the biologically active molecule alone. Additionally, the presence of the Fc fragment can significantly improve protein production. This is believed to occur, in part, because the Fc moiety of the fusion protein is designed for efficient secretion of the fusion protein, and, in part, because the fusion proteins can be produced in and secreted from host cells that naturally express the immunoglobulin such that the fusion protein is readily secreted from the

host cell. Finally, the Fc fragment can further be exploited to aid in the purification of the fused polypeptide.

[0037] In adult mammals, FcRn acts as a protective receptor that binds and salvages IgG from degradation. Numerous studies support a correlation between the affinity for FcRn binding and the serum half-life of an antibody. Surprisingly, the present invention provides that antibody fusion proteins with a mutation reducing FcRn binding exhibit comparable biological properties such as prolonged circulating half-life as corresponding fusion proteins without the mutation. Furthermore, given the role of FcRn in IgG mediated opsonization, it is contemplated that the antibody fusion protein with a mutation that reduces affinity for FcRn may exhibit reduced immunogenicity.

[0038] Consequently, the present invention provides for antibody fusion proteins comprised of two polypeptide chains. The first polypeptide chain includes a biologically active molecule linked to at least a portion of an immunoglobulin constant region, and the second polypeptide chain includes at least a portion of an immunoglobulin constant region. The amino acid sequence of the portion of the immunoglobulin constant region of one of the polypeptide chains differs from the amino acid sequence of the portion of the immunoglobulin constant region of the second polypeptide chain in that it contains a mutation in the FcRn binding site.

[0039] The invention also provides methods and compositions for expressing soluble, biologically active antibody fusion proteins with a mutation in the FcRn binding site that reduces FcRn binding. In particular, the invention provides nucleic acid molecules that encode a polypeptide chain that includes a biologically active molecule and at least a portion of an immunoglobulin constant region comprising a mutation that reduces FcRn binding. In another aspect, the invention relates to a composition of one nucleic acid encoding a polypeptide chain that includes a biologically active molecule and at least a portion of an immunoglobulin constant region, and a second nucleic acid encoding a second polypeptide chain that includes at least a portion of an immunoglobulin constant region. One of the nucleic acid sequences contains a mutation in the FcRn binding site.

[0040] The invention also provides methods for treating a disease or condition alleviated by the administration of the antibody fusion proteins by administering to a mammal an effective amount of the antibody fusion protein of the invention.

Antibody fusion protein structural variants

[0041] The invention discloses antibody fusion proteins comprised of two polypeptide chains. The first polypeptide chain includes a biologically active molecule linked to at least a portion of an immunoglobulin constant region, and the second polypeptide chain includes at least a portion of an immunoglobulin constant region. One of the polypeptide chains comprises a mutation in FcRn binding.

[0042] Figure 1 illustrates a subset of ways in which a biologically active molecule may be linked to the immunoglobulin constant region, for example, the Fc subunit, of an antibody. The biologically active molecule can be linked to the N-terminus of the immunoglobulin constant region (Figures 1A and 1B) or to the C-terminus of the immunoglobulin constant region (Figures 1C and 1D). The biologically active molecule can be linked to the immunoglobulin constant region with a mutation in FcRn binding or linked to the immunoglobulin constant region without the mutation.

[0043] The antibody fusion protein can include at least two biologically active molecules. As illustrated in Figure 2, the biologically active molecules may be linked to the N-terminus of the immunoglobulin constant region (Figure 2A) or to the C-terminus of the immunoglobulin constant region (Figure 2B), or one of the biologically active molecule can be linked to the N-terminus of the immunoglobulin constant region and the second biologically active molecule linked to the C-terminus of the immunoglobulin constant region (Figure 2C). The two biologically active molecules may be the same or different. Figures 2D-2F illustrate how two different biologically active molecules can be linked to the immunoglobulin constant regions.

[0044] The invention also features antibody fusion proteins comprised of two polypeptide chains, wherein at least one of the peptide chains comprise an antibody variable domain. Figure 3 illustrates a subset of ways in which a biologically active molecule can be linked to immunoglobulin regions comprised of an antibody variable domain. The biologically active molecule can be linked to either the N-terminus or the

C-terminus of one of the polypeptide chains comprised of an antibody variable domain (Figures 3A – 3D). Alternatively, both peptide chains can comprise an antibody variable domain (Figures 3E – 3H).

[0045] Figure 4 shows how two biologically active molecules can be linked to an antibody that include a constant region and a variable region of the heavy chain. The biologically active molecule can be linked to the N-terminus, the C-terminus, or the N-terminus and C-terminus of the immunoglobulin regions (Figures 4A – 4C). Different biologically active molecules can be linked to the immunoglobulin regions (Figures 4D – 4F).

[0046] The fusion protein of the invention can include a biologically active molecule linked to an intact antibody, for example, an IgG. Figure 5 depicts a subset of ways in which a biologically active molecule can be linked to an antibody. The biologically active molecule can be linked to the N-terminus or the C-terminus of the immunoglobulin heavy chain (Figures 5A and 5B). Alternatively, the biologically active molecule can be linked to the N-terminus or the C-terminus of the immunoglobulin light chain (Figures 5C and 5D).

[0047] Figure 6 illustrates how two biologically active molecules can be linked to an intact antibody. The biologically active molecules can be linked to the N-terminus or the C-terminus of the immunoglobulin heavy chain (Figures 6A and 6B). Alternatively, the biologically active molecule can be linked to the N-terminus or the C-terminus of the immunoglobulin light chain (Figures 6C and 6D). One of the biologically active molecules can be linked to the immunoglobulin heavy chain, and the second biologically active molecule can be linked to the immunoglobulin light chain (Figures 6E and 6F). Figure 7 shows how different biologically active molecules can be linked to the intact antibody (Figures 7A – 7F).

Antibody fusion protein biological properties

[0048] As mentioned previously, antibody fusion proteins of the invention can demonstrate comparable biological properties such as prolonged circulating half-life as corresponding fusion proteins without the mutation. For example, Figures 11 to 15 demonstrate that human Fcγ4-IFNβ with the H435A mutation displayed similar serum

stability as human Fcγ4-IFNβ without the mutation *in vivo*. Fcγ4-IFNβ with the H435A mutation intravenously injected into mice exhibited similar circulating half-life as antibody fusion proteins without the mutation (Figures 11 and 12). Similarly Fcγ4-IFNβ with the H435A mutation injected subcutaneously into mice exhibited similar circulating half-life as antibody fusion proteins without the mutation (Fig. 13 and 14).

[0049] Derivatives of the antibody fusion proteins of the invention are contemplated and can be made by altering their amino acid sequences by substitutions, additions, and/or deletions/truncations or by introducing chemical modifications that result in functionally equivalent molecules. It will be understood by one of skill in the art that certain amino acids in a sequence of any protein may be substituted for other amino acids without adversely affecting the activity of the protein. Derivatives, analogs or mutants resulting from such changes and the use of such derivatives are within the scope of the present invention.

FcRn binding site mutations

[0050] The present invention provides antibody fusion proteins with a mutation in the FcRn binding site of one polypeptide chain comprising the Fc. While the other polypeptide chain comprising the Fc retains the intrinsic binding affinity to FcRn, the mutation results in reduced binding avidity of the antibody fusion protein to the FcRn receptor. Reduced binding is characterized by low affinity with an affinity constant K_A of lower than 10^6 M^{-1}. If necessary, FcRn binding can be reduced by varying the binding conditions. Binding conditions such as the concentration of the molecules, ionic strength of the solutions, temperature, time allowed for binding can be determined by one skilled in the art.

[0051] The region of the Fc portion of IgG that binds to the FcRn receptor has been described based on X-ray crystallography (Burmeister *et al.*, 1994, Nature 372:379). IgG residues that are involved in binding to FcRn are located at the CH2-CH3 domain interface of the Fc region. The major contact sites include amino acid residues 248, 250-257, 272, 285, 288, 290, 291, 308-311, and 314 of the CH2 domain and amino acid residues 385-387, 428, and 433-436 of the CH3 domain.

[0052] Using this knowledge, the Fc fragment of the antibody fusion protein can be modified according to well recognized procedures such as site-directed mutagenesis to generate modified antibody fusion proteins with reduced affinity for FcRn. The modifications can be substitutions, additions, and/or deletions/truncations. For example, the immunoglobulin constant region of the antibody fusion protein may contain an alteration at position 435, corresponding to a histidine in the native IgG1 Fc fragment. The amino acid alteration may replace the histidine with alanine (H435A) through methods known in the art.

[0053] In addition to an alteration at position 435, the invention also contemplates antibody fusion proteins with other altered residues. For example, the antibody fusion protein may be altered at one or more of positions 233, 234, 235, 236, 252, 253, 254, 255, 288, 309, 386, 388, 400, 415, 433, 435, 436, 439 and 447, wherein the numbering of the residues in the IgG Fc region is that of the EU index as described in Kabat et al. (Sequences of Proteins of Immunological Interest, 5th Ed., Public Health Service, National Institute of Health, Bethesda, MD (1991)). Examples of modifications that reduce or abrogate binding to FcRn include, but are not limited to, the following: E233A, L234A, L235A, G236A, I253A, S254A, R255A, K288A, S415A, H433A, H435A, Y436A. It is contemplated that additional amino acid residues not listed above may also be mutated to reduce FcRn binding. Furthermore, in addition to alanine, other amino acid residues may be substituted for the wild type amino acids at the positions specific above. The mutations may be introduced singly, or combinations of two, three or more of such mutations may be introduced together. Furthermore, one of the polypeptide chains of the antibody fusion protein may be mutated to reduce FcRn binding or both polypeptide chains may be mutated. Any of the mutations described herein can be used regardless of the biologically active molecule.

Immunoglobulin regions

[0054] The antibody fusion protein of the invention includes at least a portion of an immunoglobulin constant region. Intact immunoglobulins include four protein chains that associate covalently - two heavy chains and two light chains. Each chain is further comprised of one variable region and one constant region. Depending upon

the immunoglobulin isotype, the heavy chain constant region contains 3 or 4 constant region domains (*e.g.* CH1, CH2, CH3, CH4) and a hinge region. The domains are named sequentially as follows: CH1-hinge-CH2-CH3(-CH4).

[0055] The portion of an immunoglobulin constant region can include a portion of an IgG, an IgA, an IgM, an IgD, or an IgE. In one embodiment, the immunoglobulin is an IgG. In another embodiment, the immunoglobulin is IgG1. In another embodiment, the immunoglobulin is IgG2. In another embodiment, the immunoglobulin is IgG3, while in yet another embodiment, the immunoglobulin is IgG4. The choice of appropriate immunoglobulin heavy chain constant regions is discussed in detail in U.S. Patent Nos. 5,541,087, and 5,726,044. The choice of particular immunoglobulin heavy chain constant region sequences from certain immunoglobulin classes and subclasses to achieve a particular result is considered to be within the level of one skilled in the art.

[0056] The portion of an immunoglobulin constant region can include the entire heavy chain constant region, or a fragment or analog thereof. In one embodiment, the portion of an immunoglobulin constant region is an Fc fragment. For example, an immunoglobulin Fc fragment may include 1) a CH2 domain; 2) a CH3 domain; 3) a CH4 domain; 4) a CH2 domain and a CH3 domain; 5) a CH2 domain and a CH4 domain; 6) a CH3 domain and a CH4 domain; or 7) a combination of an immunoglobulin hinge region and/or a CH2 domain and/or CH3 domain and/or a CH4 domain. In one embodiment, the immunoglobulin Fc region includes at least an immunoglobulin hinge region, while in another embodiment the immunoglobulin Fc region includes at least one immunoglobulin constant heavy region, for example, a CH2 domain or a CH3 domain, and depending on the type of immunoglobulin used to generate the Fc region, optionally a CH4 domain. In another embodiment, the Fc region includes a hinge region, a CH2 domain and a CH3 domain, and preferably lacks the CH1 domain, while in another embodiment, the Fc region includes a hinge region and a CH2 domain. In yet another embodiment, the Fc region includes a hinge region and a CH3 domain.

[0057] The immunoglobulin Fc fragment may be from any immunoglobulin class. For example, the immunoglobulin class may be IgG (IgG γ1) (γ subclasses 1, 2, 3, or

4). Other classes of immunoglobulin such as IgA (Igα), IgD (Igδ), IgE (Igε), and IgM (Igμ), can also be used. The choice of appropriate immunoglobulin heavy chain constant regions is discussed in detail in U.S. Patent Nos. 5,541,087, and 5,726,044. It is understood that a person skilled in the art will know how to choose the particular immunoglobulin heavy chain constant region from certain immunoglobulin classes and subclasses to achieve a particular result.

[0058] It is contemplated that the Fc fragment used in the generation of the fusion proteins can be adapted to the specific applications. For example, the Fc fragment can be derived from an immunoglobulin γ1 isotype or variants thereof. The use of human γ1 as the Fc fragment sequence has several advantages. For example, an Fc fragment derived from an immunoglobulin γ1 isotype can be used when targeting the fusion protein to the liver is desired. Additionally, if the antibody fusion protein is to be used as a biopharmaceutical, the Fcγ1 domain may confer effector function activities to the fusion protein. The effector function activities include the biological activities such as placental transfer and increased serum half-life. The immunoglobulin Fc fragment also provides for detection by anti-Fc ELISA and purification through binding to *Staphylococcus aureus* protein A ("Protein A").

[0059] Alternatively, the Fc fragment of the antibody fusion protein is derived from an immunoglobulin γ4 isotype. Because the immunoglobulin γ4 isotype is ineffective in mediating effector functions and displays vastly reduced binding to Fcγ receptor, it is contemplated that the antibody fusion proteins with immunoglobulin γ4 as the Fc region may exhibit reduced immune effector functions and enhanced circulating half-life when administered to a mammal.

[0060] The immunoglobulin Fc fragment may combine multiple immunoglobulin classes or subclasses. For example, the fragment may combine an IgG1 hinge region and IgG2 CH2 and CH3 domain (see, *e.g.*, U.S. Patent No. 7,148,326). In some embodiments, portions of a domain are combined from different isotypes to create a strand exchange engineered domain ("SEED") with altered dimerization properties, as described in U.S. Patent Application Publication No. 2007/0287170.

[0061] In one embodiment, the antibody fusion protein includes at least one antibody variable domain. The antibody variable domain may be heavy chain variable domain or light chain variable domain.

[0062] It is understood that portions of an immunoglobulin constant region for use in the present invention can include mutants or analogs thereof, or can include chemically modified immunoglobulin constant regions (e.g. pegylated), or fragments thereof. A person skilled in the art can prepare such variants using well-known molecular biology techniques.

Biologically active molecules

[0063] The invention relates to antibody fusion proteins that include two polypeptide chains. The first polypeptide chain includes a biologically active molecule linked to at least a portion of an immunoglobulin constant region, and the second polypeptide chain includes at least a portion of an immunoglobulin constant region. The biologically active molecule may be linked to the amino-terminus of the immunoglobulin constant region. Alternatively, the biologically active molecule may be linked to the carboxy terminus of the immunoglobulin constant region. In an embodiment, the second polypeptide chain may also include a biologically active molecule.

[0064] The invention contemplates the use of any biologically active molecule capable of exerting a biological effect when administered to a mammal. The biologically active molecule can include but is not limited to polypeptides, nucleic acids, small molecules. Other examples of biologically active molecules include but are not limited to hormones, antiviral agents, hemostatic agents, peptides, proteins, chemotherapeutics, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, and aptamers.

Cytokines

[0065] In one embodiment, the biologically active molecule is a cytokine. Cytokines are factors that support the growth and maturation of cells, including lymphocytes. Examples of cytokines include, but are not limited to, interleukin-I (IL-1),

IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, lymphokine inhibitory factor, macrophage colony stimulating factor, platelet derived growth factor, stem cell factor, tumor necrosis factor, granulocyte colony stimulating factor, and granulocyte macrophage colony stimulating factor.

[0066] In a specific embodiment, the biologically active molecule can include human interferons, for example, interferon-β. The interferon-β moiety can be a wild-type mature human interferon-β protein, or a sequence at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identical to wild-type mature human interferon-β. For example, the biologically active molecule can incorporate a human interferon-β moiety with one or more mutations, *e.g.* to improve the protein folding properties of the fusion protein, to reduce aggregation, or to improve protein expression. For example, the interferon-β moiety of the antibody fusion protein can contain alterations at one, two, three, four, or more of positions 17, 50, 57, 130, 131, 136, and 140 corresponding to the native mature interferon-β. The amino acid alterations at these positions can be generated by amino acid substitutions, amino acid deletions, or amino acid modifications through methods known in the art. Alterations introduced at these residues are believed to alleviate non-covalent aggregation. In one example, the cysteine at position 17 is substituted with either a serine (C17S), an alanine (C17A), a valine (C17V), or a methionine (C17M). In some embodiments, the phenylalanine at position 50 is replaced with histidine (F50H). In some embodiments, the leucine at position 57 is replaced by alanine (L57A), while in other embodiments, the leucine at position 130 is replaced by alanine (L130A). In some embodiments, the histidine at position 131 is replaced by alanine (H131A), while in other embodiments the lysine at position 136 is replaced by alanine (K136A). In some embodiments, the histidine at position 140 is replaced with either an alanine (H140A) or threonine (H140T). While certain amino acid substitutions have been enumerated, the invention is not limited to these alterations. Any suitable amino acid capable of conferring the appropriate properties on the fusion protein may be substituted in place of the original amino acid residues at positions 17, 50, 57, 130, 131, 136, and 140 of the native mature interferon-β. The present invention also contemplates an interferon-β moiety of the antibody fusion protein having a combination of one, two, three, four, five, six, or

seven of the alterations at positions 17, 50, 57, 130, 131, 136, and 140 as disclosed herein.

Growth factors

[0067] In one embodiment, the biologically active agent is a growth factor. The biologically active molecule can be any agent capable of inducing cell growth and proliferation. Examples of growth factors include, but are not limited to, hepatocyte growth factor, fibroblast growth factor, keratinocyte growth factor, nerve growth factor, tumor growth factor-α, epidermal growth factor, VEGF, insulin growth factor, and insulin-like growth factor I and II.

[0068] In a specific embodiment, the biologically active molecule is any agent that can induce erythrocytes to proliferate. Thus one example of a biologically active molecule contemplated by the invention is human erythropoietin.

Hormones

[0069] In one embodiment, the biologically active molecule is a hormone. Hormones alter cell growth, function, and metabolism. Examples of hormones include, but are not limited to, pituitary hormones, *e.g.*, chorionic gonadotropin, cosyntropin, menotropins, somatotropin, iorticotropin, protirelin, thyrotropin, vasopressin, lypressin; adrenal hormones, *e.g.*, beclomethasone dipropionate, betamethasone, dexamethasone, triamcinolone; pancreatic hormones, *e.g.*, glucagon, insulin; parathyroid hormone, *e.g.*, dihydrochysterol; thyroid hormones, *e.g.*, calcitonin, thyroglobulin, teriparatide acetate; steroid hormones, *e.g.*, glucocorticoids, estrogens, progestins, androgens, tetrahydrodesoxycaricosterone; gastrointestinal hormones: cholecystokinin, enteroglycan, galanin, gastrins, pentagastrin, tetragastrin, motilin, peptide YY, and secretin. In a specific embodiment, the biologically active molecule is human growth hormone.

Nucleic acids

[0070] In one embodiment, the biologically active molecule is a nucleic acid such as DNA or RNA. For example, the biologically active molecule can be a nucleic acid molecule that is used in RNA interference such as antisense RNA, short interfering

RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA). The nucleic acid molecule should be about 6 to about 60 nucleotides in length. For example, in some embodiments, the nucleic acid molecule is about 15 to about 50, about 25 to about 45, or about 30 to about 40 nucleotides in length.

[0071] The oligonucleotides can be DNA or RNA or mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, *etc.* The oligonucleotide may include other appended groups such as polypeptides (*e.g.* for targeting host cell receptors in vivo), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.* (1989) Proc. Natl. Acad. Sci. USA 86:6553; Lemaitre *et al.* (1987) Proc. Natl. Acad. Sci. USA 84:648; and WO 88/09810) or the blood-brain barrier (see, *e.g.*, WO 89/10134), hybridization-triggered cleavage agents (see, *e.g.*, Krol *et al.* (1988) BioTechniques 6:958) or intercalating agents (see, *e.g.*, Zon (1988) Pharm. Res. 5:539). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a polypeptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent.

Small molecules

[0072] The invention also contemplates the use of any therapeutic small molecule or drug as the biologically active molecule. The biologically active molecule can be, for example, a lipid molecule. The biologically active molecule can be a sugar molecule. The biologically active molecule can be a small organic molecule or a small inorganic molecule.

Production of Modified Antibody Fusion Proteins

[0073] It is understood that the present invention can exploit conventional recombinant DNA methodologies to generate the antibody fusion proteins useful in the practice of the invention. The antibody fusion constructs preferably are generated at the DNA level, and the resulting DNAs integrated into expression vectors, and expressed to produce the fusion proteins of the invention.

[0074] The invention provides nucleic acids encoding a biologically active molecule linked to at least a portion of an immunoglobulin constant region comprising a mutation in the FcRn binding site. In one embodiment, the mutation is a codon substitution replacing the histidine at position 435 of the immunoglobulin constant region with an alanine (H435A). The invention also provides compositions of nucleic acids: the first nucleic acid encodes a biologically active molecule and at least a portion of an immunoglobulin constant region; the second nucleic acid encodes at least a portion of an immunoglobulin constant region. One of the nucleic acids encodes an immunoglobulin constant region with a mutation affecting FcRn binding. In one embodiment, the mutation is a codon substitution replacing the histidine at position 435 of the immunoglobulin constant region with an alanine (H435A). The nucleic acids can also include additional sequences or elements known in the art (*e.g.*, promoters, enhancers, poly A sequences, or affinity tags).

[0075] Nucleic acids and nucleic acid compositions according to the invention can be readily synthesized using recombinant techniques well known in the art. For example, nucleic acids can be synthesized by standard methods, *e.g.*, by use of an automated DNA synthesizer.

[0076] For recombinant protein production, the nucleic acid or the nucleic acid composition is inserted into appropriate expression vehicles, *i.e.* vectors which contains the necessary elements for the transcription and translation of the inserted coding sequence. As used herein, the term "vector" is understood to mean any nucleic acid including a nucleotide sequence competent to be incorporated into a host cell and to be recombined with and integrated into the host cell genome, or to replicate autonomously as an episome. Such vectors include linear nucleic acids, plasmids, phagemids, cosmids, RNA vectors, viral vectors and the like. Non-limiting examples of a viral vector include a retrovirus, an adenovirus and an adeno-associated virus.

[0077] A useful expression vector is pdCs (Lo *et al.* (1988) Protein Engineering 11:495), which transcription utilizes the enhancer/promoter of the human cytomegalovirus and the SV40 polyadenylation signal. The enhancer and promoter sequence of the human cytomegalovirus used is derived from nucleotides -601 to +7

of the sequence provided in Boshart *et al.* (1985) Cell 41:521. The vector also contains the mutant dihydrofolate reductase gene as a selection marker (Simonsen and Levinson (1983) Proc. Nat. Acad. Sci. USA 80:2495).

[0078] An appropriate isolated host cell can be transformed or transfected with the DNA sequence of the invention, and utilized for the expression and/or secretion of the target protein. Exemplary isolated host cells for use in the invention include immortal hybridoma cells, NS/0 myeloma cells, 293 cells, Chinese hamster ovary cells, HeLa cells, and COS cells.

Methods of using the antibody fusion proteins

[0079] The invention also provides pharmaceutical compositions comprising the antibody fusion protein with a mutation in the FcRn binding site and a pharmaceutically acceptable carrier or excipient. The term "pharmaceutically acceptable excipient" is art-recognized and refers to a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent, used in formulating pharmaceutical products. Each excipient must be "acceptable" in the sense of being compatible with the subject composition and its components and not injurious to the patient. Examples of suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences by E. W. Martin. Examples of excipients can include (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

[0080] Compositions of the present invention may be administered by any route which is compatible with the particular molecules. It is contemplated that the compositions of the present invention may be provided to a mammal by any suitable means, directly (*e.g.*, locally, as by injection, implantation or topical administration to a tissue locus) or systemically *(e.g.*, parenterally or orally). Where the composition is to be provided parenterally, such as by intravenous, subcutaneous, ophthalmic, intraperitoneal, intramuscular, buccal, rectal, vaginal, intraorbital, intracerebral, intracranial, intraspinal, intraventricular, intrathecal, intracistemal, intracapsular, intranasal or by aerosol administration, the composition preferably includes part of an aqueous or physiologically compatible fluid suspension or solution. Thus, the carrier or vehicle is physiologically acceptable so that in addition to delivery of the desired composition to the patient, it does not otherwise adversely affect the patient's electrolyte and/or volume balance. The fluid medium for the agent thus can include normal physiologic saline.

[0081] The present invention provides methods of treating various cancers, viral diseases, other diseases, related conditions and causes thereof by administering the DNA, RNA or proteins of the invention to a mammal having such condition. Related conditions may include, but are not limited to inflammatory conditions or an autoimmune disease, such as multiple sclerosis, arthritis, psoriasis, lupus erythematosus; a variety of malignancies, such as acute myeloid leukemia, multiple myeloma, Hodgkin's disease, basal cell carcinoma, cervical dysplasia and osteosarcoma; a variety of viral infections, including viral hepatitis, herpes zoster and genitalis, papilloma viruses, viral encephalitis, and cytomegalovirus pneumonia; anemia; and hemostatic disorders.

[0082] The optimal dose of the fusion protein of the invention will depend upon the disease being treated and upon the existence of side effects. The optimal dosages can be determined using routine experimentation. Dosages per administration can range from 0.1 mg/m^2 -100 mg/m^2, 1 mg/m^2 -20 mg/m^2, and 2 mg/m^2 -6 mg/m^2. Administration of the fusion protein may be by periodic bolus injections, or by continuous intravenous or intraperitoneal administration from an external reservoir (for example, from an intravenous bag) or internal (for example, from a bioerodable implant). Furthermore, it is contemplated that the fusion proteins of the invention also

may be administered to the intended recipient together with a plurality of different biologically active molecules. It is contemplated, however, that the optimal combination of fusion protein and other molecules, modes of administration, dosages may be determined by routine experimentation well within the level of skill in the art.

[0083] It is contemplated that the antibody fusion protein of the invention can also be used to treat a mammal with a disease or condition in combination with at least one other known agent to treat said disease or condition.

EXAMPLES

Example 1: Construction of DNA sequences for the expression of huFcγ4h-mono-L-DI-IFNß

[0084] The huFcγ4h-mono-L-DI-IFNß (huFcγ4 hinge mutant-linker monomeric de-immunized interferon-β) antibody fusion protein is a heterodimer consisting of a human Fcγ4h chain and a human Fc-γ4h-Linker-DI-IFNß chain. The protein was produced in mammalian cells by coexpressing the human Fcγ4h chain and the human Fc-γ4h-Linker-DI-IFNß chain, the transcription units of which were contained in one single plasmid or two separate plasmids.

[0085] The DNA encoding the huFcγ4h (huFcγ4 hinge mutant) was derived from the human IgG4 genomic sequence and then engineered to contain a modified γ1 hinge region in order to minimize half-molecule formation. The formation of IgG4 half molecules that did not form covalent disulphide bonds at the hinge regions was previously reported (Angal *et al.* (1993) Mol. Immunol. 30:105).

Construction of DNA sequence encoding the Fc fragment of the human immunoglobulin-gamma 4 (Fcγ4)

[0086] The genomic sequence encoding human IgG4 was obtained from cellular DNA isolated from HeLa cells. The sequence is highly conserved with the published IgG4 sequence – Locus HUMIGCD2 (Accession K01316) in GenBank, except for the difference of 3 amino acid residues in the CH3 region. The published sequence

contains R409, E419, V422, whereas our sequence contains K409, Q419, I422. Interestingly, K409 and Q419 are also found in human IgG1, IgG2 and IgG3; and I422 is found in human IgG3. Such allotypic determinants consisting of amino acid substitutions from other IgG subclasses are known as isoallotypes, and isoallotypes of the human IgG4 gene have previously been reported (Brusco *et al.* (1998) Eur. J. Immunogenetics 25:349-355). The amino acid sequence of the Fcγ4 fragment is shown in SEQ ID NO: 1.

SEQ ID NO: 1: Peptide sequences of the huFcγ4 (γ4 hinge region underlined, and K409, Q419, I422 in bold)

ESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNA
KTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQE
EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS**K**LTVDKSRWQ**Q**GN**I**FSC
SVMHEALHNHYTQKSLSLSPGK

Modification of the hinge region of Fcγ4

[0087] The γ4 hinge region with the amino acid sequence "ESKYGPPCPSCP" **(SEQ ID NO: 7)** was replaced by a modified γ1 hinge region with the amino acid sequence "EPKSSDKTHTCPPCP" **(SEQ ID NO: 8)** (Lo *et al.* (1998) Protein Engineering 11:495-500) to minimize the formation of half-molecules (U.S. Patent No. 7,148,321).

[0088] In order to modify the hinge region of Fcγ4, the *AflII-StuI* fragment 5' CTTAAGCG**AGTCCAAATATGGTCCCCCATGCCCATCATGCCCAG** **(SEQ ID NO: 9)** containing the native γ4 hinge exon (bold) was replaced by the corresponding *AflII-StuI* fragment 5'-CTTAAGCG**AGCCCAAATCTTCTGACAAAACTCACACATGCCCACCGTGCCCAG** **(SEQ ID NO: 10)** containing the modified γ1 hinge exon (bold) with a Cys to Ser substitution (underlined) that eliminates the Cys residue that normally pairs with the light chain. Since the *StuI* sites in both the γ1 and γ4 exons are C-methylated and the *StuI* restriction endonuclease is methylation sensitive, both plasmids had to be isolated from a DNA cytosine methylase (DCM) negative strain of bacteria before they could be digested with the *StuI* enzyme. The resulting Fcγ4 with the modified γ1 hinge region was designated Fcγ4h (γ4h: gamma-4 hinge mutant).

Cloning and de-immunization of IFN-β

[0089] The coding sequence for mature IFN-β was PCR amplified from human placental DNA (Sigma, Poole, UK). The cloned PCR products were sequenced to identify an IFN-β clone, the amino acid sequence of which completely matches with the published wild type IFN-β sequence - Locus XM_005410 in GenBank.

[0090] The de-immunized IFN-β (DI-IFNß) contains three amino acid substitutions (L57A, H131A, H140A) to remove potential T helper cell epitopes and one amino acid substitution (C17S) to minimize covalent aggregation. The DNA encoding DI-IFN□ was cloned into the mammalian expression vector pdCs-huFc (Lo *et al.* (1998) Protein Engineering 11:495-500), which had been modified such that the IFN-β sequence is fused to the C-terminus of human Fcγ4h via a 15 amino acid flexible linker with the amino acid sequence $G_4SG_4SG_3SG$. The linker-DI-IFN□ was denoted as L-DI-IFN□, the sequence of which is shown in SEQ ID NO: 2 (linker underlined, and C17S, L57A, H131A, and H140A in bold).

SEQ ID NO: 2: Peptide sequences of L-DI-IFNß

GGGGSGGGGSGGGSGMSYNLLGFLQRSSNFQ**S**QKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKED
AA**A**TIYEMLQNIFAIFRQDSSSTGWNETIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSLHLK
RYYGRIL**A**YLKAKEYS**A**CAWTIVRVEILRNFYFINRLTGYLRN

Adaptation of a genomic leader as the signal peptide for secretion

[0091] A genomic signal peptide sequence (438-bp) from a mouse immunoglobulin light chain gene was used for the secretion of both the huFcγ4h and huFcγ4h-L-DI-IFNß chains. The gene sequence encoding the –2 amino acid residue (the –1 amino acid being the C-terminal residue of the signal peptide) of the signal peptide was mutagenized from a serine residue to a leucine residue (AGC to TTA) so that the DNA encoding the end of the signal peptide is CTTAAGC, where CTTAAG is a created *Afl*II site (Lo *et al.* (1998) Protein Engineering 11:495-500). In addition, the Kozak consensus sequence CCACC**ATG**G was introduced for optimal ribosome binding for translation initiation at **ATG** (Kozak *et al.* (1986) Cell 44:283-292). This was achieved by mutating the first amino acid residue after the initiation codon from AAG to GAG to give the sequence TCTAGACCACC**ATG**GAG **(SEQ ID NO: 11)**, where the Kozak consensus sequence is underlined and TCTAGA is an *Xba*I site. Therefore,

the signal peptide contains a substitution at the first amino acid residue after the initiation codon and another substitution at the amino acid residue at the –2 position. Since the signal peptide is cleaved off by signal peptidase inside the cell and does not appear in the secreted protein, these mutations do not affect the amino acid composition of the Fcγ4h and Fcγ4h-L-DI-IFNß products.

The peptide and DNA sequences of the huFcγ4h and huFcγ4h-L-DI-IFNß chains

[0092] The coding regions for the entire huFcγ4h and huFcγ4h-L-DI-IFNß chains were completely sequenced. The peptide and DNA sequences of the huFcγ4h and huFcγ4h-L-DI-IFNß chains are shown and SEQ ID NO: 3 to SEQ ID NO: 6. In order to facilitate ligation of the DNA fragments encoding huFcγ4h and L-DI-IFNß, a *Sma*I site was created by using the PGK sequence (Lo *et al.* (1998) Protein Engineering 11:495-500) found in human IgG4 (Locus CAC20457 in GenPept), and also in IgG1 and IgG2; in addition, a lysine to alanine substitution at the C-terminal residue of CH3 was introduced to minimize potential cleavage at the junction. Importantly, the resultant sequence at the CH3-L-DI-IFNß junction does not create any potential T cell epitope.

SEQ ID NO: 3: Peptide sequence of the huFcγ4h **(signal peptide underlined)**

MELPVRLLVLMFWIPASLSEPKSSDKTHTCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEK
TISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS
FFLYSKLTVDKSRWQQGNIFSCSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 4: DNA sequence of the huFcγ4h chain from the translation initiation codon to the translation stop codon (coding sequence in upper case and non-coding sequence in lower case)

ATGGAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGgtgaggagagggaagtgaggg
aggagaatggacagggagcaggagcactgaatcccattgctcattccatgtattctggcatgggtgaga
agatgggtcttatcctccagcatggggcctctggggtgaatacttgttagagggaggttccagatggga
acatgtgctataatgaagattatgaaatggatgcctgggatggtctaagtaatgcctagaagtgactag
acacttgcaattcacttttttggtaagaagagattttaggctataaaaaaatgttatgtaaaaataa
acatcacagttgaaataaaaaaaaatataaggatgttcatgaattttgtgtataactatgtatttctct
ctcattgtttcagCTTCCTTAAGCGAGCCCAAATCTTCTGACAAAACTCACACATGCCCACCGTGCCCA
Ggtaagccagcccaggcctcgccctccagctcaaggcgggacaggtgccctagagtagcctgcatccag
ggacaggccccagccgggtgctgacgcatccacctccatctcttcctcagCACCTGAGTTCCTGGGGGG
ACCATCAGTCTTCCTGTTCCCCCCAAAACCCAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTCAC
GTGCGTGGTGGTGGACGTGAGCCAGGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGATGGCGTGGA
GGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTTCAACAGCACGTACCGTGTGGTCAGCGTCCT
CACCGTCCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGCCTCCC
GTCCTCCATCGAGAAAACCATCTCCAAAGCCAAAGgtgggacccacggggtgcgagggccacatggaca

gaggtcagctcggcccaccctctgccctgggagtgaccgctgtgccaacctctgtccctacagGGCAGC
CCCGAGAGCCACAGGTGTACACCCTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTCAGCCTGA
CCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGA
ACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCG
TGGACAAGAGCAGGTGGCAGCAGGGGAACATCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACC
ACTACACGCAGAAGAGCCTCTCCCTGTCCCCGGGTAAATGA

SEQ ID NO: 5: Peptide sequence of the huFcγ4h-L-DI-IFNß (the signal peptide and the K to A substitution at the end of CH3 are underlined; and L-DI-IFNß in bold)

MELPVRLLVLMFWIPASLSEPKSSDKTHTCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEK
TISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS
FFLYSKLTVDKSRWQQGNIFSCSVMHEALHNHYTQKSLSLSPGA**GGGGSGGGGSGGGSGMSYNLLGFLQ**
RSSNFQSQKLLWQLNGRLEYCLKDRMNFDIPEEIKQLQQFQKEDAAATIYEMLQNIFAIFRQDSSSTGW
NETIVENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSLHLKRYYGRILAYLKAKEYSACAWTIVRV
EILRNFYFINRLTGYLRN

SEQ ID NO: 6: DNA sequence of the huFcγ4h-L-DI-IFN□ chain from the translation initiation codon to the translation stop codon (coding sequence in upper case and non-coding sequence in lower case)

ATGGAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGgtgaggagagggaagtgaggg
aggagaatggacagggagcaggagcactgaatcccattgctcattccatgtattctggcatgggtgaga
agatgggtcttatcctccagcatggggcctctggggtgaatacttgttagagggaggttccagatggga
acatgtgctataatgaagattatgaaatggatgcctgggatggtctaagtaatgcctagaagtgactag
acacttgcaattcacttttttggtaagaagagattttaggctataaaaaaatgttatgtaaaaataa
acatcacagttgaaataaaaaaaaatataaggatgttcatgaattttgtgtataactatgtatttctct
ctcattgtttcagCTTCCTTAAGCGAGCCCAAATCTTCTGACAAAACTCACACATGCCCACCGTGCCCA
Ggtaagccagcccaggcctcgccctccagctcaaggcgggacaggtgccctagagtagcctgcatccag
ggacaggccccagccgggtgctgacgcatccacctccatctcttcctcagCACCTGAGTTCCTGGGGGG
ACCATCAGTCTTCCTGTTCCCCCCAAAACCCAAGGACACTCTCATGATCTCCCGGACCCCTGAGGTCAC
GTGCGTGGTGGTGGACGTGAGCCAGGAAGACCCCGAGGTCCAGTTCAACTGGTACGTGGATGGCGTGGA
GGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTTCAACAGCACGTACCGTGTGGTCAGCGTCCT
CACCGTCCTGCACCAGGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGGCCTCCC
GTCCTCCATCGAGAAAACCATCTCCAAAGCCAAAGgtgggacccacggggtgcgagggccacatggaca
gaggtcagctcggcccaccctctgccctgggagtgaccgctgtgccaacctctgtccctacagGGCAGC
CCCGAGAGCCACAGGTGTACACCCTGCCCCCATCCCAGGAGGAGATGACCAAGAACCAGGTCAGCCTGA
CCTGCCTGGTCAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGA
ACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCG
TGGACAAGAGCAGGTGGCAGCAGGGGAACATCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACC
ACTACACGCAGAAGAGCCTCTCCCTGTCCCCGGGTGCAGGGGGCGGGGGCAGCGGGGGCGGAGGATCCG
GCGGGGGCTCGGGTATGAGCTACAACTTGCTTGGATTCCTACAAAGAAGCAGCAATTTTCAGAGTCAGA
AGCTCCTGTGGCAATTGAATGGGAGGCTTGAATATTGCCTCAAGGACAGGATGAACTTTGACATCCCTG
AGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGACGCCGCAGCCACCATCTATGAGATGCTCCAGA
ACATCTTTGCTATTTTCAGACAAGATTCATCTAGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCC
TGGCTAATGTCTATCATCAGATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGAAAGAAGATT
TCACCAGGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATTATGGGAGGATTCTGGCCTACCTGA
AGGCCAAGGAGTACAGTGCCTGTGCCTGGACCATAGTCAGAGTGGAAATCCTAAGGAACTTTTACTTCA
TTAACAGACTTACAGGTTACCTCCGAAACTGA

Example 2: Construction of DNA sequences for the expression of huFcγ4h-mono-L-DI-IFNß variants containing H435A substitution in γ4

[0093] DNA sequences for the expression of huFcγ4h-mono-L-DI-IFNß variants containing the H435A substitution (Kabat numbering) in γ4 were constructed. These include DNA sequences encoding the heterodimers huFcγ4h(H435A)/huFcγ4h-L-DI-IFNß, huFcγ4h/huFcγ4h(H435A)-L-DI-IFNß and huFcγ4h(H435A)/huFcγ4h(H435A)-L-DI-IFNß. The mutation from the CAC codon to GCG encoding the H435A substitution in the naked huFcγ4h chain or the huFcγ4h-L-DI-IFNß fusion protein chain was introduced by overlapping PCR (Daugherty *et al.* (1991) Nucleic Acids Res. 19:2471-2476) with mutagenic primers, using forward primer 5'-G GCTCTGCACAACGCGTACACGCAGAAGAG **(SEQ ID NO: 12)**, where GCG encodes the alanine substitution, and reverse primer 5'-CTCTTCTGCGTGTACGCGTT GTGCAGAGCC **(SEQ ID NO: 13)**, where CGC is the anti-codon of the alanine substitution.

Example 3: Expression of Fusion Proteins

[0094] The huFcγ4h-mono-L-DI-IFNß heterodimer was produced in mammalian cells by coexpressing the human Fcγ4h chain and the human Fcγ4h-Linker-DI-IFNß chain, the transcription units of which were contained in one single plasmid or two separate plasmids. For rapid analysis of protein expression, the plasmid pdCs-Fcγ4h and pdCs-Fc Fcγ4h-Linker-DI-IFNß or variants were introduced into human kidney 293T cells (GenHunter Corporation, Nashville, TN) by transient transfection using lipofectamine (Invitrogen, Carlsbad, CA).

[0095] Mouse myeloma NS/0 and Chinese hamster ovary cells were used to obtain stably transfected clones which express the huFcγ4h-mono-L-DI-IFNß heterodimer. For high level expression, the plasmid pdCs containing both the human Fcγ4h chain and the human Fcγ4h-Linker-DI-IFNß chain transcription units was introduced into the mouse myeloma NS/0 cells by electroporation. NS/0 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and penicillin/streptomycin. About $5x10^6$ cells were washed once with PBS and resuspended in 0.5 ml PBS. 10 µg of linearized plasmid DNA were then incubated with the cells in a Gene Pulser Cuvette (0.4 cm electrode gap, BioRad) on ice for 10 min. Electroporation was performed using a Gene Pulser

(BioRad, Hercules, CA) with settings at 0.25 V and 500 μF. Cells were allowed to recover for 10 min on ice, after which they were resuspended in growth medium and plated onto two 96 well plates. Stably transfected clones were selected by their growth in the presence of 100 nM methotrexate (MTX), which was added to the growth medium two days post-transfection. The cells were fed every 3 days for two to three more times, and MTX-resistant clones appeared in 2 to 3 weeks. Supernatants from clones were assayed by anti-Fc ELISA to identify high producers. High producing clones were isolated and propagated in growth medium containing 100 nM MTX. The growth medium typically used was H-SFM or CD medium (Invitrogen, Carlsbad, CA).

[0096] For routine characterization by gel electrophoresis, the huFc-IFNß fusion protein secreted into the medium was captured on Protein A Sepharose beads (Repligen, Cambridge, MA) and then eluted by boiling the sample in protein sample buffer, with or without a reducing agent such as ß-mercaptoethanol. The samples were analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and the protein bands were visualized by Coomassie staining. Figure 8 shows the huFcγ4h-mono-L-DI-IFNß produced by transient coexpression of human Fcγ4h chain and the human Fcγ4h-Linker-DI-IFNß in 293T cells. Lane 2 of the SDS-PAGE analysis under reducing conditions (left panel) shows that both the Fcγ4h chain and the Fcγ4h-Linker-DI-IFNßchain were expressed, and the naked Fc was expressed at a higher level than the fusion protein. When these two chains were coexpressed at these levels, one would expect that the non-reduced gel should show a ratio of naked Fc homodimer : Fc/Fc-IFNß heterodimer : Fc-IFNß homodimer according to the binomial $a^2 + 2ab + b^2$, where a and b are the relative expression levels of the naked Fc chain and the fusion protein chain, respectively, as seen on the reduced gel. Surprisingly, SDS-PAGE analysis under non-reducing conditions (middle panel) shows that very little Fc-IFNß homodimer ("$(Fc\text{-}X)_2$") was produced, in sharp contrast to the more normal pattern (ratio according to $a^2 + 2ab + b^2$) that was obtained with coexpression of Fc and Fc-IL-2 analyzed also under non-reducing conditions (right panel). Stable clones coexpressing the Fcγ4h chain and the Fcγ4h-Linker-DI-IFNß chain had a similar lack of the Fc-IFNß homodimer (Figure 9),

suggesting that the production of the Fc-IFNß homodimer in the cell is unfavorable, probably due to protein folding since IFNß itself tends to aggregate.

Example 4: Purification of huFcγ4h-mono-L-DI-IFNß

[0097] Purification of Fc-containing fusion proteins was performed based on the affinity of the Fc protein moiety for Protein A. Briefly, cell supernatant containing the fusion protein was loaded onto a pre-equilibrated Protein A Sepharose column and the column was washed extensively in same buffer (150 mM sodium phosphate, 100 mM NaCl at neutral pH). Bound protein was eluted at a low pH (pH 2.5 - 3) in the same buffer and eluate fractions were immediately neutralized. The huFcγ4h-mono-L-DI-IFNß heterodimer could be readily separated from the Fcγ4h homodimer based on the affinity of IFNß protein for Cibacron Blue 3GA (Blue Sepharose Fast Flow column; Pharmacia). Culture supernatant containing the expressed fusion protein was adjusted to 1 M NaCl and loaded onto the Blue Sepharose Fast Flow column which had been pre-equilibrated with Buffer A (Buffer A: 20 mM Sodium Phosphate (pH 7.2) 1 M NaCl). The column was washed with 10 column volumes of Buffer A, followed by 15 column volumes of a 1 : 1 mixture of Buffer A : Buffer B (Buffer B: 20 mM Sodium Phosphate (pH 7.2), 50% (v/v) Ethylene Glycol). The heterodimer was eluted in 100 % Buffer B and 1 ml fractions were collected into tubes containing 0.5 ml Buffer A. The purified huFcγ4h-mono-L-DI-IFNß was analyzed by size exclusion chromatography (SEC) and further confirmed by a Western blot probed with an anti-IFNß antibody.

Example 5: Expression of immunoglobulin- and huFc-mono-ligand variants containing single substitution that abrogates FcRn binding

[0098] A huFc-mono-L-DI-IFNß variant derived from an Fc-SEED (see WO2007/110205) was produced in the form of huFc-AG2(H435A)/huFc-GA2-L-DI-IFNß. Additional variants that were produced include IgG subclass and ligand variants, such as huFcγ1(H435A)/huFcγ1-IL2 and deimmunized KS-γ1(H435A)/KS-γ1-IL2, a class of whole IgG fusion proteins known as immunocytokines (Davis *et al.* (2003) Cancer Immunol. Immunother. 52:297-308).

[0099] In addition to H435, H310 was also reported to be involved in FcRn binding (Kim *et al.* (1999) Eur. J. Immunol. 29:2819-2825). Hence another huFcγ4h-mono-L-DI-IFNß variant containing the H310A substitution was produced in the form of a huFcγ4h(H310A)/huFcγ4h-L-DI-IFNßheterodimer.

Example 6. Activity of huFc-IFNß proteins in cell-based bioassays

[00100] The activity of the Fc-IFNß fusion proteins was determined in an antiviral assay. Viral replication is often toxic to cells, resulting in cell lysis, an effect known as a cytopathic effect (CPE). Interferons act on pathways that inhibit viral proliferation, protecting cells from CPE. The antiviral activity of IFNß could be assayed by measuring the extent to which CPE is reduced (CPER), as described in "*Lymphokines and Interferons: A Practical Approach,*" edited by M.J. Clemens, A.G. Morris, and A.J.H. Gearin, I.R.L. Press, Oxford, 1987. The antiviral activities of purified Fcγ4h-mono-L-DI-IFNß and Fcγ4h-mono-L-DI-IFNß variant containing the H435A substitution were compared to Rebif using the human epithelial lung carcinoma line A549 (ATCC # CCL-185) as a host for the encephalomyocarditis virus (EMCV; ATCC # VR 129B). The effective dose (ED50) was set as the amount of protein that led to 50% CPER (i.e. 50% of the cells are protected), determined relative to uninfected control cells. On a molar basis, both Fcγ4h-mono-L-DI-IFNß and Fcγ4h-mono-L-DI-IFNß variant were found to be at least as potent as Rebif in this CPE assay (Figure 10).

Example 7. Pharmacokinetics of huFc-IFNß proteins

[00101] The pharmacokinetics (PK) of huFc-IFNß heterodimeric fusion proteins and variants were determined in Balb/c mice (n=3). For intravenous administration, 25 µg of the heterodimeric fusion protein were injected into the tail vein of each mouse. Blood was collected into heparin-coated tubes immediately following injection (t = 0 min), and at 30 min, 1 hr, 2 hrs, 4 hrs, 8 hrs, 24 hrs, 48 hrs, 72 hrs and 96 hrs post-injection by retro-orbital bleeding. For subcutanenous administration, 50 µg of the heterodimeric fusion protein were injected per mouse. Blood was collected into heparin-coated tubes 1, 2, 4, 8, 24, 48, 72 and 96 hrs post-injection by retro-orbital bleeding. Cells were removed by centrifugation (4 min at 12,500 g) and the concentration of the fusion protein in the plasma was determined by an anti-huFc

ELISA consisting of an anti-(H&L) (AffiniPure Goat anti-Human IgG (H+L), Jackson Immuno Research Laboratories, West Grove, PA) capture and anti-Fc (HRP-conjugated $(Fab')_2$ dimer goat anti-human IgG Fc, Jackson Immuno Research Laboratories, West Grove, PA) detection, and an anti-huFc-IFNß ELISA consisting of an anti-(H&L) capture and anti-huIFNß (Goat anti-human IFNß biotinylated, R&D Systems, Minneapolis, MN) detection. Furthermore, the integrity of the circulating fusion protein was confirmed by an immunoblot of the PK serum samples probed with an anti-huFc antibody or an anti-huIFNß antibody.

[00102] Figures 11 and 12 compare the pharmacokinetic profiles of intravenously administered huFcγ4h-mono-L-DI-IFNß and an huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution in the form of the huFc4h(H435A)/huFcγ4h-L-DI-IFNß heterodimer, as determined by anti-huFc ELISA and anti-huFc-IFNß ELISA, respectively. The huFcγ4h-mono-L-DI-IFNß has a circulating half-life of 48 hr, which is many times longer than that of IFNß, which was in minutes. Surprisingly, the huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution has a circulating half-life of 46 hr, which is essentially identical to that of huFcγ4h-mono-L-DI-IFNß within experimental error. The H435A variant also has an AUC (area under the curve) about two-thirds of that of the wild-type, which is several hundred times that of IFNß. The anti-huFc-IFNß ELISA detected specifically the fusion protein and not any cleaved fragments without the N-terminal Fc or C-terminal IFNß moieties. Furthermore, Western blot analyses of the intravenous PK samples under reducing conditions using either anti-huFc antibody or anti-huIFNß antibody as probes confirmed that the fusion proteins stayed intact in vivo (Figure 17). In the top left section of Figure 17, the upper arrow denotes the huFcγ4h-L-DI-IFNß polypeptide chain, and the bottom arrow denotes the huFcγ4h chain. Similarly, in the bottom left section of Figure 17, the upper arrow denotes the huFcγ4h-L-DI-IFNß polypeptide chain, and the bottom arrow denotes the huFcγ4h(H435A) chain.

[00103] Figures 16 and 13 compare the pharmacokinetic profiles of the same two molecules administered subcutaneously. The huFcγ4h-mono-L-DI-IFNß and the huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution have a similar circulating half-life, which is many times longer than that of IFNß. Surprisingly, the huFcγ4h-mono-L-DI-IFNß variant containing the H435A substitution has a circulating

half-life of 46 hr, which is essentially identical to that of huFcγ4h-mono-L-DI-IFNß within experimental error. The H435A variant also has an AUC (area under the curve) about two-thirds of that of the wild-type, which is several hundred times that of IFNß The anti-hu Fc ELISA and anti-hu IFNß ELISA detected the two ends of the fusion protein molecule. Furthermore, Western blot analyses of the subcutaneous PK samples under reducing conditions using either anti-hu Fc antibody or anti-hu IFNß antibody as probes confirmed that the fusion proteins stayed intact in vivo (Figure 15). In the top left panel of Figure 15, the upper arrow denotes the huFcγ4h-L-DI-IFNß polypeptide chain, and the bottom arrow denotes the huFcγ4h chain. Similarly, in the bottom left panel of Figure 15, the upper arrow denotes the huFcγ4h-L-DI-IFNß polypeptide chain, and the bottom arrow denotes the huFcγ4h(H435A) chain.

Example 8. Reduced immunogenicity of immunoglobulin- and huFc-mono-ligand variants containing single substitution that abrogates FcRn binding

[00104] To demonstrate the reduced immunogenicity of immunoglobulin- or huFc-mono-ligand variants containing a single substitution that abrogates FcRn binding of only one polypeptide chain, mice were immunized with huFcγ4h-mono-L-DI-IFNß or huFcγ4h-mono-L-DI-IFNß variant containing the H435A mutation. Antibody titers were compared at appropriate times after immunization.

[00105] Two groups of female, 8-weeks-old Balb/C mice (5 mice per group) were injected subcutaneously with 33 µg/mouse of huFcγ4h-mono-L-DI-IFNß or huFcγ4h-mono-L-DI-IFNß variant containing the H435A mutation, respectively. On day 15, mice received a subcutaneous boost injection of 33 µg/mouse of huFcγ4h-mono-L-DI-IFNß or huFcγ4h-mono-L-DI-IFNß variant containing the H435A mutation. Mouse antibody titers were determined on Day 26 by ELISA. This consisted of coating the wells of a 96-well plate with 1µg of goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA, cat # 115-005-062) by incubation overnight at 4°C in PBS followed by washing four times with PBS 0.05% Tween. Next, the goat anti-mouse IgG (H+L)-coated plate was blocked with 1% milk, washed four times with PBS 0.05% Tween, dried, and stored at -20°C. For capturing mouse antibodies, serum was initially diluted 1:1000 followed by serial dilutions of 1:2 in PBS 0.05% Tween and added to the goat anti-mouse IgG (H+L)-coated wells for one

hour at 37°C. The wells were then washed four times with PBS 0.05% Tween. The captured antibodies were detected by adding the immunogens, huFcγ4h-mono-L-DI-IFNß or huFcγ4h-mono-L-DI-IFNß variant containing the H435A mutation at 0.5 µg/ml, and a 1:20,000 dilution of F(ab)2 Goat anti-hu-IgG-Fc-HRP (Jackson ImmunoResearch Laboratories, cat # 109-036-098) in PBS 0.05% Tween. The plate was incubated for one hour at room temperature followed by four washes with PBS 0.05% Tween. Signal detection was carried out using 100 µl of 3',3',5',5'-Tetramethylbenzidine (TMB), and after 15 minutes, the reaction was stopped with 100 µl of 2N H_2SO_4.

[00106] Figure 15 compares the antibody titers of mice that were immunized with huFcγ4h-mono-L-DI-IFNß or huFcγ4h-mono-L-DI-IFNß variant containing the H435A mutation. Mice that received the huFcγ4h-mono-L-DI-IFNß variant containing the H435A mutation (diamonds) had lower titer than mice immunized with huFcγ4h-mono-L-DI-IFNß (circles) indicating that that the variant had reduced immunogenicity. The comparison between groups was analyzed by Sigma-Plot using Paired-t-test. The difference between two groups was statistically significant (Normality test: p=0.288, t=5.422 with 7 degrees of freedom (p=<0.001)).

Example 9. Determining immunogenicity by in vitro T cell assay

[00107] To show that the huFc-mono-ligand variant containing the H435A mutation is less immunogenic than the wild-type counterpart we use a human T cell proliferation assay that involves culturing and maturation of monocyte derived dendritic cells (DC), loading and presentation of antigen on DC, and determination of antigen-induced response in CD4+ T cells. Human peripheral blood mononuclear cells (PBMC) from healthy donors serve as a source of DC and autologous T cells. Typically PBMC are isolated by Ficoll-Hypaque gradient from leukopack samples and monocytes are purified using MACS CD14 isolation kit (Miltenyi Biotec Inc. Auburn, CA) and cultured with GM-CSF and IL-4 for 5 days. Immature DC are loaded with the different antigens, such as huFcγ4h-mono-L-DI-IFNß, the huFcγ4h-mono-L-DI-IFNß variant containing the H435A mutation, as well as tetanus toxoid. Since IFNß has a very potent immunosuppressive effect, the huFcγ4h-mono-L-DI-IFNß and the huFcγ4h-mono-L-DI-IFNß variant have to be heat treated at a moderately high

temperature so that the more heat-sensitive IFNß moiety is inactivated while the more heat stable Fc remains in its native form. A useful temperature range for this selective inactivation is from 56°C to 65°C. Alternatively, fusion proteins containing inactive IFNß moiety can be produced recombinantly through amino acid substitutions.

[00108] After the DC are incubated with the antigens for 24 hours, they are stimulated for 24 hours with TNF-α, IL-1β, IL-6, and PGE2 to induce DC maturation. Autologous CD4+ T cells that are prepared from the same human PBMC using MACS CD4 isolation kit (Miltenyi Biotec Inc. Auburn, CA) are labeled with carboxyfluorescein diacetate and succinimidyl ester (CFSE) using CellTrace CFSE Proliferation Kit (Invitrogen). DC and T cells are then co-cultured for 6-7 days, after which the percentage of dividing T cells are determined on FACS. Autologous T cell proliferation stimulated by the DC-presented peptides from the huFcγ4h-mono-L-DI-IFNß variant are expected to be lower than those against the corresponding wild-type Fc fusion protein.

Patent Claims:

1. An antibody fusion protein comprising a first and a second heavy chain domain, each comprising a -CH2 - CH3 domain of an IgG immunoglobulin including a hinge region, thus constituting with each other a Fc fragment, wherein

(i) the first heavy chain domain is fused via its C-terminal to human interferon and
(ii) the second heavy chain domain is not fused to a biologically active molecule, and

comprises a H435 mutation of the amino acid sequence within said heavy chain constant region, wherein this mutation results in a partial or complete loss of binding FcRn.

2. An antibody fusion protein of claim 1, wherein the IgG immunoglobulin is IgG2 or IgG4.

3. The antibody fusion protein of claim 2, wherein the hinge region is presented by an IgG1 hinge region.

4. An antibody fusion protein of any of the claims 1 - 3, wherein the mutation in said heavy chain constant region is H435A.

5. An antibody fusion protein of any of the claims 1 - 4, wherein the interferon is human Interferon-ß (IFNß).

6. An antibody fusion protein of claim 5, wherein the amino acid sequence of human IFNß is altered by amino acid substitutions, wherein these substitutions are selected from the group consisting of C17S, C17A, C17V, C17M, F50H, L57A, L130A, H131A, K136A, H140A, and H140T.

7. An antibody fusion protein of claim 3, wherein the amino acid sequence of the heavy chain domain fused to human IFNß is designated as huFcy4-L-DI- IFNß and specified by SEQ ID NO:5 without the signal peptide sequence at the N-terminal.

8. A nucleic acid molecule encoding an antibody fusion protein of any of the claims 1 - 7.

9. A pharmaceutical composition suitable for the treatment of cancer, viral infections, anemia or multiple sclerosis comprising the antibody fusion protein of any of the claims 1 or 7 and a pharmaceutically acceptable carrier, excipient or diluents.

10. Use of an antibody fusion protein of any of the claims 1 - 7 for the manufacture of a medicament for the treatment of cancer, viral infections, anemia, or multiple sclerosis.