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(54) Title: DUAL CYTOKINE FUSION PROTEINS COMPRISING MULTI-SUBUNIT CYTOKINES

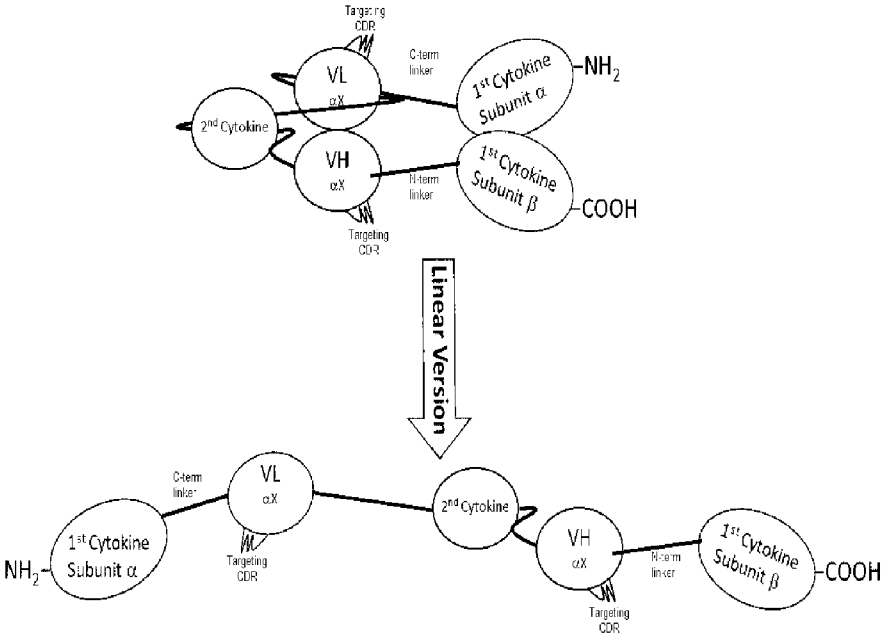

FIG. 1

(57) Abstract: The application relates to a dual cytokine fusion protein composition, pharmaceutical composition, and/or formulation thereof comprising the alpha and beta multi-subunits cytokines, such as IL-12 or IL-27, fused to a single chain variable fragment scaffolding system and a second cytokine, where the second cytokine is linked in the hinge region of the scFv. The application also relates to methods of using the dual cytokine fusion protein composition for treating cancer, inflammatory diseases or disorders, and immune and immune mediated diseases or disorders.

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DUAL CYTOKINE FUSION PROTEINS COMPRISING MULTI-SUBUNIT CYTOKINES

CROSS-REFERENCE TO RELATED APPLICATION

[001] The present application claims the benefit of priority to U.S. Provisional application number 63/265,339, filed December 13, 2021, the entire contents of which is incorporated herein by reference in its entirely.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[002] The contents of the electronic sequence listing (039451-00083_Sequence-Listing.xml; Size: 76,662 bytes; and Date of Creation: December 13, 2022) are herein incorporated by reference in its entirety.

FIELD OF INVENTION

[003] The present disclosure relates to the field of biotechnology, and more specifically, to a novel dual cytokine fusion protein comprising Interleukin-12 ("IL-12") or Interleukin-IL-27 in combination with other inflammatory and immune regulating cytokines, methods of treating inflammatory and immune disease or conditions, and/or methods of treating cancer.

INTRODUCTION

[004] IL-12 is a 70 kDa heterodimeric cytokine that is the prototypic T helper 1 (Th1) polarizing cytokine (Mossman, 1989; Athie-Morales, 2004). IL-12 exerts potent anti-tumor immunity through activating CD8+ T cells (Henry, 2008; Vacaflores, 2017; Chowdhury, 2011), NK cells (Martinović, 2015; Parihar, 2002; Zhang, 2008), CD4+ T cells (Yoo, 2002; Vacaflores1, 2016), and to a limited degree, monocytes (Coma, 2006). Therefore IL-12 predominantly enhances antigen specific T cell activation, while partially bridging to the innate immune system through moderate stimulation of both NK cells and monocytes.

[005] Interferon-alpha (IFNα-2a) is monomeric Type I interferon that directly induces dendritic cell maturation (Simmons, 2012; Gessani, 2014; Padovan, 2002) and enhances CD8+ T cell cytotoxic function (Hiroishi, 2000; Kolumam, 2005; Lu, 2019). Therefore, IFNα-2a exhibits greater function on the innate immune system compared to it's more limited effects on the adaptive immune response.

[006] The anti-tumor effects of IL-12 have been evaluated preclinically and clinically (Brunda, 1993; Atkins, 1997), where due to toxicity, direct intratumor injection was evaluated and found to be superior to clinical administration (Herpen, 2004; Li S. , 2005; Sabel, 2004).

[007] Similarly, IFNα-2a has been evaluated preclinically and in the pegylated form clinically (Lyrdal, 2009; Sunela, 2009; Medrano, 2017), and approved for use in some cancers (How, 2020).

[008] Therefore, given the need for both priming the anti-tumor immune response (Nemunaitis, 2005), and stimulation of CD8+, CD4+ and NK cells to drive robust anti-tumor function, the inventor has found that combining both IFNα-2a and IL-12 on a targeting dual cytokine scaffold system (known as a "Diakine™, ("DK"), which was generally described in co-pending application U.S. Application number 17/110,104) to target these two cytokines into the tumor microenvironment, enhances *in situ* priming via inducing the differentiation of M2 monocytes to functional antigen presenting cells (Vidyarthi, 2018), and enhances T and NK cell function. The combination of these two cytokines bridges stimulation between the adaptive and innate immune systems.

[009] Interleukin 10 (IL-10) is a non-covalent homo-dimeric cytokine with structural similarities to Interferon g (IFNg). The IL-10 receptor consists of two molecules of the IL10 receptor 1 (IL10R1) and two molecules of the IL-10 receptor 2 (IL10R2) (Moore, 2001). The IL-10 receptor is expressed on the surface of most hematopoietic cells and is highly expressed on macrophages and T-cells.

[0010] While IL-10 has been reported to be both an immunosuppressive (Schreiber, 2000) and immunostimulatory cytokine (Mumm, 2011), clinical evaluation of IL-10 for treating Crohn's patients resulted in an inverse dose response (Fedorak, 2000; Schreiber, 2000), whereas treating cancer patients with PEGylated IL-10 resulted in dose titratable potent anti-tumor responses (Naing, 2018).

[0011] IL-10 has been reported to suppress IL-2 driven IFNg production secreted by both NK and CD4+T cells (Scott, 2006), but it has also been reported to act as a cofactor for IL-2 induced CD8+ T cell proliferation (Groux, 1998).

[0012] The inventor has also found that combining both IL-10 and IL-12 on a targeting DK cytokine scaffold system to target these two cytokines into the tumor microenvironment, enhances NK and T cell function.

[0013] IL-27 is a member of the IL-12 family and is a heterodimeric cytokine comprised of two subunits, p28 and Epstein Barr virus-induced-gene 3 ("EIB3"). Il-27 is known to induce IL-10. IL-28 is a type 3 interferon that elicits IFNα-2a release stimulating CD8+ T-cells. IL-28 includes two isoforms, IL-28A and IL-28B. IL-29, which shares sequence homology to IL-28, is also a type 3 interferon that is involved in both the innate and adaptive immune response.

[0014] The inventor also found that other cytokine combinations including, but not limited to, IL-12 with interleukin-28 (IL-28) or interleuking-29 (IL-29), interleukin-27 (IL-27) with IFNα-2a, IL-28 or IL-29, may be incorporated into the dual cytokine scaffolding system described herein (see, *e.g.*, FIG. 1). Briefly, the dual cytokine scaffolding system permits multi-subunit cytokines (*e.g.*, including but not limited to IL-12, IL-27) to be fused to the terminal ends of a scaffolding system comprising an antigen binding domain or single chain variable region (scFv) of a human anti-HIV or human anti-ebola monoclonal antibody, in combination with a second cytokine (*e.g.*, IL-2, IL-4, IFNa-2a, IL-10), where the second cytokine is fused in the hinge region of the antigen binding domain or scFv.

[0015] The inventor also found that the dual cytokine scaffolding system permits multi-subunit cytokines (e.g., IL-12, IL-27) to be fused to the terminal ends of the scaffolding system comprising an antigen binding domain or scFv of a human anti-HIV or human anti-ebola monoclonal antibody, in combination with a monomeric cytokine (e.g., IFN-α, IL-28, IL-29) or another multi-subunit cytokine (e.g., IL-10, IL-12, IL-27).

[0016] As previously described in U.S. Patent 10,858,412, a scaffolding system comprising non-immunogenic variable heavy ("VH") and variable light ("VL") regions, resulted in the production of a half-life extended IL-10 variant molecules that properly folded and remained functionally active. The incorporation of the IL-10 into the scaffolding system showed enhanced IL-10 function on both inflammatory cells (*e.g.*, monocytes/macrophages/dendritic cells) and immune cells (*e.g.*, $CD8^+$ T-cells). *See,* U.S. Patent 10,858,412; filed on March, 6, 2020 as U.S. Application 16/811,718, incorporated by reference in its entirety. This application improves on the earlier discovery of an IL-10 based dual cytokine scaffolding system by substituting other multi-subunit based cytokines (*e.g.*, IL-12, IL-27 to name a few) in place of the IL-10 and further incorporating a second cytokine into the new fusion protein that additively

or synergistically enhances the biology of the multi-subunit cytokine (*e.g.*, IL-12 or IL-27) to treat inflammatory diseases, immune diseases, and/or cancer.

SUMMARY OF VARIOUS ASPECTS OF THE INVENTION

[0017] The present disclosure generally relates to a dual cytokine fusion protein.

[0018] Thus in a first aspect, the present disclosure relates to a dual cytokine fusion protein comprising a first cytokine that is a multi-subunit cytokines, such as but not limited to IL-10, IL-12 or IL-27 and variants thereof, where each of the subunits is fused to either a variable heavy ("VH") or a variable light ("VL") regions of scFv or antigen binding fragment obtained from a monoclonal antibody, and a second cytokine, wherein the second cytokine is linked in between the VH and VL regions of the scFv or antigen binding fragment. In certain embodiments, the first cytokine is any multi-subunit cytokine, such as but not limited to IL-10, IL-12 or IL-27, or functional variants thereof that include one or more amino acid substitution(s) that enhance the function of IL-10, IL-12 or IL-27. The fusion protein further includes a second cytokine, which is a cytokine that works in tandem with the multi-subunit cytokine (e.g., IL-10, IL-12 or IL-27) such that there is an additive or synergistic effect when the first and second cytokines are targeted to a specific antigen by the VH and VL regions of the scFv or antigen binding fragment. These second cytokines may be any cytokine, which includes, amongst others, IL-6, IL-4, IL-1, IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, IL-10, IL-15, IL-21 IL-26, IL-27, IL-28, IL-29, GM-CSF, G-CSF, interferons-α, -β, -γ, TGF-β, or tumor necrosis factors -α, -β, basic FGF, EGF, PDGF, IL-4, IL-11, or IL-13. The dual cytokine fusion protein may also include engraftment or replacement of the complement determining regions ("CDRs") of the scFv with CDRs from any targeting antibody that directs the dual cytokine fusion protein to a target antigen.

[0019] In yet another aspect, the present disclosure relates to a dual cytokine fusion protein of formula (Ia) or (Ib):

$$NH_2\text{-}(R^1)\text{–}(X^1)\text{–}(Z_n)\text{–}(X^2)\text{–}(R^2)\text{-}COOH \quad \text{(Formula Ia);}$$

$$NH_2\text{-}(R^2)\text{–}(X^1)\text{–}(Z_n)\text{–}(X^2)\text{–}(R^1)\text{-}COOH \quad \text{(Formula Ib);}$$

wherein

"R^1" is an alpha subunit from any multi-subunit first cytokine, preferably either IL-12-alpha subunit (p35) or IL-27 alpha subunit (p28), more preferably a subunit of SEQ ID No: 1 or 5 or 17 or 19;

"R^2" is a beta subunit from any multi-subunit first cytokine, preferably either IL-12-beta subunit (p40) or IL-27 beta subunit (EBI3), more preferably a subunit of SEQ ID No: 3 or 7 or 18 or 20;

wherein when R^1 is an alpha subunit of the first cytokine, R^2 is a beta subunit of the first cytokine; or when R^1 is p35, R^2 is p40; or when R^1 is p28, R^2 is EBI3; or when R^1 is SEQ ID No: 1, 17, or 19, R^2 is SEQ ID No: 3, 18, or 20; or when R^1 is SEQ ID No: 5, R^2 is SEQ ID No: 7;

"X^1" is a VL or VH region obtained from a first monoclonal antibody; "X^2" is a VH or VL region obtained from the first monoclonal antibody; wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"Z" is any cytokine that enhances the biological function of the multi-subunit cytokine, preferably IFNα-2a, IL-28, IL-29; and

"n" is an integer selected from 0-2.

In one embodiment, the VH and VL is in the form of a scFv obtained from a human anti-ebola antibody. In another embodiment, the 6 CDRs (CDRs 1-3 from the VH and CDRs 1-3 from the VL) of the scFv obtained from the human anti-ebola antibody are replaced or engrafted with 6 CDR from a second monoclonal antibody that allows the dual cytokine fusion protein to be directed to a specific target, such as, but not limited to enzymes, receptors, extracellular proteins, or intracellular protein, such as those associated with a tumors (*e.g.*, tumor associated antigens (TAAs)), inflammatory response, or autoimmune diseases. The second antibody may include, but not limited to, epidermal growth factor receptor (EGFR); CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD19; CD20; CD22; CD47;CD123; GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin; BCMA; PSA; PSMA; CEA; GPC3; SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1.

[0020] In yet another aspect, the present disclosure relates to a dual cytokine fusion protein comprising IL-12, said fusion protein being Formula (IIa) or (IIb):

NH_2-(p35)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(p40)-COOH (Formula IIa);

NH_2-(p40)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(p35)-COOH (Formula IIb);

wherein

"p35" is an alpha subunit of IL-12 having a sequence of SEQ ID No; 1, 17 or 19;

"p40" is a beta alpha subunit of IL-12 having a sequence of SEQ ID No; 3, 18, or 20;

"L" is any linker;

X^1" is a VL or VH region obtained from a first monoclonal antibody; "X^2" is a VH or VL region obtained from the first monoclonal antibody; wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"Z" is a cytokine selected from IL-6, IL-4, IL-1, IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, IL-10 monomer, IL-15, IL-21 IL-26, IL-27, IL-28, IL-29, GM-CSF, G-CSF, interferons-α, -β, -γ, TGF-β, or tumor necrosis factors -α, -β, basic FGF, EGF, PDGF, IL-4, IL-11, or IL-13; and

"n" is an integer selected from 0-2.

In one embodiment, the VH and VL is in the form of a scFv obtained from a human anti-ebola antibody. In another embodiment, the 6 CDRs (CDRs 1-3 from the VH and CDRs 1-3 from the VL) of the scFv obtained from the human anti-ebola antibody are replaced or engrafted with 6 CDR from a second monoclonal antibody that allows the dual cytokine fusion protein to be directed to a specific target, such as, but not limited to enzymes, receptors, extracellular proteins, or intracellular protein, such as those associated with a tumors (*e.g.*, tumor associated antigens (TAAs)), inflammatory response, or autoimmune diseases. The second antibody may include, but not limited to, epidermal growth factor receptor (EGFR); CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD19; CD20; CD22; CD47;CD123; GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin; BCMA; PSA; PSMA; CEA; GPC3; SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1.

[0021] In yet another aspect, the present disclosure relates to a dual cytokine fusion protein comprising IL-27, said fusion protein being Formula (IIIa) or (IIIb):

NH_2-(p28)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(EBI3)-COOH (Formula IIIa);

NH_2-(EBI3)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(p28)-COOH (Formula IIIb);

wherein

"p28" is an alpha subunit of IL-27 having a sequence of SEQ ID No; 5;

"EBI3" is a beta alpha subunit of IL-27 having a sequence of SEQ ID No; 7;

"L" is any linker;

X^1" is a VL or VH region obtained from a first monoclonal antibody; "X^2" is a VH or VL region obtained from the first monoclonal antibody; wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"Z" is a cytokine selected from IL-6, IL-4, IL-1, IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, IL-10 monomer, IL-15, IL-21 IL-26, IL-27, IL-28, IL-29, GM-CSF, G-CSF, interferons-α, -β, -γ, TGF-β, or tumor necrosis factors -α, -β, basic FGF, EGF, PDGF, IL-4, IL-11, or IL-13; and

"n" is an integer selected from 0-2.

In one embodiment, the VH and VL is in the form of a scFv obtained from a human anti-ebola antibody. In another embodiment, the 6 CDRs (CDRs 1-3 from the VH and CDRs 1-3 from the VL) of the scFv obtained from the human anti-ebola antibody are replaced or engrafted with 6 CDR from a second monoclonal antibody that allows the dual cytokine fusion protein to be directed to a specific target, such as, but not limited to enzymes, receptors, extracellular proteins, or intracellular protein, such as those associated with a tumors (*e.g.*, tumor associated antigens (TAAs)), inflammatory response, or autoimmune diseases. The second antibody may include, but not limited to, epidermal growth factor receptor (EGFR); CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD19; CD20; CD22; CD47;CD123; GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin; BCMA; PSA; PSMA; CEA; GPC3; SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1.

[0022] In yet another aspect, the present disclosure relates to a dual cytokine fusion protein comprising two multi-subunit proteins, said fusion protein being Formula (IV):

NH_2-(R^1)-(L_a)-(X^1)-(L_a)-(W^1)-(L_b)-(W^2)-(L_a)-(X^2)-(L_a)-(R^2)-COOH (Formula IV); wherein

"R^1" is an alpha subunit of a first cytokine, such as IL-12 or IL-27 or a first monomer of a homodimeric cytokine, such as IL-10, wherein R^1 is preferably p40;

"R^2" is a beta alpha subunit of the first cytokine, such as IL-12 or IL-27 or a second monomer of the homodimeric cytokine, such as IL-10, wherein R^2 is preferably p35;

"L_a" is any linker; preferably $(GGGGS)_4$ of SEQ ID No.: 45, or $(GGGGS)_5$ of SEQ ID No.: 44;

"L_b" is any linker; preferably GGGSGGG of SEQ ID No.: 43 or of $(GGGGS)_3$ of SEQ ID No.: 46;

X^1" is a VL or VH region obtained from a first monoclonal antibody; "X^2" is a VH or VL region obtained from the first monoclonal antibody; wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"W^1" is an alpha subunit of a first cytokine, such as IL-12 or IL-27 or a first monomer of a homodimeric cytokine, such as IL-10, preferably a first monomer of IL-10;

"W^2" is a beta alpha subunit of the first cytokine, such as IL-12 or IL-27 or a second monomer of the homodimeric cytokine, such as IL-10, preferably a second monomer of IL-10.

In one embodiment, the VH and VL is in the form of a scFv obtained from a human anti-ebola antibody. In another embodiment, the 6 CDRs (CDRs 1-3 from the VH and CDRs 1-3 from the VL) of the scFv obtained from the human anti-ebola antibody are replaced or engrafted with 6 CDR from a second monoclonal antibody that allows the dual cytokine fusion protein to be directed to a specific target, such as, but not limited to enzymes, receptors, extracellular proteins, or intracellular protein, such as those associated with a tumors (*e.g.*, tumor associated antigens (TAAs)), inflammatory response, or autoimmune diseases. The second antibody may include, but not limited to, epidermal growth factor receptor (EGFR); CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD19; CD20; CD22; CD47;CD123; GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin; BCMA; PSA; PSMA;

CEA; GPC3; SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1.

[0023] In yet another aspect, the present disclosure relates to a dual cytokine fusion protein comprising two multi-subunit proteins, said fusion protein being Formula (V):

NH_2-(P35)-(L_a)-(X^1)-(L_a)-($IL10_{monomer}$)-(L_b)-($IL10_{monomer}$)-(L_a)-(X^2)-(L_a)-(P40)-COOH (Formula Va);

NH_2-(P40)-(L_a)-(X^1)-(L_a)-($IL10_{monomer}$)-(L_b)-($IL10_{monomer}$)-(L_a)-(X^2)-(L_a)-(P35)-COOH (Formula Vb);

wherein

"p35" is an alpha subunit of IL-12 having a sequence of SEQ ID No: 1, 17, or 19;

"p40" is a beta alpha subunit of IL-12 having a sequence of SEQ ID No; 3, 18, or 20;

"L_a" is any linker; preferably $(GGGGS)_4$ of SEQ ID No.: 45, or $(GGGGS)_5$ of SEQ ID No.: 44;

"L_b" is any linker; preferably GGGSGGG of SEQ ID No.: 43 or $(GGGGS)_3$ of SEQ ID No.: 46;

X^1" is a VL or VH region obtained from a first monoclonal antibody; "X^2" is a VH or VL region obtained from the first monoclonal antibody; wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"$IL10_{monomer}$" is monomer of IL-10 having a sequence of SEQ ID No: 1, 3, 5, 7, 14, or 16, preferably SEQ ID No: 16;

In one embodiment, the VH and VL is in the form of a scFv obtained from a human anti-ebola antibody. In another embodiment, the 6 CDRs (CDRs 1-3 from the VH and CDRs 1-3 from the VL) of the scFv obtained from the human anti-ebola antibody are replaced or engrafted with 6 CDR from a second monoclonal antibody that allows the dual cytokine fusion protein to be directed to a specific target, such as, but not limited to enzymes, receptors, extracellular proteins, or intracellular protein, such as those associated with a tumors (*e.g.*, tumor associated antigens (TAAs)), inflammatory response, or autoimmune diseases. The second antibody may include, but not limited to EGFR; CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD19; CD20; CD22; CD47;CD123; GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5),

VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin; BCMA; PSA; PSMA; CEA; GPC3; SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1.

[0024] In other aspects, the present disclosure relates to a nucleic acid molecule that encodes the multi-subunit dual cytokine fusion protein. These would include those that encode the dual cytokine fusion protein represented by formula (Ia), (Ib), (IIa), (IIb), (IIIa), (IIIb), (IV), (Va) and (Vb)

[0025] In other aspects, the present disclosure relates to methods of making and purifying the dual cytokine fusion protein. In one embodiment, the method of making the dual cytokine fusion protein includes recombinantly expressing the nucleic acid encoding the dual cytokine fusion protein.

[0026] In other aspects, the present disclosure relates to a method of treating cancer comprising administering to a subject in need thereof, an effective amount of the dual cytokine fusion protein.

[0027] In other aspects, the present disclosure relates to a method of treating inflammatory diseases or conditions comprising administering to a subject in need thereof, an effective amount of the dual cytokine fusion protein. Preferably, the inflammatory disease is Crohn's disease, psoriasis, and/or rheumatoid arthritis.

[0028] In other aspects, the present disclosure relates to a method of treating immune diseases or conditions comprising administering to a subject in need thereof, an effective amount of the dual cytokine fusion protein.

[0029] In other aspects, the present disclosure relates to method of treating, inhibiting, and/or alleviating sepsis and/or septic shock and associated symptoms thereof.

[0030] The above simplified summary of representative aspects serves to provide a basic understanding of the present disclosure. This summary is not an extensive overview of all contemplated aspects, and is intended to neither identify key or critical elements of all aspects nor delineate the scope of any or all aspects of the present disclosure. Its sole purpose is to present one or more aspects in a simplified form as a prelude to the more detailed description of the disclosure that follows. To the accomplishment of the foregoing, the one or more aspects of the present disclosure include the features described and exemplarily pointed out in the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] FIG. 1 is a schematic diagram of a dual cytokine fusion protein in folded and linear form.

[0032] FIG. 2 is a schematic diagram that is representative of a dual cytokine fusion protein embodied in the present disclosure, wherein the dual cytokine fusion protein comprises terminally linked IL-12α (p35) and IL-12β (p40) subunits, where a second cytokine is incorporated into the linker of a scFv between the VH and VL from an anti-X antibody (*e.g.*, from a human anti-ebola antibody). The 6 CDRs (3 from the VH and 3 from the VL) may be optionally substituted or engrafted with 6 CDRs from a second antibody (*e.g.*, such as those that target TAAs, such as an anti-HER2, anti-EGFR, anti-VEGFR1, or anti-VEGFR2 antibody).

[0033] FIG. 3 is a schematic diagram that is representative of a dual cytokine fusion protein embodied in the present disclosure, wherein the dual cytokine fusion protein comprises terminally linked IL-27α (p28) and IL-27β (EBI3) subunits, where a second cytokine is incorporated into the linker of a scFv between the VH and VL from an anti-X antibody (*e.g.*, from a human anti-ebola antibody). The 6 CDRs (3 from the VH and 3 from the VL) may be optionally substituted or engrafted with 6 CDRs from a second antibody (*e.g.*, such as those that target TAAs, such as, but not limited to, an anti-HER2, an anti-HER3, anti-EGFR, anti-VEGFR1, or anti-VEGFR2 antibody).

[0034] FIG. 4 is a schematic diagram that is representative of a dual cytokine fusion protein comprising IL-27 and IL-28 termed "$DK27^{28}$".

[0035] FIG. 5 is a schematic diagram that is representative of a dual cytokine fusion protein comprising IL-27 and IL-29 termed "$DK27^{29}$".

[0036] FIG. 6 is a schematic diagram representing one of the previously disclosed IL-10 fusion protein constructs where IL-10 monomers are terminally linked to a scaffolding comprising a scFv described in U.S. Patent 10,858,412.

[0037] FIG. 7 is a schematic diagram that is a representative examples of a dual cytokine fusion protein comprising two multi-subunit (or dimeric) cytokines, in particular, a dual cytokine fusion protein comprising IL-12 and IL-10 termed $DK12^{10}$.

[0038] FIG. 8 is a schematic diagram that is a representative examples of a dual cytokine fusion protein comprising two multi-subunit (or dimeric) cytokines, in particular, a dual cytokine fusion protein comprising IL-27 and IL-10 termed $DK27^{10}$.

[0039] FIG 9 is a graph showing that $DK12^{10}$ (EGFR) using standard linkers, such as $(GGGGS)_3$, between IL-10 and the scFv and between IL-12 and the scFv have partial cytolytic effect on target cancer cells in combination with BiTE.

[0040] FIG 10 is a graph showing that $DK12^{10}$ (EGFR) using extended linkers between IL-10 and the scFv and between IL12 and the scFv have improved cytolytic effect on target cancer cells in combination with BiTE.

DETAILED DESCRIPTION

[0041] Exemplary aspects are described herein in the context of a dual cytokine fusion protein comprising a multi-subunit first cytokine (such as IL-12 or IL-27), methods of making the dual cytokine fusion protein comprising a multi-subunit first cytokine (such as IL-12 or IL-27), and methods of using the dual cytokine fusion protein comprising a multi-subunit first cytokine (such as IL-12 or IL-27) for treating inflammatory diseases or conditions, immune diseases or conditions, treating and/or preventing cancer. Those of ordinary skill in the art will realize that the following description is illustrative only and is not intended to be in any way limiting. Other aspects will readily suggest themselves to those skilled in the art having the benefit of this disclosure. Reference will now be made in detail to implementations of the exemplary aspects as illustrated in the accompanying drawings. The same reference indicators will be used to the extent possible throughout the drawings and the following description to refer to the same or like items.

[0042] Although a number of methods and materials similar or equivalent to those described herein can be used in the practice of the various described embodiments, the preferred materials and methods are described herein.

[0043] Unless otherwise indicated, the embodiments described herein employ conventional methods and techniques of molecular biology, biochemistry, pharmacology, chemistry, and immunology, well known to a person skilled in the art. Many of the general techniques for designing and fabricating the dual cytokine fusion proteins comprising the multi-subunit first cytokine (such as IL-12 or IL-27), as well as the assays for testing the expression and function of dual cytokine fusion proteins comprising the multi-subunit first cytokine (such as IL-12 or IL-27), are well known methods that are readily available and detailed in the art. *See, e.g.*, Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Handbook of Experimental

Immunology, Vols. I-IV (D. M. Weir and C. C. Blackwell eds., Blackwell Scientific Publications); A. L. Lehninger, Biochemistry (Worth Publishers, Inc., current addition). N-terminal aldehyde based PEGylation chemistry is also well known in the art.

Definitions

[0044] The following terms will be used to describe the various embodiments discussed herein, and are intended to be defined as indicated below.

[0045] As used herein in describing the various embodiments, the singular forms “a”, “an” and “the” include plural referents unless the content clearly dictates otherwise.

[0046] The term “about”, refers to a deviance of between 0.0001-5% from the indicated number or range of numbers. In one embodiment, the term “about”, refers to a deviance of between 1-10% from the indicated number or range of numbers. In one embodiment, the term “about”, refers to a deviance of up to 25% from the indicated number or range of numbers. In a more specific embodiment, the term “about” refers to a difference of 1-25% in terms of nucleotide sequence homology or amino acid sequence homology when compared to a wild-type sequence.

[0047] The term “multi-subunit cytokine” refers to a cytokine protein comprising at least an alpha subunit and a beta subunit to make a heterodimer or two monomers to make a homodimer. As reference, a multi-subunit cytokine may include, amongst other, IL-10, IL-12 or IL-27. Other multi-subunit cytokines are known by those of skill in the art and may be substituted into the terminal ends of the dual cytokine fusion proteins described herein.

[0048] The term “interleukin-12” or “IL-12” refers to a protein comprising an alpha (p35) and beta (p40) subunit, non-covalently joined to form a heterodimer. As used herein, unless otherwise indicated “interleukin-12” and “IL-12” refers to any form of IL-12, including but not limited to human; mouse, or variant forms. For example, the term “wild-type” or “native” would thus correspond to an amino acid sequence that is most commonly found in nature for the alpha and beta subunits. In one embodiment, p35 is a sequence of SEQ ID No: 1, 17 or 19 and p40 is a sequence of SEQ ID No: 3, 18, or 20.

[0049] The term “interleukin-27” or “IL-27” refers to a protein comprising an alpha (p28) and beta (EBI3) subunit, non-covalently joined to form a heterodimer. As used herein, unless otherwise indicated “interleukin-12” and “IL-12” refers to any form

of IL-12, including but not limited to human; mouse, or variant forms. For example, the term “wild-type” or “native” would thus correspond to an amino acid sequence that is most commonly found in nature for the alpha and beta subunits. In one embodiment, p28 is a sequence of SEQ ID No: 5 and EBI3 is a sequence of SEQ ID No: 7.

[0050] The term “interleukin-10” or “IL-10” refers to a protein comprising two monomers that joined to form a homodimer. As used herein, unless otherwise indicated “interleukin-10” and “IL-10” refers to any form of IL-10, including but not limited to human; mouse, or variant forms. For example, the term “wild-type” or “native” would thus correspond to an amino acid sequence that is most commonly found in nature for the alpha and beta subunits. In one embodiment, human IL-10 is a sequence of SEQ ID No: 31, mouse IL-10 is a sequence of SEQ ID No: 37, viral forms of IL-10 include EBV IL-10 having SEQ ID No: 33, and CMV IL-10 having SEQ ID No: 35.

[0051] The terms “variant,” “analog” and “mutein” refer to biologically active derivatives of the reference molecule, that retain a desired activity, such as, for example, anti-inflammatory activity. Generally, the terms “variant,” “variants,” “analog” and “mutein” as it relates to a polypeptide refers to a compound or compounds having a native polypeptide sequence and structure with one or more amino acid additions, substitutions (which may be conservative in nature), and/or deletions, relative to the native molecule. As such, the terms “IL-12 variant”, “variant IL-12,” “IL-12 variant molecule,” “IL-27 variant”, “variant IL-27,” “IL-27 variant molecule,” and grammatical variations and plural forms thereof are all intended to be equivalent terms that refer to an IL-12 or IL-27 amino acid (or nucleic acid) sequence that differs from wild-type IL-12 or IL-27. The difference in amino acid sequence for IL-12 or IL-27 may be additions, deletions, or substitutions within the alpha, beta, or both subunits such that there is anywhere from 1-25% in sequence identity or homology. These variant forms include modifications to the glycosylation (deglycosylated or aglycosylated) forms thereof to the protein. IL-10 variant forms may include those have increased or decreased binding affinity when compared to wild-type IL-10. Thus in one embodiment, a variant form of IL-10 having increased or higher binding affinity includes an IL-10 variant (internally denoted as DV07) of SEQ ID No.:41 or an IL-10 variant having decreased or lower binding affinity (internally denoted as DV06) of SEQ ID No: 39.

[0052] The term “fusion protein” refers to a combination or conjugation of two or more proteins or polypeptides that results in a novel arrangement of proteins that do not normally exist naturally. The fusion protein is a result of covalent linkages of the two or more proteins or polypeptides. The two or more proteins that make up the fusion protein may be arranged in any configuration from amino-terminal end (“NH_2”) to carboxy-terminal end (“COOH”). Thus, for example, the carboxy-terminal end of one protein may be covalently linked to either the carboxy terminal end or the amino terminal end of another protein. Exemplary fusion proteins may include combining (from N-terminal to C-terminal) an alpha subunit of IL-12 to an antibody VH domain to a second cytokine (such as IFN-alpha, IL-28, or IL-29) to a VL domain (such that the VH and VL domains form a VH/VL pair) to a beta subunit of IL-12. Another exemplary fusion protein may include combining (from N-terminal to C-terminal) an alpha subunit of IL-27 to an antibody VH domain to a second cytokine (such as IFN-alpha, IL-28, or IL-29) to a VL domain (such that the VH and VL domains form a VH/VL pair) to a beta subunit of IL-27. Yet another exemplary fusion protein may include combining (from N-terminal to C-terminal) an alpha subunit of IL-12 to an antibody VH domain to a first monomer of a homodimeric cytokine (such as IL-10) to a second monomer of the homodimeric cytokine (such as IL-10) to a VL domain (such that the VH and VL domains form a VH/VL pair) to a beta subunit of IL-12. In one preferred embodiment, a representative forms of a multi-subunit dual cytokine fusion protein may include heterodimeric cytokines such as IL-12 or IL-27 in combination with monomeric cytokines such as IL-6, IL-4, IL-1, IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, IL-15, IL-21 IL-26, IL-27, IL-28, IL-29, GM-CSF, G-CSF, interferons-α, -β, -γ, TGF-β, or tumor necrosis factors -α, -β, basic FGF, EGF, PDGF, IL-4, IL-11, or IL-13. In another embodiment, a multi-subunit dual cytokine fusion protein may include a heterodimer cytokine such as IL-12 or IL-27 in combination with a homodimeric cytokine such as IL-10, IL-10 variants, mouse IL-10, DV07 (SEQ ID No:41), or DV06 (SEQ ID No:39).

[0053] The term “homolog,” “homology,” “homologous” or “substantially homologous” refers to the percent identity between at least two polynucleotide sequences or at least two polypeptide sequences. Sequences are homologous to each other when the sequences exhibit at least about 50%, preferably at least about 75%, more preferably at least about 80%-85%, preferably at least about 90%, and

most preferably at least about 95%-98% sequence identity over a defined length of the molecules.

[0054] The term “sequence identity” refers to an exact nucleotide-by-nucleotide or amino acid-by-amino acid correspondence. The sequence identity may range from 100% sequence identity to 50% sequence identity. A percent sequence identity can be determined using a variety of methods including but not limited to a direct comparison of the sequence information between two molecules (the reference sequence and a sequence with unknown percent identity to the reference sequence) by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the reference sequence, and multiplying the result by 100. Readily available computer programs can be used to aid in the identification of percent identity.

[0055] The terms “subject,” “individual” or “patient” are used interchangeably herein and refer to a vertebrate, preferably a mammal. Mammals include, but are not limited to, murine, rodent, simian, human, farm animals, sport animals, and certain pets.

[0056] The term “administering” includes routes of administration which allow the active ingredient of the application to perform their intended function.

[0057] A “therapeutically effective amount” as it relates to, for example, administering the dual cytokine fusion proteins described herein, refers to a sufficient amount of dual cytokine fusion proteins to promote certain biological activities. These might include, for example, suppression of myeloid cell function, enhanced Kupffer cell activity, and/or lack of any effect on $CD8^+$ T cells or enhanced $CD8^+$ T-cell activity as well as blockade of mast cell upregulation of Fc receptor or prevention of degranulation. Thus, an “effective amount” will ameliorate or prevent a symptom or sign of the medical condition. Effective amount also means an amount sufficient to allow or facilitate diagnosis.

[0058] The term “treat” or “treatment” refers to a method of reducing the effects of a disease or condition. Treatment can also refer to a method of reducing the underlying cause of the disease or condition itself rather than just the symptoms. The treatment can be any reduction from native levels and can be, but is not limited to, the complete ablation of the disease, condition, or the symptoms of the disease or condition.

Dual Cytokine Fusion Protein Structure

[0059] The present disclosure provides an improvement on an embodiment of an IL-10 fusion protein previously described in U.S. Patent 10,858,412 (filed as U.S. Application No. 16/811,718), which is incorporated by reference in its entirety. FIG. 6 is a schematic diagram representing one of the previously disclosed IL-10 fusion protein constructs described in U.S. Patent 10,858,412. FIG. 1 provides a general schematic representation of how the present application improves on the original IL-10 fusion protein. The improvement on the IL-10 fusion protein includes (1) substituting the IL-10 monomers with alpha and beta subunits of a multi-subunit cytokine and (2) incorporating a second cytokine molecule between the VH and VL domains of a scFv (i.e. in the hinge region of the scFv). The dual cytokine fusion protein of the present application may be constructed on a scaffolding system comprising a VH and VL (scFv) featuring an alpha and beta subunit of a multi-subunit cytokine, where the alpha subunit is fused on the N-terminus and the beta subunit is sued to the C-terminus (or vice versa). According the term fused to the “terminal ends” will mean that either an alpha (or beta) subunit will be located on the N-terminal end and the beta (or alpha) subunit will be located on the C-terminal end of the fusion protein. The scaffolding system will comprises a scFv, preferably obtained from an antibody that is specific for HIV or ebola, preferably the scFv is obtained from a human anti-ebola antibody. The dual cytokine fusion protein includes a scFv (preferably obtained from a human anti-ebola antibody) having 6 complementarity-determining regions (“CDRs”), where there are 3 CDRs (CDR 1-3) in the VH and 3 CDRs (CDR 1-3) in the VL. Optionally, the VH and VL regions are capable of targeting the dual cytokine fusion protein to a specific antigen. This may be accomplished by substituting the 6 CDR regions of the VH and VL pair (3 CDRs in the VH and 3 CDRs in the VL) with 6 CDR regions from a VH and VL of a receptor or antigen targeting antibody, or antigen binding fragment thereof. This process is also generally known as CDR grafting or CDR engraftment. Those of skill in the art are capable of substituting and optimizing the engraftment or grafting of the 6 CDR into the scFv framework regions or into scFv scaffolding described herein. These are well known and practiced techniques used by those of skill in the art. The 6 CDR regions from, for example the scFv obtained from a human anti-ebola antibody are substitutable with 6 CDRs from any monoclonal antibody, which any person of skill would be capable of determining

based on the specific target of interest. For example, the specific target may include, but not limited to, enzymes, receptors, extracellular proteins, or intracellular protein, such as those associated with a tumors (*e.g.*, tumor associated antigens (TAAs)), inflammatory response, or autoimmune diseases. Thus, the 6 CDRs targeting the specific target may be any antibody, including but not limited to, those that are specific for EGFR; CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD19; CD20; CD22; CD47;CD123; GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin; BCMA; PSA; PSMA; CEA; GPC3; SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1. The aforementioned list is representative of the possible targeting antibodies that may be incorporated into the dual cytokine fusion protein of the present disclosure, and a person of skill in the art would be able to recognize that the CDRs from other targeting antibodies, especially ones that target surface markers on cancers or inflammatory tissues, may be engrafted into the scaffolding. In a preferred embodiment, the antibody is an anti-HER2, anti-HER3, anti-EGFR, anti-VEGFR1, anti-VEGFR2, anti-BCMA, anti-PSA, anti-PSMA, anti-CD19, anti-CD20, anti-CD22, anti-CEA, anti-GPC3, or anti-CD14 antibody.

[0060] In a first aspect, the present application relates to a dual cytokine fusion protein comprising IL-12 or IL-27 and at least one other cytokine, whereby the dual cytokine fusion protein has a combined or synergistic functionality when compared to IL-12 or IL-27 and the other cytokine fusion individually. FIG. 1 is a representative diagram of the improved dual cytokine fusion protein comprising the alpha and beta subunits of the multi-subunit cytokine fused to the terminal ends of the fusion protein. In particular, the improved dual cytokine fusion protein adapts the same or substantially same scaffolding system made up of a VH and VL scFv whereby alpha and beta subunits of IL-12 or IL-27, for example, terminate the dual fusion protein at the amino and carboxy terminal ends (see, *e.g.*, FIG. 2-5). In certain embodiments the IL-12 subunit is an alpha subunit or p35 of SEQ ID No: 1, 17, or 19 or a beta subunit or p40 of SEQ ID No: 3, 18, or 20 wherein the amino acid subunit fused to the scFv lacks the signal peptide or leader sequence. In other embodiments the IL-27 subunit is an alpha subunit or p28 of SEQ ID No: 5 or a beta subunit of and EBI3 of SEQ ID

No: 7, wherein the amino acid fused to the scFv lacks the signal peptide or leader sequence. In certain embodiments, modifications to one or both subunits of IL-12 or IL-27 maybe include additions, deletions, or substitutions. These modifications may include modifications that alter binding affinity (increase or decrease), alter glycosylation sites, or decrease immunogenicity. In certain embodiments, the glycosylation sites in IL-27, preferably in EIB3 are modified, more preferably amino acid positions 55-57 and/or 105-107 of SEQ ID No: 7, even more preferably position 57 and/or 107 of SEQ ID No: 7, where threonine is substituted. The second cytokine is conjugated to the dual cytokine fusion protein by being fused between the VH and VL regions of the scFv, which is the hinge region of the scFv (see, *e.g.* FIG. 1-5). The dual cytokine fusion protein is capable of forming a functional protein complex whereby the alpha and beta subunits heterodimerize along with the pairing of the VH and VL regions to form a pair that associate together to form a scFv complex.

[0061] In certain embodiments, the dual cytokine fusion protein comprising the multi-subunit cytokine is a structure having formula Ia or Ib

NH_2-(R^1)−(X^1)−(Z_n)−(X^2)−(R^2)-COOH (Formula Ia);

NH_2-(R^2)−(X^1)−(Z_n)−(X^2)−(R^1)-COOH (Formula Ib)

wherein

"R^1" is an alpha subunit of a first cytokine sequence selected from SEQ ID No: 1, 17, 19 or 5;

"R^2" is a beta subunit of a first cytokine sequence selected from SEQ ID No: 3, 18, 20, or 7;

wherein when R^1 is SEQ ID No: 1, 17 or 19, R^2 is SEQ ID No: 3, 18, or 20 or when R^1 is SEQ ID No: 5, R^2 is SEQ ID No:7;

"X^1" is a VL or VH region obtained from a first monoclonal antibody;

"X^2" is a VH or VL region obtained from the first monoclonal antibody;

wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL

"Z" is a cytokine;

"n" is an integer selected from 0-2.

[0062] In another embodiment, the dual cytokine fusion protein is a structure having formula IIa or IIb

NH_2-(p35)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(p40)-COOH (Formula IIa);

NH_2-(p40)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(p35)-COOH (Formula IIb);

wherein

"p35" is a sequence of SEQ ID No: 1, 17, or19;

"p40" is a sequence of SEQ ID No: 3, 18, or 20;

"L" is a linker;

"X^1" is a VL or VH region obtained from a first monoclonal antibody;

"X^2" is a VH or VL region obtained from the first monoclonal antibody;

wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"Z" is a cytokine selected from IL-6, IL-4, IL-1, IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, IL-15, IL-21, IL-26, IL-27, IL-28, IL-29, GM-CSF, G-CSF, interferons-α, -β, -γ, TGF-β, or tumor necrosis factors -α, -β, basic FGF, EGF, PDGF, IL-4, IL-11, or IL-13;

"n" is an integer selected from 0-2.

[0063] In another embodiment, the dual cytokine fusion protein is a structure having formula IIIa or IIIb

NH_2-(p28)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(EBI3)-COOH (Formula IIIa);

NH_2-(EBI3)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(p28)-COOH (Formula IIIb);

wherein

"p28" is a sequence of SEQ ID No: 5;

"EBI3" is a sequence of SEQ ID No: 7;

"L" is a linker;

"X^1" is a VL or VH region obtained from a first monoclonal antibody;

"X^2" is a VH or VL region obtained from the first monoclonal antibody;

wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"Z" is a cytokine selected from IL-6, IL-4, IL-1, IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, IL-15, IL-21, IL-26, IL-28, IL-29, GM-CSF, G-CSF, interferons-α, -β, -γ, TGF-β, or tumor necrosis factors -α, -β, basic FGF, EGF, PDGF, IL-4, IL-11, or IL-13;

"n" is an integer selected from 0-2.

[0064] In another embodiment, the VH and VL regions are from an antibody, antibody fragment, or antigen binding fragment thereof. The antigen binding fragment includes, but is not limited to, a scFv, Fab, $F(ab')_2$, V-NAR, diabody, or nanobody. Preferably the VH and VL, are from a single chain variable fragment ("scFv").

[0065] In another embodiment, the dual cytokine fusion protein comprising a first multi-subunit cytokine (*e.g.*, IL-12 or IL-27) includes a VH and VL pair from a single

antibody. The VH and VL pair act as a scaffolding onto which first multi-subunit cytokine may be attached such that the alpha and beta subunits of the multi-subunit cytokine may be able to heterodimerize into a functioning cytokine. A person of skill in the art will therefore appreciate that the VH and VL scaffolding used in the fusion protein may be selected based on the desired physical attributes needed for proper heterodimerization of the first multi-subunit cytokine (*e.g.,* IL-12 or IL-27) and/or the desire to maintain VH and VL targeting ability. Likewise, a person of skill will also understand that the 6 CDRs within the VH and VL pair (3 CDRs from the VH and 3 CDRs from VL) may also be substituted with 6 CDRs from other antibodies to obtain a specifically targeted fusion protein. In one embodiment, 3 CDRs from a VH and 3 CDRs from a VL (i.e., a VH and VL pair) of any monoclonal antibody may be engrafted into a scaffolding system. It is also envisioned that if the fusion protein is not intended to target any specific antigen, a VH and VL pair may be selected as the scaffolding that does not target any particular antigen (or is an antigen in low abundance *in vivo*), such as the VH and VL pair from a human anti-HIV and/or human anti-Ebola antibody. The fusion protein may comprise a range of 1-4 variable regions. In another embodiment, the variable regions may be from the same antibody or from at least two different antibodies. The amino acid sequence encoding the multi-subunit cytokines will be fused to the scFv scaffolding without the signal peptides (or leader sequence).

[0066] In yet another aspect, the dual cytokine fusion protein may comprise two multi-subunit proteins. For example, the dual cytokine fusion protein may comprise a first cytokine that is a heterodimer (such as but not limited to IL12 or IL27) and then a second homodimeric cytokine (such as but not limited to IL10). The second homodimeric cytokine will be capable of being fused between the VH and VL regions. A representative image of a two multi-subunit dual cytokine fusion protein is provided in Figures 7 and 8. Thus, in one embodiment of the invention, said fusion protein will have a generic formula of Formula (IV):

$$\mathbf{NH_2\text{-}(R^1)\text{-}(L_a)\text{-}(X^1)\text{-}(L_b)\text{-}(W^1)\text{-}(L_c)\text{-}(W^2)\text{-}(L_b)\text{-}(X^2)\text{-}(L_a)\text{-}(R^2)\text{-}COOH} \quad \textbf{(Formula IV)}$$

wherein

"R^1" is an alpha subunit of a first cytokine, such as IL-12 or IL-27 or a first monomer of a homodimeric cytokine, such as IL-10, preferably (p40);

"R^2" is a beta alpha subunit of the first cytokine, such as IL-12 or IL-27 or a second monomer of the homodimeric cytokine, such as IL-10, preferably p35;

"L_a" is any linker; preferably $(GGGGS)_3$ of SEQ ID No: 46, $(GGGGS)_4$ of SEQ ID No: 45, or $(GGGGS)_5$ of SEQ ID No: 44;

"L_b" is any linker; preferably $(GGGGS)_3$ of SEQ ID No: 46, $(GGGGS)_4$ of SEQ ID No: 45, or $(GGGGS)_5$ of SEQ ID No: 44;

"Lc" is any linker; preferably GGGSGGG of SEQ ID No: 43 or $(GGGGS)_3$ of SEQ ID No: 46;

X^1" is a VL or VH region obtained from a first monoclonal antibody; "X^2" is a VH or VL region obtained from the first monoclonal antibody; wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"W^1" is an alpha subunit of a first cytokine, such as IL-12 or IL-27 or a first monomer of a homodimeric cytokine, such as IL-10, preferably a first monomer of IL-10 of SEQ ID No: 31, 33, 35, 37, 39, or 41;

"W^2" is a beta alpha subunit of the first cytokine, such as IL-12 or IL-27 or a second monomer of the homodimeric cytokine, such as IL-10, preferably a second monomer of IL-10 of SEQ ID No: 31, 33, 35, 37, 39, or 41.

In one embodiment, the VH and VL is in the form of a scFv obtained from a human anti-ebola antibody. In another embodiment, the 6 CDRs (CDRs 1-3 from the VH and CDRs 1-3 from the VL) of the scFv obtained from the human anti-ebola antibody are replaced or engrafted with 6 CDR from a second monoclonal antibody that allows the dual cytokine fusion protein to be directed to a specific target, such as, but not limited to enzymes, receptors, extracellular proteins, or intracellular protein, such as those associated with a tumors (*e.g.*, tumor associated antigens (TAAs)), inflammatory response, or autoimmune diseases. The second antibody may include, but not limited to EGFR; CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD19; CD20; CD22; CD47;CD123; GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin; BCMA; PSA; PSMA; CEA; GPC3; SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-

I1; or SR-J1. In a more preferred embodiment, the dual cytokine fusion protein comprising two multi-subunit proteins is represented by Formula Va or Vb

NH_2-(P35)-(L_a)-(X^1)-(L_b)-($IL10_{monomer}$)-(L_c)-($IL10_{monomer}$)-(L_b)-(X^2)-(L_a)-(P40)-COOH (Formula Va);

NH_2-(P40)-(L_a)-(X^1)-(L_a)-($IL10_{monomer}$)-(L_c)-($IL10_{monomer}$)-(L_b)-(X^2)-(L_a)-(P35)-COOH (Formula Vb);

wherein

"p35" is an alpha subunit of IL-12 having a sequence of SEQ ID No; 1, 17, 19;

"p40" is a beta alpha subunit of IL-12 having a sequence of SEQ ID No; 3, 18, 20;

"L_a" is any linker; preferably $(GGGGS)_3$ of SEQ ID No: 46, $(GGGGS)_4$ of SEQ ID No: 45, or $(GGGGS)_5$ of SEQ ID No: 44;

"L_b" is any linker; preferably $(GGGGS)_3$ of SEQ ID No: 46, $(GGGGS)_4$ of SEQ ID No: 45, or $(GGGGS)_5$ of SEQ ID No: 44;

"L_c" is any linker; preferably GGGSGGG of SEQ ID No: 43 or $(GGGGS)_3$ of SEQ ID No: 46

X^1" is a VL or VH region obtained from a first monoclonal antibody; "X^2" is a VH or VL region obtained from the first monoclonal antibody; wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"$IL10_{monomer}$" is monomer of IL-10 having a sequence of SEQ ID No: 31, 33, 35, 37, 39, or 41, preferably SEQ ID No: 41.

the first monoclonal antibody being an anti-ebola antibody (US Published Application 2018/0180614, incorporated by reference in its entirety, especially mAbs described in Tables 2, 3, and 4), which may be engrafted with 6 CDRs from a second antibody having specificity for any one of EGFR; CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD19; CD20; CD22; CD47;CD123; GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin; BCMA; PSA; PSMA; CEA; GPC3; SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1.

Tables 2a-2d are different combinations of a dual cytokine fusion protein comprising IL-12 and IL-10 as represented by Formula IV.

Table 2a (Embodiments of $DK12^{10}$ Combination 1)

(R^1)	(X^1)	(W^1)	(W^2)	(X^2)	(R^2)
P40	VH EGFR	IL10 monomer[1]	IL10 monomer[1]	VL EGFR	P35
	VH HER2			VL HER2	
	VH HER3			VL HER3	
	VH VEGFR2			VL VEGFR2	
	VH VEGFR1			VL VEGFR1	
	VH PDGFR			VL PDGFR	
	VH EpCAM			VL EpCAM	
	VH CD14			VL CD14	
	VH CD52			VL CD52	
	VH PD-L1			VL PD-L1	
	VH PD-1			VL PD-1	
	VH TIM3			VL TIM3	
	VH BTLA			VL BTLA	
	VH LAG3			VL LAG3	
	VH CTLA4			VL CTLA4	
	VH CD19			VL CD19	
	VH CD20			VL CD20	
	VH CD22			VL CD22	
	VH CD47			VL CD47	
	VH CD123			VL CD123	
	VH GD-2			VL GD-2	
	VH ICAM1			VL ICAM1	
	VH ICAM2			VL ICAM2	
	VH ICAM3			VL ICAM3	
	VH ICAM4			VL ICAM4	
	VH VCAM			VL VCAM	
	VH FAPa			VL FAPa	
	VH 5T4			VL 5T4	
	VH Trop2			VL Trop2	
	VH EDB-FN			VL EDB-FN	
	VH TGFb			VL TGFb	
	VH Trap			VL Trap	
	VH MAdCAM			VL MAdCAM	
	VH b7 integrin			VL b7 integrin	
	VH a4b7			VL a4b7	
	VH a4			VL a4	
	VH of anyone of SR[2]			VL of anyone of SR[2]	
	VH BCMA			VL BCMA	
	VH PSA			VL PSA	
	VH PSMA			VL PSMA	
	VH CEA			VL CEA	

[1] R1, X1, X2, and R2 may be combined with an IL-10 monomer selected wild-type human IL10, EBV IL10, CMV IL10, mouse IL10, high affinity IL10 (known as DV07), low affinity IL10 (known as DV06) having SEQ ID Nos: 31, 33, 35, 37, 39, and 41, respectively. P40 and P35 having sequences of SEQ ID Nos: 18 and 17, respectively.

[2] SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1.

	VH GPC3			VL GPC3	

Table 2b (Embodiments of DK12[10] Combination 2)					
(R^1)	(X^1)	(Z^1)	(Z^2)	(X^2)	(R^2)
P40	VL EGFR	IL10 monomer[1]	IL10 monomer[1]	VH EGFR	P35
	VL HER2			VH HER2	
	VL HER3			VH HER3	
	VL VEGFR2			VH VEGFR2	
	VL VEGFR1			VH VEGFR1	
	VL PDGFR			VH PDGFR	
	VL EpCAM			VH EpCAM	
	VL CD14			VH CD14	
	VL CD52			VH CD52	
	VL PD-L1			VH PD-L1	
	VL PD-1			VH PD-1	
	VL TIM3			VH TIM3	
	VL BTLA			VH BTLA	
	VL LAG3			VH LAG3	
	VL CTLA4			VH CTLA4	
	VL CD19			VH CD19	
	VL CD20			VH CD20	
	VL CD22			VH CD22	
	VL CD47			VH CD47	
	VL CD123			VH CD123	
	VL GD-2			VH GD-2	
	VL ICAM1			VH ICAM1	
	VL ICAM2			VH ICAM2	
	VL ICAM3			VH ICAM3	
	VL ICAM4			VH ICAM4	
	VL VCAM			VH VCAM	
	VL FAPa			VH FAPa	
	VL 5T4			VH 5T4	
	VL Trop2			VH Trop2	
	VL EDB-FN			VH EDB-FN	
	VL TGFb			VH TGFb	
	VL Trap			VH Trap	
	VL MAdCAM			VH MAdCAM	
	VL b7 integrin			VH b7 integrin	
	VL a4b7			VH a4b7	
	VL a4			VH a4	
	VL of anyone of SR[2]			VH of anyone of SR[2]	
	VL BCMA			VH BCMA	
	VL PSA			VH PSA	
	VL PSMA			VH PSMA	
	VL CEA			VH CEA	
	VL GPC3			VH GPC3	

Table 2c (Embodiments of DK12[10] Combination 3)

(R^1)	(X^1)	(Z^1)	(Z^2)	(X^2)	(R^2)
P35	VH EGFR	IL10 monomer[1]	IL10 monomer[1]	VL EGFR	P40
	VH HER2			VL HER2	
	VH HER3			VL HER3	
	VH VEGFR2			VL VEGFR2	
	VH VEGFR1			VL VEGFR1	
	VH PDGFR			VL PDGFR	
	VH EpCAM			VL EpCAM	
	VH CD14			VL CD14	
	VH CD52			VL CD52	
	VH PD-L1			VL PD-L1	
	VH PD-1			VL PD-1	
	VH TIM3			VL TIM3	
	VH BTLA			VL BTLA	
	VH LAG3			VL LAG3	
	VH CTLA4			VL CTLA4	
	VH CD19			VL CD19	
	VH CD20			VL CD20	
	VH CD22			VL CD22	
	VH CD47			VL CD47	
	VH CD123			VL CD123	
	VH GD-2			VL GD-2	
	VH ICAM1			VL ICAM1	
	VH ICAM2			VL ICAM2	
	VH ICAM3			VL ICAM3	
	VH ICAM4			VL ICAM4	
	VH VCAM			VL VCAM	
	VH FAPa			VL FAPa	
	VH 5T4			VL 5T4	
	VH Trop2			VL Trop2	
	VH EDB-FN			VL EDB-FN	
	VH TGFb			VL TGFb	
	VH Trap			VL Trap	
	VH MAdCAM			VL MAdCAM	
	VH b7 integrin			VL b7 integrin	
	VH a4b7			VL a4b7	
	VH a4			VL a4	
	VH of anyone of SR^2			VL of anyone of SR^2	
	VH BCMA			VL BCMA	
	VH PSA			VL PSA	
	VH PSMA			VL PSMA	
	VH CEA			VL CEA	
	VH GPC3			VL GPC3	

Table 2d (Embodiments of $DK12^{10}$ Combination 4)					
(R^1)	(X^1)	(Z^1)	(Z^2)	(X^2)	(R^2)
P35	VL EGFR	IL10 monomer[1]	IL10 monomer[1]	VH EGFR	P40
	VL HER2			VH HER2	
	VL HER3			VH HER3	

	VL VEGFR2			VH VEGFR2	
	VL VEGFR1			VH VEGFR1	
	VL PDGFR			VH PDGFR	
	VL EpCAM			VH EpCAM	
	VL CD14			VH CD14	
	VL CD52			VH CD52	
	VL PD-L1			VH PD-L1	
	VL PD-1			VH PD-1	
	VL TIM3			VH TIM3	
	VL BTLA			VH BTLA	
	VL LAG3			VH LAG3	
	VL CTLA4			VH CTLA4	
	VL CD19			VH CD19	
	VL CD20			VH CD20	
	VL CD22			VH CD22	
	VL CD47			VH CD47	
	VL CD123			VH CD123	
	VL GD-2			VH GD-2	
	VL ICAM1			VH ICAM1	
	VL ICAM2			VH ICAM2	
	VL ICAM3			VH ICAM3	
	VL ICAM4			VH ICAM4	
	VL VCAM			VH VCAM	
	VL FAPa			VH FAPa	
	VL 5T4			VH 5T4	
	VL Trop2			VH Trop2	
	VL EDB-FN			VH EDB-FN	
	VL TGFb			VH TGFb	
	VL Trap			VH Trap	
	VL MAdCAM			VH MAdCAM	
	VL b7 integrin			VH b7 integrin	
	VL a4b7			VH a4b7	
	VL a4			VH a4	
	VL of anyone of SR[2]			VH of anyone of SR[2]	
	VL BCMA			VH BCMA	
	VL PSA			VH PSA	
	VL PSMA			VH PSMA	
	VL CEA			VH CEA	

The IL-12 subunits, P40 and P35, as noted in Tables 2a-2d above, may be selected from wild type, deglycosylated, or aglycosyated forms of IL-12. In addition, the IL-12 may be derived from human IL-12 or mouse IL-12 or any variant of IL-12 that retains, enhances, or decreases the function of IL-12, when compared to wild type IL-12. The IL-10 monomers listed in Table 2a-2d above, may be selected from human IL-10, EBV IL-10, CMV IL-10, high affinity variant forms of IL-10 (such as DV07, SEQ ID 41), or

low affinity variant forms of IL-10 (such as DV06, SEQ ID 39). Moreover, IL-10 monomers may be any variant of IL-10 that retains, enhances, or decreases the function of IL-10, when compared to wild type IL-10.

[0067] In another one embodiment, Tables 3a-3d are different combinations of a dual cytokine fusion protein comprising IL-27 and IL-10 as represented by Formula IV.

Table 3a (Embodiments of DK27[10] Combination 1)					
(R^1)	(X^1)	(Z^1)	(Z^2)	(X^2)	(R^2)
P28	VH EGFR	IL-10 Monomer[3]	IL-10 Monomer[3]	VL EGFR	EBI3
	VH HER2			VL HER2	
	VH HER3			VL HER3	
	VH VEGFR2			VL VEGFR2	
	VH VEGFR1			VL VEGFR1	
	VH PDGFR			VL PDGFR	
	VH EpCAM			VL EpCAM	
	VH CD14			VL CD14	
	VH CD52			VL CD52	
	VH PD-L1			VL PD-L1	
	VH PD-1			VL PD-1	
	VH TIM3			VL TIM3	
	VH BTLA			VL BTLA	
	VH LAG3			VL LAG3	
	VH CTLA4			VL CTLA4	
	VH CD19			VL CD19	
	VH CD20			VL CD20	
	VH CD22			VL CD22	
	VH CD47			VL CD47	
	VH CD123			VL CD123	
	VH GD-2			VL GD-2	
	VH ICAM1			VL ICAM1	
	VH ICAM2			VL ICAM2	
	VH ICAM3			VL ICAM3	
	VH ICAM4			VL ICAM4	
	VH VCAM			VL VCAM	
	VH FAPa			VL FAPa	
	VH 5T4			VL 5T4	
	VH Trop2			VL Trop2	
	VH EDB-FN			VL EDB-FN	
	VH TGFb			VL TGFb	
	VH Trap			VL Trap	
	VH MAdCAM			VL MAdCAM	
	VH b7 integrin			VL b7 integrin	
	VH a4b7			VL a4b7	
	VH a4			VL a4	
	VH of anyone of SR[2]			VL of anyone of SR[2]	
	VH BCMA			VL BCMA	
	VH PSA			VL PSA	

[3] R1, X1, X2, and R2 may be combined with an IL-10 monomer selected wild-type human IL10, EBV IL10, CMV IL10, mouse IL10, high affinity IL10 (known as DV07), low affinity IL10 (known as DV06) having SEQ ID Nos: 31, 33, 35, 37, 39, and 41, respectively. P40 and P35 having sequences of SEQ ID Nos: 18 and 17, respectively

	VH PSMA			VL PSMA	
	VH CEA			VL CEA	
	VH GPC3			VL GPC3	

Table 3b (Embodiments of DK27[10] Combination 2)					
(R^1)	(X^1)	(Z^1)	(Z^2)	(X^2)	(R^2)
P28	VL EGFR	IL-10 Monomer[3]	IL-10 Monomer[3]	VH EGFR	EBI3
	VL HER2			VH HER2	
	VL HER3			VH HER3	
	VL VEGFR2			VH VEGFR2	
	VL VEGFR1			VH VEGFR1	
	VL PDGFR			VH PDGFR	
	VL EpCAM			VH EpCAM	
	VL CD14			VH CD14	
	VL CD52			VH CD52	
	VL PD-L1			VH PD-L1	
	VL PD-1			VH PD-1	
	VL TIM3			VH TIM3	
	VL BTLA			VH BTLA	
	VL LAG3			VH LAG3	
	VL CTLA4			VH CTLA4	
	VL CD19			VH CD19	
	VL CD20			VH CD20	
	VL CD22			VH CD22	
	VL CD47			VH CD47	
	VL CD123			VH CD123	
	VL GD-2			VH GD-2	
	VL ICAM1			VH ICAM1	
	VL ICAM2			VH ICAM2	
	VL ICAM3			VH ICAM3	
	VL ICAM4			VH ICAM4	
	VL VCAM			VH VCAM	
	VL FAPa			VH FAPa	
	VL 5T4			VH 5T4	
	VL Trop2			VH Trop2	
	VL EDB-FN			VH EDB-FN	
	VL TGFb			VH TGFb	
	VL Trap			VH Trap	
	VL MAdCAM			VH MAdCAM	
	VL b7 integrin			VH b7 integrin	
	VL a4b7			VH a4b7	
	VL a4			VH a4	
	VL of anyone of SR[2]			VH of anyone of SR[2]	
	VL BCMA			VH BCMA	
	VL PSA			VH PSA	
	VL PSMA			VH PSMA	
	VL CEA			VH CEA	
	VL GPC3			VH GPC3	

Table 3d (Embodiments of DK27[10] Combination 4)

Table 3c (Embodiments of DK27[10] Combination 3)

(R^1)	(X^1)	(Z^1)	(Z^2)	(X^2)	(R^2)
	VL EGFR			VH EGFR	
	VL HER2			VH HER2	
	VL HER3			VH HER3	
	VL VEGFR2			VH VEGFR2	
	VL VEGFR1			VH VEGFR1	
	VL PDGFR			VH PDGFR	
	VL EpCAM			VH EpCAM	
	VL CD14			VH CD14	
	VL CD52			VH CD52	
	VL PD-L1			VH PD-L1	
	VL PD-1			VH PD-1	
	VL TIM3			VH TIM3	
	VL BTLA			VH BTLA	
	VL LAG3			VH LAG3	
	VL CTLA4			VH CTLA4	
	VL CD19			VH CD19	
	VL CD20			VH CD20	
	VL CD22			VH CD22	
	VL CD47			VH CD47	
	VL CD123			VH CD123	
	VL CD2			VH CD2	
	VL ICAM1			VH ICAM1	
	VL ICAM2			VH ICAM2	
EBI3	VL ICAM3	IL-10 Monomer[3]	IL-10 Monomer[3]	VH ICAM3	P28
	VL ICAM4			VH ICAM4	
	VL VCAM			VH VCAM	
	VL FAPa			VH FAPa	
	VL 5T4			VH 5T4	
	VL Trop2			VH Trop2	
	VL EDB-FN			VH EDB-FN	
	VL TGFb			VH TGFb	
	VL Trap			VH Trap	
	VL MAdCAM			VH MAdCAM	
	VL b7 integrin			VH b7 integrin	
	VL a4b7			VH a4b7	
	VL a4			VH a4	
	VL of anyone of SR[2]			VH of anyone of SR[2]	
	VL BCMA			VH BCMA	
	VL PSA			VH PSA	
	VL PSMA			VH PSMA	
	VL CEA			VH CEA	
	VL GPC3			VH GPC3	

[0068] The IL-27 subunits, P28 and EBI3, as noted in Tables 3a-3d above, may be selected from wild type, deglycosylated, or aglycosyated forms of IL-27. In addition, the IL-27 may be derived from human IL-27 or mouse IL-27 or any variant of IL-27 that retains, enhances, or decreases the function of IL-27, when compared to wild type IL-27. The IL-10 monomers listed in Table 2a-2d above, may be selected from human IL-10, EBV IL-10, CMV IL-10, high affinity variant forms of IL-10 (such as DV07, SEQ ID 41), or low affinity variant forms of IL-10 (such as DV06, SEQ ID 39). Moreover, IL-10 monomers may be any variant of IL-10 that retains, enhances, or decreases the function of IL-10, when compared to wild type IL-10.

[0069] In another embodiment, the target specificity of the antibody variable chains or VH and VL pair or the 6 CDRs of the VH and VL pair may include, but not limited to those targeting proteins, cellular receptors, and/or tumor associated antigens. In another embodiment, the CDR regions from any VH and VL pair may be engrafted into the dual cytokine scaffolding system described above (schematically represented by FIG. 1-5). In yet another embodiment, the variable regions or VH and VL pair or the 6 CDRs of the VH and VL pair are obtained from antibodies that target antigens associated with various diseases (*e.g.*, cancer) or those that are not typically found or rarely found in the serum of a healthy subject, for example variable regions from antibodies directed to EGFR, PDGFR, VEGFR1, VEGFR2, Her2Neu, FGFR, GPC3, or other tumor associated antigens, MAdCAM, ICAM, VCAM, CD14 or other inflammation associated cell surface proteins, HIV and/or Ebola. Thus, in one embodiment, the variable regions are obtained or derived from anti-EGFR, anti-MAdCAM, anti-HIV (Chan et al, J. Virol., 2018, 92(18):e006411-19), anti-ICAM, anti-VCAM, anti-CD14, or anti-Ebola (US Published Application 2018/0180614, incorporated by reference in its entirety, especially mAbs described in Tables 2, 3, and 4) antibodies, for example. In another embodiment, the variable regions are obtained or derived from antibodies capable of enriching the concentration of cytokines, such as IL-12 or IL-27 in combination with IFN-alpha, IL-28, or IL-29, to a specific target area so as to enable IL-12 and IL-27 to elicit its biological effect more effectively. Such an antibody might include those that target overexpressed or upregulated receptors or antigens in certain diseased regions or those that are specifically expressed in certain impacted areas. For example, the variable regions or CDRs might be obtained from antibodies specific for EGFR; CD14; CD52; various immune check point targets,

such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD19; CD20; CD22; CD47;CD123; GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin; BCMA; PSA; PSMA; CEA; GPC3; SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1 to name a few.

[0070] The dual cytokine fusion protein or dual cytokine fusion protein complex may also have an antigen targeting functionality. The dual cytokine fusion protein or dual cytokine fusion protein complex will comprise a VH and VL pair that is able to associate together to form an antigen binding site or ABS. The variable regions may be further modified (*e.g.*, by addition, subtraction, or substitution) by altering one or more amino acids that reduce antigenicity in a subject. Other modifications to the variable region may include amino acids substitutions, deletions, or additions that are found outside of the 6 CDR regions of the VH and VL regions and serve to increase stability and expression of the VH and VL regions of the scFv. A person of skill in the art would be capable of determining other modifications that stabilize the scFv and/or to optimize the sequence for purposes of expression.

[0071] The VH and VL pair form a scaffolding onto which CDR regions obtained for a plurality of antibodies may be substituted or engrafted. Such antibody CDR regions include those antibodies known and described above. The CDR regions in the above described VH and VL scaffolding will include the following number of amino acid positions available for CDR engraftment/insertion:

Heavy chain CDR1	3-7 amino acids
Heavy chain CDR2	7-11 amino acids
Heavy chain CDR3	7-11 amino acids
Light chain CDR1	9-14 amino acids
Light chain CDR2	5-9 amino acids
Light chain CDR3	7-11 amino acids

In a preferred embodiment, the dual cytokine fusion protein comprising a first multi-subunit cytokine (*e.g.*, IL-12 or IL-27) will include a VH and VL pair is derived from a human anti-ebola antibody (US Published Application 2018/0180614, incorporated by reference in its entirety, especially mAbs described in Tables 2, 3, and 4) whereby the 6 CDR regions from the human anti-ebola antibody are removed and engrafted with a VH and VL pair of a specific targeting antibody, such as but not limited to antibodies that target EGFR; CD14; CD52; various immune check point targets, such as but not

limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD19; CD20; CD22; CD47;CD123; GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin; BCMA; PSA; PSMA; CEA; GPC3; SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1. In an embodiment, the 6 human anti-ebola CDR regions are substituted with 6 CDR regions from anti-EGFR, anti-MAdCAM, anti-VEGFR1, anti-VEGFR2, anti-PDGFR, or anti-CD14. In a preferred embodiment, a second cytokine, such as but not limited to IL-28, IL-29, IFNα, is linked in the hinge region between the VH and VL of the scFv obtained from a human anti-ebola antibody. The aforementioned engraftment strategy may also be applied to the dual cytokine fusion protein comprising two multi-subunit cytokines as represented by Formula IV and Va and Vb recited above.

[0072] In yet another embodiment, the second cytokine, is fused between the VH and VL of a scFv, as depicted in FIG 1-5. The second cytokine is conjugated between the VH or VL region such that the second cytokine retains its functional properties. In one embodiment, the second cytokine is different from the first multi-subunit cytokine (*e.g.*, IL-12 or IL-27). In one embodiment, the second cytokine is IL-6, IL-4, IL-1, IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, IL-15, IL-21, IL-26, IL-27, IL-28, IL-29, GM-CSF, G-CSF, interferons-α, -β, -γ, TGF-β, or tumor necrosis factors -α, -β, basic FGF, EGF, PDGF, IL-4, IL-11, or IL-13. In a preferred embodiment, the second cytokine in the dual cytokine fusion protein comprising IL-28, IL-29 or IFN-alpha.

[0073] In some embodiments, the second cytokine may be another multi-subunit cytokine, such as IL-10. In this case, the second cytokine will also be fused between the VH and VL of the scaffolding system (see Fig. 7 and 8 for representative structures). Formulae IV, Va and Vb, described above, serve to describe these types of fusion proteins.

In still other embodiments, the dual cytokine fusion protein comprising a first multi-subunit cytokine (*e.g.*, IL-12 or IL-27) incorporates linkers. A person of skill in the art knows that linkers or spacers are used to achieve proper spatial configuration of the various fusion protein parts and therefore may select the appropriate linker to use in the formation of the dual cytokine fusion protein comprising the first multi-subunit cytokine (*e.g.*, IL-12 or IL-27). In a more preferred embodiment, the linker or spacer

may be a random amino acid sequence SEQ ID Nos.: 43, 44, 45, 46, 47, and 48. Any of the above combinations in Tables 2a-2d or 3a-3d may be combined with linker ("L_a") and "(L_b") selected from $(GGGGS)_3$, $(GGGGS)_4$, or $(GGGGS)_5$ having SEQ ID Nos: 46, 45, and 47 respectively, and a linker ("L_c") of GGGSGGG or $(GGGGS)_3$ corresponding to SEQ ID Nos: 43 and 46, respectively. In one preferred embodiment, Formula IV may include a combination of linkers as follows in Table 4.

Table 4: Possible Linker Combinations in Formula IV

IL-12 p40	L_a	VH	L_b	IL-10 monomer 1	L_c	IL-10 monomer 2	L_b	VL	L_a	IL-12 p35
X	GGGGS x 5	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 5	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 5	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 4	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 4	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 3	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 3	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 3	X

X	GGGGS x 5	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGSGGG	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGSGGG	X	GGGGS x 4	X	GGGGS x 5	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGSGGG	X	GGGGS x 3	X	GGGGS x 5	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGS x 3	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGS x 3	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGS x 3	X	GGGGS x 5	X	GGGGS x 5	X

X	GGGGS x 5	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 4	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 5	X

X	GGGGS x 4	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 4	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X
X	GGGGS x 5	X	GGGGS x 5	X	GGGGS x 3	X	GGGGS x 5	X	GGGGS x 5	X
X	GGGGS x 4	X	GGGGS x 4	X	GGGGS x 3	X	GGGGS x 4	X	GGGGS x 5	X
X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 3	X	GGGGS x 5	X
$(GGGGS)_3$ = SEQ ID No: 46, $(GGGGS)_4$ = SEQ ID No: 45, $(GGGGS)_5$ = SEQ ID No: 44										

In yet another preferred embodiment, Formula IV may include a combination of preferred linkers as follows in Table 4

Table 5: Preferred Formula IV Linker Combinations			
Combination	**"L_a"**	**"L_b"**	**"L_c"**
1	$(GGGGS)_3$	$(GGGGS)_3$	GGGSGGG
2	$(GGGGS)_3$	$(GGGGS)_3$	$(GGGGS)_3$
3	$(GGGGS)_3$	$(GGGGS)_5$	GGGSGGG
4	$(GGGGS)_4$	$(GGGGS)_3$	GGGSGGG
5	$(GGGGS)_4$	$(GGGGS)_4$	GGGSGGG
6	$(GGGGS)_4$	$(GGGGS)_5$	GGGSGGG
7	$(GGGGS)_4$	$(GGGGS)_3$	$(GGGGS)_3$
8	$(GGGGS)_4$	$(GGGGS)_4$	$(GGGGS)_3$
9	$(GGGGS)_4$	$(GGGGS)_5$	$(GGGGS)_3$
10	$(GGGGS)_5$	$(GGGGS)_3$	GGGSGGG
11	$(GGGGS)_5$	$(GGGGS)_4$	GGGSGGG
12	$(GGGGS)_5$	$(GGGGS)_5$	GGGSGGG
13	$(GGGGS)_5$	$(GGGGS)_3$	$(GGGGS)_3$
14	$(GGGGS)_5$	$(GGGGS)_4$	$(GGGGS)_3$
15	$(GGGGS)_5$	$(GGGGS)_5$	$(GGGGS)_3$
(GGGGS)3 = SEQ ID No: 46 (GGGGS)4 = SEQ ID No: 45 (GGGGS)5 = = SEQ ID No: 44			

[0074] Preferred dual cytokine fusion proteins comprising two multi-subunit cytokines include those recited in SEQ ID Nos: 21-30.

[0075] In other aspects, the present disclosure relates to nucleic acid molecules that encode for the dual cytokine fusion protein comprising a first multi-subunit cytokine (*e.g.*, IL-12 or IL-27) and a second cytokine. These would include those nucleic acid sequence that encode a dual cytokine fusion protein represented by formulas Ia, Ib, IIa, IIb, IIIa, IIIb, VI, Va, or Vb. One embodiment therefore includes a nucleic acid sequence that encodes a protein that shares 70% to 99% sequence homology thereof. The polynucleotide sequences that encode for the dual cytokine fusion protein comprising a first multi-subunit cytokine (*e.g.*, IL-12 or IL-27) and a second cytokine (e.g, IL-10, IFN-alpha, IL-28, IL-29) may also include modifications that do not alter the functional properties of the described dual cytokine fusion protein. Such modifications will employ conventional recombinant DNA techniques and methods. For example, the addition or substitution of specific amino acid sequences may be introduced into a first multi-subunit cytokine (*e.g.*, IL-12 or IL-27) sequence at the nucleic acid (DNA) level using site-directed mutagenesis methods employing synthetic oligonucleotides, which methods are also well known in the art. In a preferred embodiment, the nucleic acid molecules encoding the dual cytokine fusion protein may include insertions, deletions, or substitutions (*e.g.*, degenerate code) that do not alter the functionality of the first multi-subunit cytokine (*e.g.*, IL-12 or IL-27), the second cytokine, or the VH or VL regions of the scFv. The nucleotide sequences encoding the dual cytokine fusion proteins described herein may differ from the amino acid sequences due to the degeneracy of the genetic code and may be 70-99%, preferably 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%, homologous to the aforementioned sequences.

[0076] The nucleotide sequences encoding the dual cytokine fusion proteins described herein may further comprise well known sequences that aid in, for example, the expression, production, or secretion of the proteins. Such sequences may include, for example a leader sequence, signal peptide, and/or translation initiation sites/sequence (*e.g.* Kozak consensus sequence). The nucleotide sequences described herein may also include one of more restriction enzyme sites that allow for insertion into various expression systems/vectors.

[0077] In another embodiment, the nucleotide sequences encoding the dual cytokine fusion protein may be used directly in gene therapy. In one embodiment, the dual cytokine fusion protein of the present application can be delivered by any method know in the art, including direct administration of the gene in a vector encoding the dual cytokine fusion protein. Gene therapy may be accomplished using plasmid DNA or a viral vector, such as an adeno-associated virus vector, an adenovirus vector, a retroviral vector, etc. In some embodiments, the viral vectors of the application are administered as virus particles, and in others they are administered as plasmids (*e.g.* as “naked” DNA).

[0078] Other methods for the delivery of the nucleotide sequences include those which are already known in the art. These would include the delivery of the nucleotide sequences, such as but not limited to DNA, RNA, siRNA, mRNA, oligonucleotides, or variants thereof, encoding the dual cytokine fusion protein by a cell penetrating peptide, a hydrophobic moiety, an electrostatic complex, a liposome, a ligand, a liposomal nanoparticle, a lipoprotein (preferably HDL or LDL), a folate targeted liposome, an antibody (such as Folate receptor, transferrin receptor), a targeting peptide, or by an aptamer. The nucleotide sequences encoding dual cytokine fusion protein may be delivered to a subject by direct injection, infusion, patches, bandages, mist or aerosol, or by thin film delivery. The nucleotide (or the protein) may be directed to any region that is desired for targeted delivery of a cytokine stimulus. These would include, for example, the lung, the GI tract, the skin, liver, brain though intracranial injection, deep seated metastatic tumor lesions via ultrasound guided injections.

[0079] In another aspect, the present disclosure relates to methods of preparing and purifying the dual cytokine fusion protein. For example, nucleic acid sequences that encode the dual cytokine fusion protein described herein may be used to recombinantly produce the fusion proteins. For example, using conventional molecular biology and protein expression techniques, the dual cytokine fusion protein described herein may be expressed and purified from mammalian cell systems. These systems include well known eukaryotic cell expression vector systems and host cells. A variety of suitable expression vectors may be used and are well known to a person skilled in the art, which can be used for expression and introduction of the dual cytokine fusion proteins. These vectors include, for example, pUC-type vectors, pBR-type

vectors, pBI-type vectors, pGA-type, pBinl9, pBI121, pGreen series, pCAMBRIA series, pPZP series, pPCV001, pGA482, pCLD04541, pBIBAC series, pYLTAC series, pSB11, pSB1, pGPTV series, and viral vectors and the like can be used. Well known host cell systems include but not limited to expression in CHO cells.

[0080] The expression vectors harboring the dual cytokine fusion protein may also include other vector componentry required for vector functionality. For example, the vector may include signal sequences, tag sequences, protease identification sequences, selection markers and other sequences regulatory sequences, such as promoters, required for proper replication and expression of the dual cytokine fusion protein. The particular promoters utilized in the vector are not particularly limited as long as they can drive the expression of the dual cytokine fusion protein in a variety of host cell types. Likewise, the type of Tag promoters are not be limited as long as the Tag sequence makes for simpler or easier purification of expressed dual cytokine fusion protein easier. These might include, for example, 6-histidine, GST, MBP, HAT, HN, S, TF, Trx, Nus, biotin, FLAG, myc, RCFP, GFP and the like can be used. Protease recognition sequences are not particularly limited, for instance, recognition sequences such as Factor Xa, Thrombin, HRV, 3C protease can be used. Selected markers are not particularly limited as long as these can detect transformed rice plant cells, for example, neomycin-resistant genes, kanamycin-resistant genes, hygromycin-resistant genes and the like can be used.

[0081] The dual cytokine fusion protein described above may also include additional amino acid sequences that aid in the recovery or purification of the fusion proteins during the manufacturing process. These may include various sequence modifications or affinity tags, such as but not limited to protein A, albumin-binding protein, alkaline phosphatase, FLAG epitope, galactose-binding protein, histidine tags, and any other tags that are well known in the art. *See, e.g.*, Kimple et al (Curr. Protoc. Protein Sci., 2013, 73:Unit 9.9, Table 9.91, incorporated by reference in its entirety). In one aspect, the affinity tag is an histidine tag having an amino acid sequence of HHHHHH (SEQ ID No.: 42). The histidine tag may be removed or left intact from the final product. In another embodiment, the affinity tag is a protein A modification that is incorporated into the fusion protein (*e.g.*, into the VH region of the fusion proteins described herein). A person of skill in the art will understand that any dual cytokine fusion protein sequence described herein can be modified to incorporate a protein A

modification by inserting amino acid point substitutions within the antibody framework regions as described in the art.

[0082] In another aspect, the protein and nucleic acid molecules encoding dual cytokine fusion protein may be formulated as a pharmaceutical composition comprising a therapeutically effective amount of the dual cytokine fusion protein and a pharmaceutical carrier and/or pharmaceutically acceptable excipients. The pharmaceutical composition may be formulated with commonly used buffers, excipients, preservatives, stabilizers. The pharmaceutical compositions comprising the dual cytokine fusion protein is mixed with a pharmaceutically acceptable carrier or excipient. Various pharmaceutical carriers are known in the art and may be used in the pharmaceutical composition. For example, the carrier can be any compatible, non-toxic substance suitable for delivering the dual cytokine fusion protein compositions of the application to a patient. Examples of suitable carriers include normal saline, Ringer's solution, dextrose solution, and Hank's solution. Carriers may also include any poloxamers generally known to those of skill in the art, including, but not limited to, those having molecular weights of 2900 (L64), 3400 (P65), 4200 (P84), 4600 (P85), 11,400 (F88), 4950 (P103), 5900 (P104), 6500 (P105), 14,600 (F108), 5750 (P123), and 12,600 (F127). Carriers may also include emulsifiers, including, but not limited to, polysorbate 20, polysorbate 40, polysorbate 60, and polysorbate 80, to name a few. Non-aqueous carriers such as fixed oils and ethyl oleate may also be used. The carrier may also include additives such as substances that enhance isotonicity and chemical stability, *e.g.*, buffers and preservatives, see, *e.g.*, Remington's Pharmaceutical Sciences and U.S. Pharmacopeia: National Formulary, Mack Publishing Company, Easton, Pa. (1984). Formulations of therapeutic and diagnostic agents may be prepared by mixing with physiologically acceptable carriers, excipients, or stabilizers in the form of lyophilized powders, slurries, aqueous solutions or suspensions, for example.

[0083] The pharmaceutical composition will be formulated for administration to a patient in a therapeutically effective amount sufficient to provide the desired therapeutic result. Preferably, such amount has minimal negative side effects. In one embodiment, the amount of dual cytokine fusion protein administered will be sufficient to treat or prevent inflammatory diseases or condition. In another embodiment, the amount of dual cytokine fusion protein administered will be sufficient to treat or prevent

immune diseases or disorders. In still another embodiment, the amount of dual cytokine fusion protein administered will be sufficient to treat or prevent cancer. The amount administered may vary from patient to patient and will need to be determined by considering the subject's or patient's disease or condition, the overall health of the patient, method of administration, the severity of side-effects, and the like.

[0084] An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the method route and dose of administration and the severity of side effects. The appropriate dose administered to a patient is typically determined by a clinician using parameters or factors known or suspected in the art to affect treatment or predicted to affect treatment. Generally, the dose begins with an amount somewhat less than the optimum dose and it is increased by small increments thereafter until the desired or optimum effect is achieved relative to any negative side effects. Important diagnostic measures include those of symptoms of, *e.g.*, the inflammation or level of inflammatory cytokines produced.

[0085] The method for determining the dosing of the presently described dual cytokine fusion protein will be substantially similar to that described in U.S. Patent 10,858,412. Generally, the presently described dual cytokine fusion protein will have a dosing in the range of 0.5 microgram/kilogram to 100 micrograms/kilogram. The dual cytokine fusion protein may be administered daily, three times a week, twice a week, weekly, bimonthly, or monthly. An effective amount of therapeutic will impact the level of inflammation or disease or condition by relieving the symptom. For example, the impact might include a level of impact that is at least 10%; at least 20%; at least about 30%; at least 40%; at least 50%; or more such that the disease or condition is alleviated or fully treated.

[0086] Compositions of the application can be administered orally or injected into the body. Formulations for oral use can also include compounds to further protect the dual cytokine fusion protein from proteases in the gastrointestinal tract. Injections are usually intramuscular, subcutaneous, intradermal or intravenous. Alternatively, intra-articular injection or other routes could be used in appropriate circumstances. Parenterally administered dual cytokine fusion protein are preferably formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutical carrier and/or pharmaceutically acceptable excipients. In other

embodiments, compositions of the application may be introduced into a patient's body by implantable or injectable drug delivery system.

Testing the Dual Cytokine Fusion Protein

[0087] A plurality of screening assays are known and available to those of skill in the art to test for the desired biological function. In one embodiment, the desired biological function includes, but are not limited to, reduced anti-inflammatory response, reduce T-cell stimulation, enhanced T-cell function, enhanced Kupffer cell functionality and reduced mast cell degranulation.

[0088] For example, it is known that IL-10 exposure primes T cells to generate and secrete more IFNγ upon T cell receptor stimulation. Simultaneously, IL-10 exposure prevents the secretion of TNFα, IL-6 and other pro-inflammatory cytokines secreted from monocytes/macrophages in response to LPS. IL-10 also suppresses $FoxP3^{+}CD4^{+}$ T_{reg} proliferation. In one embodiment, the dual cytokine fusion protein that maximize monocyte/macrophage suppression but lack T cell effects, including both stimulatory and suppressive responses, will be positively selected. In one embodiment, screening for dual cytokine fusion proteins that possess increased anti-inflammatory effects will be positively selected for the treatment of autoimmune, anti-inflammatory disease or both. In another embodiments, dual cytokine fusion proteins that enhance Kupffer cell scavenging and lack T_{reg} suppression will also be selected to develop for treatment of Non-alcoholic Steatotic Hepatitis (NASH) and/or Non-alcoholic Fatty Liver Disease (NAFLD). In yet another embodiment, dual cytokine fusion proteins that maximize T cell biology, including both stimulatory and suppressive responses, and also possesses enhanced Kupffer cell scavenging, will be selected to develop for the treatment of cancer. Various assays and methods of screening the dual cytokine fusion proteins are previously described in co-pending U.S. Patent 10,858,412, which is incorporated by reference in its entirety. *See,* U.S. Application 16/811, 718 Specification at pages 39-42.

Methods of Treating and/or Preventing Using the Dual Cytokine

[0089] In other aspects, the present disclosure relates to methods of treating and/or preventing malignant diseases or conditions or cancer comprising administering to a subject in need thereof a therapeutically effective amount of the dual cytokine fusion protein comprising a first multi-subunit cytokine (*e.g.*, IL-12 or IL-

27) and a second cytokine (such as IFNalpha, IL28, IL29, and IL10). In a preferred embodiment, the dual cytokine fusion protein comprises IL-12 or IL-27 and IL-10 or variants thereof, IFN-alpha, IL28, or IL-29 and variants thereof as the second cytokine. In other embodiments, the 6 CDR regions of the human anti-ebola scFv are substituted with 6 CDRs from an anti-Her2/Neu; an anti-PDGFR; anti-VEGFR1 and anti-VEGFR2, an anti-FGFR; an anti-CD19, an anti-CD20, an anti-CD22, an anti-BCMA, an anti-PSA, an anti-PSMA, an anti-HER3; an anti-EGFR, an anti-CEA, or an anti-GPC3. Preferably the 6 CDRs are obtained from anti-EGFR, or anti-HER2. In another preferred embodiment, the second cytokine is an IL-28, IL-29, IL-10, or IFN-alpha, wherein the IL-10 is either DV07 or DV06.

[0090] In still other aspects, the present disclosure relates to methods of treating and/or preventing inflammatory diseases or conditions comprising administering to a subject in need thereof a therapeutically effective amount of the dual cytokine fusion protein. In a preferred embodiment, the inflammatory diseases or disorders include, but are not limited to Crohn's disease, psoriasis, and rheumatoid arthritis ("RA").

[0091] In yet another aspect, the present disclosure relates to methods of treating and/or preventing immune diseases or conditions comprising administering to a subject in need thereof a therapeutically effective amount of the dual cytokine fusion protein.

[0092] In other embodiments, the present disclosure also contemplates methods of co-administration or treatment with a second therapeutic agent, *e.g.*, a cytokine, steroid, chemotherapeutic agent, antibiotic, anti-inflammatory agents, or radiation, are well known in the art. These might include combination treatments with other therapeutic agents, such as but not limited to one or more the following: chemotherapeutics, interferon-β, for example, IFNβ-1α and IFN-β-1 β; a protein that simulates myelin basic protein; corticosteroids; IL-1 inhibitors; TNF inhibitors; anti-TNFα antibodies, anti-IL-6 antibodies, IL-1br-Ig fusion, anti-IL-23 antibodies, antibodies to CD40 ligand and CD80; antagonists of IL-12 and IL-23, *e.g.*, antagonists of a p40 subunit of IL-12 and IL-23 (*e.g.*, inhibitory antibodies against the p40 subunit); IL-22 antagonists; small molecule inhibitors, *e.g.*, methotrexate, leflunomide, sirolimus (rapamycin) and analogs thereof, *e.g.*, CCI-779; Cox-2 and cPLA2 inhibitors; NSAIDs; p38 inhibitors; TPL-2; Mk-2; NFkβ inhibitors; RAGE or soluble RAGE; P-selectin or

PSGL-1 inhibitors (*e.g.*, small molecule inhibitors, antibodies thereto, *e.g.*, antibodies to P-selectin); estrogen receptor beta (ERB) agonists or ERB-NFkβ antagonists.

[0093] Additionally, the combination treatment useful for administration with the dual cytokine fusion protein may include TNF inhibitors include, *e.g.*, chimeric, humanized, effectively human, human or in vitro generated antibodies, or antigen-binding fragments thereof, that bind to TNF; soluble fragments of a TNF receptor, *e.g.*, p55 or p75 human TNF receptor or derivatives thereof, *e.g.*, 75 kdTNFR-IgG (75 kD TNF receptor-IgG fusion protein, ENBREL™), p55 kD TNF receptor-IgG fusion protein; and TNF enzyme antagonists, *e.g.*, TNFα converting enzyme (TACE) inhibitors. Other combination treatment with anti-inflammatory agents/drugs that includes, but not limited to standard non-steroidal anti-inflammatory drugs (NSAIDs) and cyclo-oxygenase-2 inhibitors. NSAID may include aspirin, celecoxib, diclofenac, diflunisal, etodolac, ibuprofen, indomethacin, ketoprofen, ketorolac, nabumetone, naproxen, oxaprozin, piroxicam, salsalate, sulindac, and/or tolmetin. The cyclo-oxygenase-2 inhibitor employed in compositions according to the application could, for example, be celecoxib or rofecoxib.

[0094] Additional therapeutic agents that can be co-administered and/or co-formulated with the dual cytokine fusion protein include one or more of: interferon-β, for example, IFN β-1α and IFN β-1β; COPAXONE®; corticosteroids; IL-1 inhibitors; TNF antagonists (*e.g.*, a soluble fragment of a TNF receptor, *e.g.*, p55 or p75 human TNF receptor or derivatives thereof, *e.g.*, 75 kdTNFR-IgG; antibodies to CD40 ligand and CD80; and antagonists of IL-12 and/or IL-23, *e.g.*, antagonists of a p40 subunit of IL-12 and IL-23 (*e.g.*, inhibitory antibodies that bind to the p40 subunit of IL-12 and IL-23); methotrexate, leflunomide, and a sirolimus (rapamycin) or an analog thereof, *e.g.*, CCI-779. Other therapeutic agents may include Imfimzi or Atezolizumb.

[0095] For purposes of treating NASH, for example, the dual cytokine fusion protein may be combined with cholesterol lowering agents, such as statins and non-statin drugs. These agents include, but are not limited to simvastatin, atorvastatin, rosuvastatin, lovastatin, pravastatin, gemfibrozil, fluvastatin, cholestyramine, fenofibrate, cholesterol absorption inhibitors, bile acid-binding resins or sequestrants, and/or microsomal triglyceride transfer protein (MTP) inhibitors.

[0096] Representative chemotherapeutic agents that may be co-administered with the dual cytokine fusion protein described herein may include for following non-

exhaustive list: include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamime nitrogen mustards such as chiorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2′,2″-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside (Ara-C); cyclophosphamide; thiotepa; taxoids, *e.g.* paclitaxel (TAXOL® Bristol-Myers Squibb Oncology, Princeton, N.J.) and doxetaxel (Taxotere™, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as

cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; Xeloda® Roche, Switzerland; ibandronate; CPT11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and antiandrogens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

EXAMPLES

Example 1: IL-12 and IL-IFN-alpha Dual Cytokine Fusion Protein *in vitro* Study

[0097] To evaluate the *in vitro* effects of targeting two cytokines to a tumor, a dual cytokine fusion protein comprising IL-12 and IFN-alpha (*see* FIG. 2 as a representative diagram of the structure, termed "DKα^{12}"), are constructed from the following components:

(a) p35 and p40 subunits are terminally fused to a human anti-ebola scFv engrafted with 6 CDRs from any one of anti-EGFR, anti-HER2, anti-VEGFR1, anti-VEGFR1, or anti-CD14 antibody; and

(b) an IFN-alpha (SEQ ID No: 9);

where the IFN-alpha is conjugated or linked in the hinge (or linker) region between the VH and VL of the human anti-ebola scFv engrafted with the 6 CDRs from anti-EGFR, anti-HER2, anti-VEGFR1, anti-VEGFR1, or anti-CD14 antibody.

[0098] DK$\alpha^{12}$is generated to evaluate the combined effects of these two cytokines—IL-12 and IFN-alpha—on induction of IFNγ from NK, CD4$^+$ and CD8$^+$ T cells.

[0099] Peripheral blood monocytes, NK, CD4$^+$ and CD8$^+$ T cells were isolated by magnetic bead positive selection to evaluate the function of DKα^{12} , and then evaluated in *in vitro* testing. A series of cellular *in vitro* assays were used to mimic or model immunological function at different time points in the exposure cycle of a

molecule injected subcutaneously in the human body. These assays are described in U.S. Patent 10,858,412 and in U.S. Application 17/110,104.

[00100] First, the effects of IL-12 alone, IFN-alpha alone, the combined effects of IL-12 and IFN-alpha, and IL-12 incorporated into the single cytokine scFv scaffolding system (*see, e.g.*, FIG. 6, where p35 and p40 subunits substitute for the IL-10 monomers on the terminal ends of the scaffolding system) are compared to the effects of the DKα^{12}on monocytes/macrophages. IFN-alpha alone and when incorporated into DKα^{12} inhibit LPS mediated induction of IL-10 but induce the release of TRAIL. Treatment with IFN-alpha increases cell surface expression of MHC I and CD80. IL-12 exerts no effect on monocytes.

[00101] Second, the effects of IL-12 alone, IFN-alpha alone, the combined effects of IL-12 and IFN-alpha, and IL-12 incorporated into the single cytokine scFv scaffolding system (*see, e.g.*, FIG. 6, where p35 and p40 subunits substitute for the IL-10 monomers on the terminal ends of the scaffolding system) are compared to the effects of DKα^{12} on T cells. The T-cell assay has been used to directly elucidate the primary function of IL-10 on CD8^{+} T cells, predominantly the potentiation of IFNγ that is only released upon T cell receptor engagement (Chan, 2015; Mumm J. , 2011; Emmerich, 2012). This same assay is also applicable to other cytokines, such as IL-12 and may be used to identify T-cell stimulation. IL-12 alone or when incorporated into DKα^{12} induces IFN-alpha secretion in this assay. Treatment of T cells with IFN-alpha induces no secretion of IFN-alpha. Treatment of CD8+ T cells with IFN-alpha leads to appreciable proliferation. Treatment with IFN-alpha and IL-12 in combination or when coupled in the DKα^{12} leads to both an increase in T cell proliferation and significantly enhances IFN-alpha secretion.

<u>Example 2: IL-12 and IFN-alpha Dual Cytokine Fusion Protein *in vivo* Study</u>

[00102] Targeting a high affinity IL-10 variant (termed-DV07) via an anti-EGFR scFv (wherein DV07 is fused to a scFv comprising VH and VL obtained from a human anti-ebola ScFv scaffolding comprising 6 engrafted anti-EGFR CDRs;"Degfr:DV07") into the tumor microenvironment by virtue of generating a stably expressed human EGFR CT26 murine colorectal tumor cell line, was previously shown to exhibit superior anti-tumor function when compared with PEG-rHuIL-10. *See,* U.S. Patent 10,858,412. Using the same *in vivo* tumor study, DKα^{12} is evaluated and compared to Degfr:DV07 in human EGFR expressing CT26 cell murine tumor cell line.

[00103] CT26 (hEGFR$^+$) tumor bearing B cell k.o. Balb/C mice, with an average of 100mm^3 tumors were treated with test articles, doses and frequencies as provided shown in Table 5. All test articles were administered subcutaneously in the scruff. All articles were dosed daily for 15 days.

Table 5: Test Articles, Doses and Frequencies			
No.	**Test article**	**Dose**	**Frequency**
1	Vehicle	100 μl (control)	Daily
2	Degfr:IL-12	1 mg/kg	Daily
3	DKα^{12}	1 mg/kg	Daily
4	DKα^{12}	2 mg/kg	Daily
5	DKα^{12}	4 mg/kg	Daily

The length and width of tumors were measured every three days by electronic calipers and tumor volume was calculated ((LxW^2)/2)). In this example, the terms "Degfr:DV07" is human EGFR targeted DV07; DKα^{12}egfr is abbreviated as "DKα^{12}" and is human IFN-alpha coupled with IL-12 via the Cetuximab CDR grafted anti-ebola scFv scaffold.

Methods

[00104] *In vitro cell culture*: CT26$^{(hEGFR+)}$ tumor cells (ATCC) are grown to 70% confluency in complete RPMI, 10% FCS, and 10ug/mL puromycin. Cells are carried for no more than 3 passages *in vitro* prior to implantation. Cells are removed from cell culture plate using Accutase (Biolegend) and washed in complete RPMI spinning for 10 minutes at 400g at 4°C.

[00105] *Tumor Implantation*: Tumor cells are implanted at 1-2 x10^5 cells/mouse in 100 μL with or without 50% growth factor reduced Matrigel, 50-100% RPMI subcutaneous in the right flank of B cell knockout or wild-type mice.

Results

[00106] *Comparison of Degfr:IL-12 and DKα^{12} on tumor growth*: Targeting IL-12 to the tumor microenvironment via binding to the EGFR present on the stably transfected tumor cells was previously show to be effective. *See* U.S. Patent 10,858,412. Using the same tumor system, Degfr:IL-12 versus DKα^{12} is compared.

[00107] Tumors are measured three times a week (Table 2). Female Balb/C B cell knockout mice with 75mm^3 CT26$^{(hEGFR+)}$ tumors are treated subcutaneously with the test articles and various dosing frequencies For this experiment, the CT26$^{(hEGFR+)}$ cells are implanted at 1-2x10^5 cells in 0-50% growth factor reduced Matrigel to limit immunization of the mice against tumor antigens.

[00108] The anti-tumor effect of Degfr:IL-12 at 1 mg/kg is compared to the same dose of DKα^{12} as well as 2 and 4 mg/kg doses.

[00109] *Safety Assessment of DKα^{12}*: To test the safety of DKα^{12} dosing the weight of tumor bearing mice treated with DKα^{12} is evaluated.

[00110] *Effect of DKα^{12} dosing on survival*: The survivability of CT26$^{(hegfr+)}$ tumor bearing mice to DKα^{12} is assessed.

Example 3: IL-27 and IL-28 or IL-29 Dual Cytokine Fusion Protein

[00111] To evaluate the *in vitro* effects of targeting two cytokines to a tumor, a dual cytokine fusion protein comprising (1) IL-27 and IL-28 (*see* FIG. 4 as a representative diagram of the structure, termed "DK28^{27}") and (2) IL-27 and IL-29 (*see* FIG. 5 as a representative diagram of the structure, termed "DK29^{27}") are constructed from the following components:

DK28^{27}:

(a) p28 and EBI3 subunits are terminally fused to a human anti-ebola scFv engrafted with 6 CDRs from any one of anti-EGFR, anti-HER2, anti-VEGFR1, anti-VEGFR1, or anti-CD14 antibody; and

(b) an IL-28 (SEQ ID No: 11);

where the IL-28 is conjugated or linked in the hinge (or linker) region between the VH and VL of the human anti-ebola scFv engrafted with the 6 CDRs from anti-EGFR, anti-HER2, anti-VEGFR1, anti-VEGFR1, or anti-CD14 antibody.

DK29^{27}:

(a) p28 and EBI3 subunits are terminally fused to a human anti-ebola scFv engrafted with 6 CDRs from any one of anti-EGFR, anti-HER2, anti-VEGFR1, anti-VEGFR1, or anti-CD14 antibody; and

(b) an IL-29 (SEQ ID No: 16);

where the IL-29 is conjugated or linked in the hinge (or linker) region between the VH and VL of the human anti-ebola scFv engrafted with the 6 CDRs from anti-EGFR, anti-HER2, anti-VEGFR1, anti-VEGFR1, or anti-CD14 antibody.

[00112] Both DK28^{27} and DK29^{27} are generated to evaluate the combined effects of dual cytokines—IL-27 with IL-28 and IL-28 with IL-29—on induction of IFNγ from NK, CD4^{+} and CD8^{+} T cells. The procedures and assays described above (Example 1) are repeated with these dual cytokines on both monocytes/macrophages, T-cells (CD4+ and CD8+), and NK cells.

Example 4: IL-27 with IL-28 and IL-27 with IL-29 Dual Cytokine Fusion Protein *in vivo* Study

[00113] Using the same *in vivo* tumor study, both $DK28^{27}$ and $DK29^{27}$ are evaluated and compared to single targeted cytokine in human EGFR expressing CT26 cell murine tumor cell line. The procedures and assays described above (Example 2) are repeated with these dual cytokines.

Example 5: IL-12 with IL-10 Dual Cytokine Fusion Protein in an in vitro cell killing assay combined with CD19 BiTE

[00114] This study was designed to evaluate whether linker lengths have an effect on the cytotoxic function of CD8+ T cells in a molecule that combines IL-12 and IL-10 (DV07) onto an anti-ebola scaffolding engrafted with CDRs that target EGFR (internally designated as $DK12^{10}$EGFR) when combined with a CD19 Bispecific T-Cell Engager (CD19 BiTE).

[00115] CD8+ T cells were isolated from fresh donor Leukopaks via anti-CD8+ magnetic bead isolation per the manufacturer's suggested protocol (Miltenyi).

[00116] The isolated CD8+ T cells were plated at $2x10^6$ cells/well and exposed for 2 days in various concentrations (0 or 200 ng/mL) of $DK12^{10}$EGFR in AIMV. Following the 2 days of exposure to the various concentrations of $DK12^{10}$EGFR with standard linkers or with $DK12^{10}$EGFR having extended linkers on both the IL10 and IL-12 side, the CD8+ T cells were harvested, counted, washed, and finally resuspended in the corresponding concentration of $DK12^{10}$EGFR. Concurrently, Raji cells, which constitutively express Green Florescent Protein (GFP), were counted, washed and resuspended in varying concentrations (0 and 0.1 (data not shown), or 1 ng/mL) of CD19 BiTE. The CD8+ cells (effector) and Raji-GFP cells (target) are then combined at a 10:1 effector to target ratio. The mixture of effector and target cells, which are exposed to (1) no treatment, (2) CD19 BiTE alone, (3) $DK2^{10}$EGFR alone, or (4) the combination of CD19 BiTE and $DK2^{10}$EGFR, were monitored over 2 days using an IncuCyte. Following the 2-day exposure, the percentage of GFP disappearance is measured as an indicator of cytotoxicity.

[00117] CD19 BiTE, also known as Blinatumomab, is currently the only FDA approved BiTE therapy. We have shown (data not provided) that combining CD19 BiTE with other dual cytokine fusion proteins including IL10 and IL2 (internally termed

DK210) enhances CD19 BiTE cytotoxicity. Here we use the same assay system to determine whether combining CD19 BiTE with (1) DK12^{10}EGFR having standard linkers (e.g., SEQ ID No: 46) would be improved over (2) DK12^{10}EGFR having extended linkers (e.g., SEQ ID No:44) would drive normal, healthy human donor derived CD8+ T cells to cytolyze target cancer cells. See Fig. 9 and 10.

[00118] The assessment of healthy human donor derived CD8+ T cells to respond to CD19 BiTE with both DK12^{10}EGFR having standard linkers versus DK12^{10}EGFR having extended linkers suggests that the extended linkers improve the overall capability of combining the two modalities to enhance CD8+ T cell cytolysis of target Raji cells.

[00119] This written description uses examples to disclose aspects of the present disclosure, including the preferred embodiments, and also to enable any person skilled in the art to practice the aspects thereof, including making and using any devices or systems and performing any incorporated methods. The patentable scope of these aspects is defined by the claims, and may include other examples that occur to those skilled in the art. Such other examples are intended to be within the scope of the claims if they have structural elements that do not differ from the literal language of the claims, or if they include equivalent structural elements with insubstantial differences from the literal language of the claims. Aspects from the various embodiments described, as well as other known equivalents for each such aspect, can be mixed and matched by one of ordinary skill in the art to construct additional embodiments and techniques in accordance with principles of this application.

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CLAIMS

1. A dual cytokine fusion protein comprising at least a first and a second monomeric subunit of a first cytokine fused to a single chain variable region (scFv) or an antigen binding fragment of a monoclonal antibody, wherein each monomeric subunit is fused at a terminal end of the fusion protein; and a second cytokine, wherein the second cytokine is fused between the VH and VL regions of the scFv or antigen binding fragment.

2. The dual cytokine fusion protein according to claim 1, wherein the first cytokine is IL-12 or IL-27.

3. The dual cytokine protein according to claim 1, wherein the first monomeric subunit is a p35 subunit and the second monomeric subunit is a p40 subunit.

4. The dual cytokine protein according to claim 1, wherein the first monomeric subunit is SEQ ID No: 1, 17, 19 and the second monomeric subunit is SEQ ID No: 3, 18, 20.

5. The dual cytokine protein according to claim 1, wherein the first monomeric subunit is a p28 subunit and the second monomeric subunit is a EBI3 subunit.

6. The dual cytokine protein according to claim 1, wherein the first monomeric subunit is SEQ ID No: 5 and the second monomeric subunit is SEQ ID No: 7.

7. A dual cytokine fusion protein of formula (Ia) or (Ib)

$$NH_2\text{-}(R^1)\text{-}(X^1)\text{-}(Z_n)\text{-}(X^2)\text{-}(R^2)\text{-}COOH \quad \text{(Formula Ia);}$$

$$NH_2\text{-}(R^2)\text{-}(X^1)\text{-}(Z_n)\text{-}(X^2)\text{-}(R^1)\text{-}COOH \quad \text{(Formula Ib)}$$

wherein

"R^1" is an alpha subunit of a first cytokine sequence selected from SEQ ID No: 1, 17, 19 or 5;

"R^2" is a beta subunit of a first cytokine sequence selected from SEQ ID No: 3, 18, 20, or 7;

wherein when R^1 is SEQ ID No: 1 17, or 19, R^2 is SEQ ID No: 3, 18, 20 or when R^1 is SEQ ID No: 5, R^2 is SEQ ID No:7;

"X^1" is a VL or VH region obtained from a first monoclonal antibody;

"X^2" is a VH or VL region obtained from the first monoclonal antibody;

wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL

"Z" is a cytokine;

"n" is an integer selected from 0-2.

8. The dual cytokine fusion protein according to claim 7, wherein X^1 and X^2 are obtained from the first monoclonal antibody specific for epidermal growth factor receptor (EGFR); CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD20; CD47;GD-2; VEGFR1, VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; SR-J1; HIV, or Ebola.

9. The dual cytokine fusion protein according to claim 7, wherein the VL and VH are obtained from the first monoclonal antibody that is an anti-HIV or anti-Ebola antibody.

10. The dual cytokine fusion protein according to claim 9, wherein the VL and VH from the anti-HIV or anti-Ebola monoclonal antibody include 6 CDRs that are engrafted (substituted) with 6 CDRs from a second antibody.

11. The dual cytokine fusion protein according to claim 10, wherein the second antibody is a monoclonal antibody selected from epidermal growth factor receptor (EGFR); CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD20; CD47;GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1.

12. The dual cytokine fusion protein according to claim 10, wherein the 6 engrafted CDRs from the second monoclonal antibody comprise 6 CDRs from an anti-EGFR antibody, an anti-HER2 antibody, an anti-VEGFR1 antibody, or an anti-VEGFR2 antibody wherein the 6 CDRs comprise CDR 1-3 from the VL and CDR 1-3 from VH.

13. The dual cytokine fusion protein according to claim 7, wherein Z is a cytokine selected from IL-6, IL-4, IL-1, IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, IL-15, IL-21, IL-26, IL-

28, IL-29, GM-CSF, G-CSF, interferons-α, -β, -γ, TGF-β, or tumor necrosis factors -α, -β, basic FGF, EGF, PDGF, IL-4, IL-11, or IL-13.

14. The dual cytokine fusion protein according to claim 7, wherein Z is a interferon-α, IL-28, or IL-29.

15. The dual cytokine fusion protein according to claim 7, wherein Z is an integer of 1.

16. The dual cytokine fusion protein according to claim 7, further comprising linkers between each of R^1, X^1, Z, X^2, and R^2.

17. A dual cytokine fusion protein comprising IL-12, wherein the fusion protein is Formula (IIa) or (IIb)

NH_2-(p35)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(p40)-COOH (Formula IIa);

NH_2-(p40)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(p35)-COOH (Formula IIb);

wherein

"p35" is a sequence of SEQ ID No: 1, 17, or 19;

"p40" is a sequence of SEQ ID No: 3, 18, or 20;

"L" is a linker;

"X^1" is a VL or VH region obtained from a first monoclonal antibody;

"X^2" is a VH or VL region obtained from the first monoclonal antibody;

wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"Z" is a cytokine selected from IL-6, IL-4, IL-1, IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, IL-15, IL-21, IL-26, IL-27, IL-28, IL-29, GM-CSF, G-CSF, interferons-α, -β, -γ, TGF-β, or tumor necrosis factors -α, -β, basic FGF, EGF, PDGF, IL-4, IL-11, or IL-13;

"n" is an integer selected from 0-2.

18. The IL-12 fusion protein according to claim 17, wherein the VL and VH are obtained from the first antibody specific for epidermal growth factor receptor (EGFR); CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD20; CD47;GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin SR-A1; SR-A3;

SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; SR-J1; HIV, or Ebola.

19. The IL-12 fusion protein according to claim 18, wherein the VL and VH are obtained from the first antibody specific for HIV or Ebola.

20. The IL-12 fusion protein according to claim 19, wherein the VL and VH from the anti-HIV or anti-Ebola include 6 CDRs that are grafted (substituted) with 6 CDRs from a second antibody.

21. The IL-12 fusion protein according to claim 20, wherein the second antibody is an antibody selected from epidermal growth factor receptor (EGFR); CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD20; CD47;GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1.

22. The IL-12 fusion protein according to claim 20, wherein the 6 engrafted CDRs from the second antibody comprise 6 CDRs from an antibody selected from an anti-EGFR antibody, an anti-VEGFR1 or VEGFR2 antibody, an anti-HER2 antibody, or an ant-CD14 antibody, wherein the 6 CDRs comprise CDRs 1-3 from the VL and CDRs 1-3 from VH.

23. A dual cytokine fusion protein comprising IL-27, said fusion protein is Formula (IIIa) or (IIIb)

NH_2-(p28)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(EBI3)-COOH (Formula IIIa);

NH_2-(EBI3)-(L)-(X^1)-(L)-(Z_n)-(L)-(X^2)-(L)-(p28)-COOH (Formula IIIb);

wherein

"p28" is a sequence of SEQ ID No: 5;

"EBI3" is a sequence of SEQ ID No: 7;

"L" is a linker;

"X^1" is a VL or VH region obtained from a first monoclonal antibody;

"X^2" is a VH or VL region obtained from the first monoclonal antibody;

wherein when X^1 is a VL, X^2 is a VH or when X^1 is a VH, X^2 is a VL;

"Z" is a cytokine selected from IL-6, IL-4, IL-1, IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, IL-15, IL-21, IL-26, IL-28, IL-29, GM-CSF, G-CSF, interferons-α, -β, -γ, TGF-β, or tumor necrosis factors -α, -β, basic FGF, EGF, PDGF, IL-4, IL-11, or IL-13;

"n" is an integer selected from 0-2.

24. The IL-27 fusion protein according to claim 23, wherein the VL and VH are obtained from the first antibody specific for epidermal growth factor receptor (EGFR); CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD20; CD47;GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; SR-J1; HIV, or Ebola.

25. The IL-27 fusion protein according to claim 24, wherein the VL and VH are obtained from the first antibody specific for HIV or Ebola.

26. The IL-27 fusion protein according to claim 25, wherein the VL and VH from the anti-HIV or anti-Ebola include 6 CDRs that are grafted (substituted) with 6 CDRs from a second antibody.

27. The IL-27 fusion protein according to claim 26, wherein the second antibody is an antibody selected from epidermal growth factor receptor (EGFR); CD14; CD52; various immune check point targets, such as but not limited to PD-L1, PD-1, TIM3, BTLA, LAG3 or CTLA4; CD20; CD47;GD-2; VEGFR1; VEGFR2; HER2; PDGFR; EpCAM; ICAM (ICAM-1, -2, -3, -4, -5), VCAM, FAPα; 5T4; Trop2; EDB-FN; TGFβ Trap; MAdCAM, β7 integrin subunit; α4β7 integrin; α4 integrin SR-A1; SR-A3; SR-A4; SR-A5; SR-A6; SR-B; dSR-C1; SR-D1; SR-E1; SR-F1; SR-F2; SR-G; SR-H1; SR-H2; SR-I1; or SR-J1.

28. The IL-27 fusion protein according to claim 27, wherein the 6 engrafted CDRs from the second antibody comprise 6 CDRs from an anti-EGFR antibody, an anti-

VEGFR1 or VEGFR2 antibody, an anti-HER2 antibody, or an ant-CD14 antibody, wherein the 6 CDRs comprise CDRs 1-3 from the VL and CDRs 1-3 from VH.

29. A method of treating cancer comprising administering to a subject in need thereof, an effective amount of the fusion protein according to claims 1, 2, or 7.

30. The method according to claim 29, wherein the fusion protein comprises VL and VH regions from a first antibody selected from anti-HIV or anti-Ebola and wherein the 6 CDR regions of the first antibody are engrafted with 6 CDR regions from second antibody selected from an anti-EGFR antibody, an anti-HER2 antibody, an anti-VEGFR1 antibody, or an anti-VEGFR2 antibody, or anti-CD14.

31. The method according to claim 30, wherein the first antibody is an anti-Ebola antibody and the second antibody is selected from an anti-EGFR antibody, an anti-HER2 antibody, an anti-VEGFR1 antibody, an anti-VEGFR2 antibody, or an anti-CD14 antibody.

32. The method according to claim 29, wherein the first cytokine is an IL-12 or IL-27.

33. The method according to claim 32, wherein the cytokine or “Z” is IL-12.

34. The method according to claim 24, wherein “Z” has a “n” value of 1.

35. The method according to claim 21, wherein the fusion protein is administered at 0.01 ng/ml to 100 ng/ml.

36. The method according to claim 21, wherein the fusion protein is administered at 0.01 ng/ml to 10 ng/ml.

37. A method of treating inflammatory disease comprising administering to a subject in need thereof, an effective amount of the fusion protein according to claims 1, 2, or 7.

38. The method according to claim 37, wherein the fusion protein comprises VL and VH regions from a first antibody selected from anti-HIV or anti-Ebola and wherein the 6 CDR regions of the first antibody are engrafted with 6 CDR regions from an anti-

HER2 antibody, an anti-VEGFR1, an anti-VEGFR2, an anti-EGFR antibody, or anti-CD14 antibody.

39. The method according to claim 37, wherein “Z” has a “n” value of 1.

40. The method according to claim 37, wherein the fusion protein is administered at 0.01 ng/ml to 100 ng/ml.

41. The method according to claim 37, wherein the inflammatory disease is sepsis, Crohn’s disease, rheumatoid arthritis, psoriasis, and/or inflammatory bowel disease (IBD).

42. A method of treating sepsis, septic shock, and/or symptoms associated thereof comprising administering to a subject in need thereof, an effective amount of the fusion protein according to claims 1, 2 or 7.

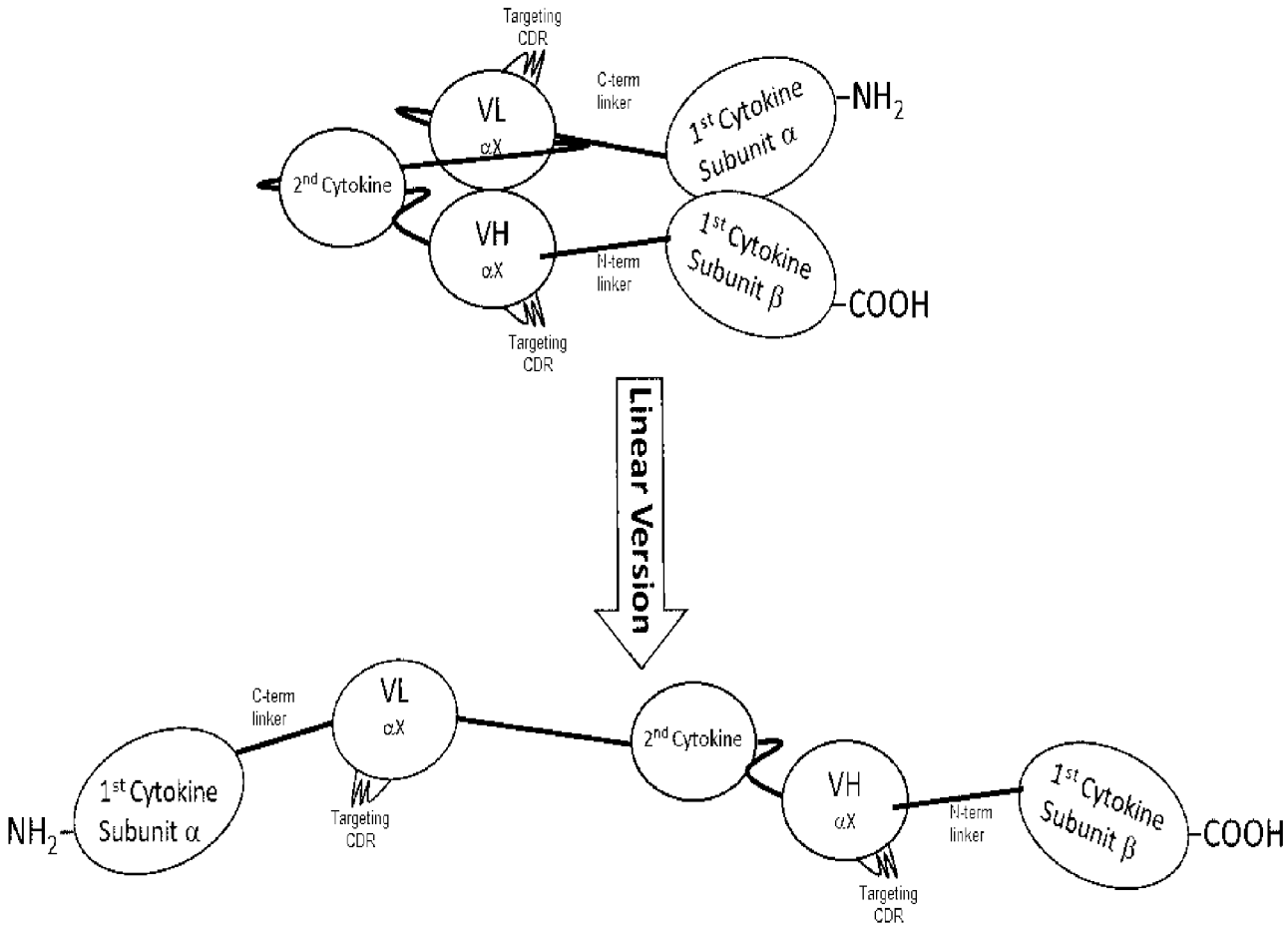

Targeting CDR
C-term linker
VL αX
1st Cytokine Subunit α
NH2
2nd Cytokine
VH αX
N-term linker
1st Cytokine Subunit β
COOH
Targeting CDR
Linear Version
C-term linker
VL αX
2nd Cytokine
1st Cytokine Subunit α
NH2
Targeting CDR
VH αX
N-term linker
1st Cytokine Subunit β
COOH
Targeting CDR

FIG. 1

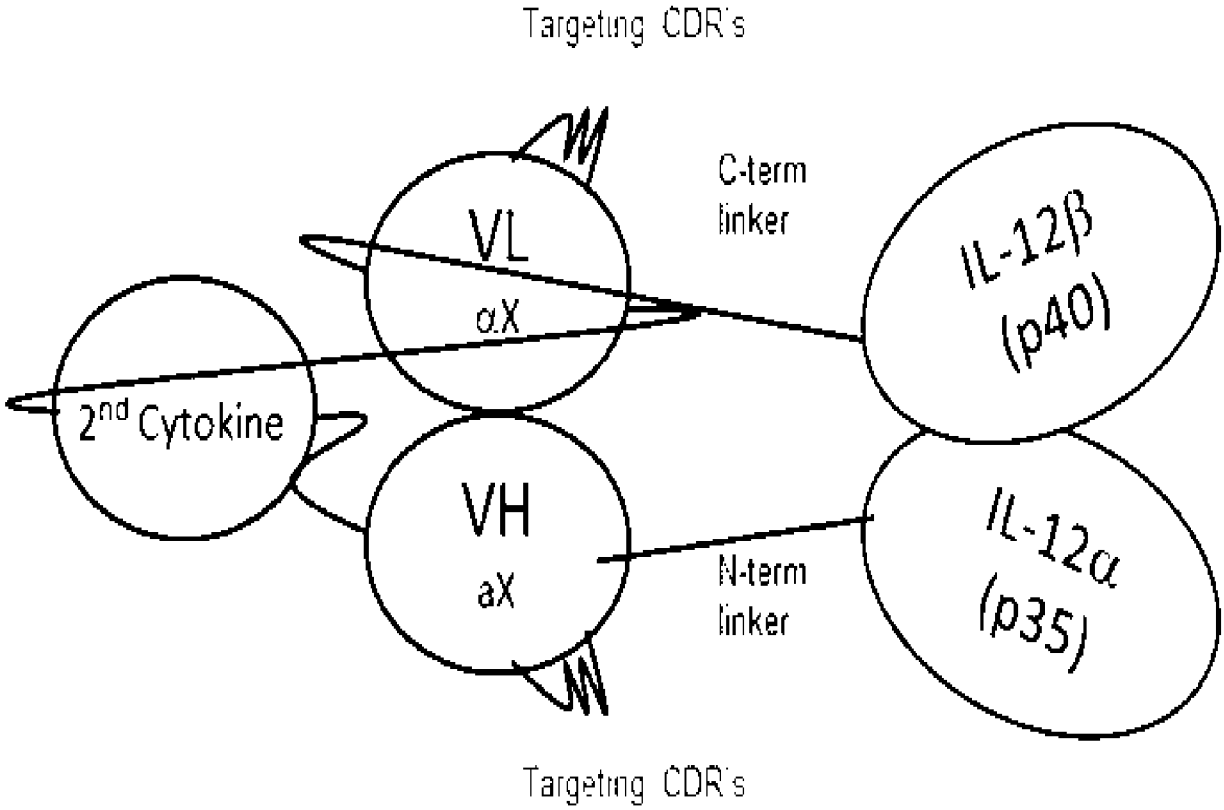

Targeting CDR's
VL
αX
C-term
linker
IL-12β
(p40)
2nd Cytokine
VH
αX
N-term
linker
IL-12α
(p35)
Targeting CDR's

FIG. 2

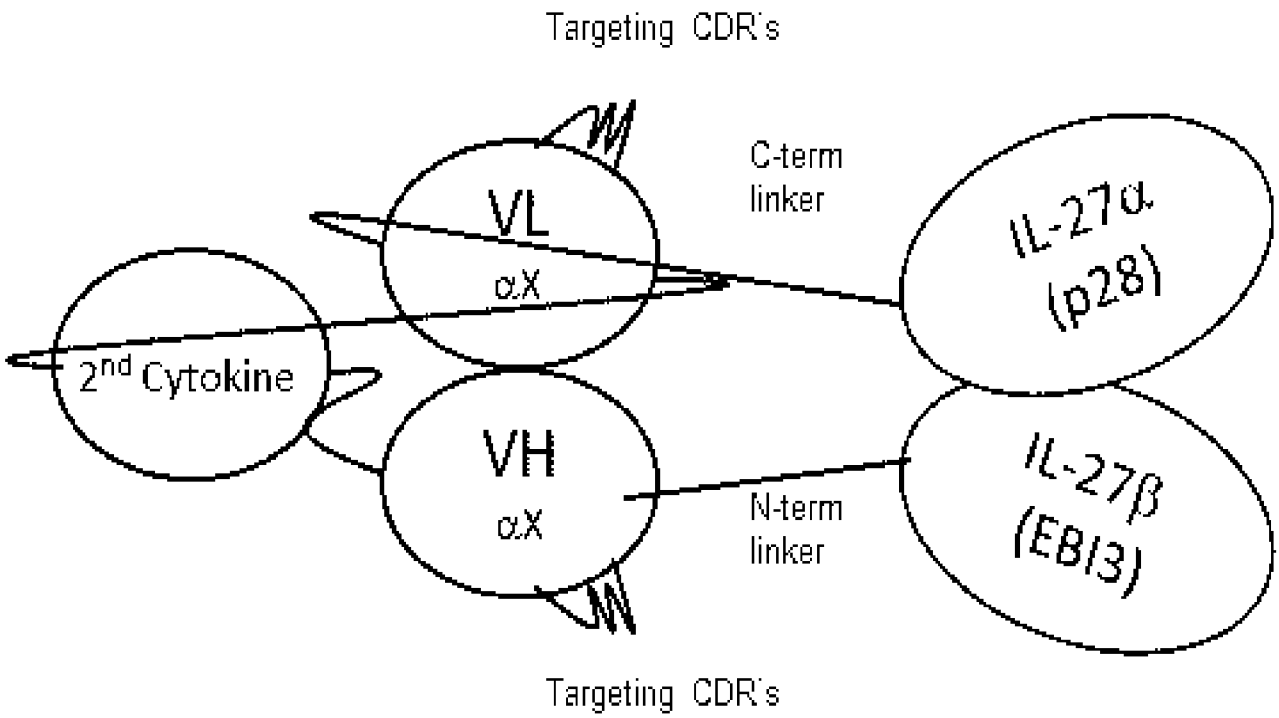

Targeting CDR's
VL
αX
C-term
linker
IL-27α
(p28)
2nd Cytokine
VH
αX
N-term
linker
IL-27β
(EBI3)
Targeting CDR's

FIG. 3

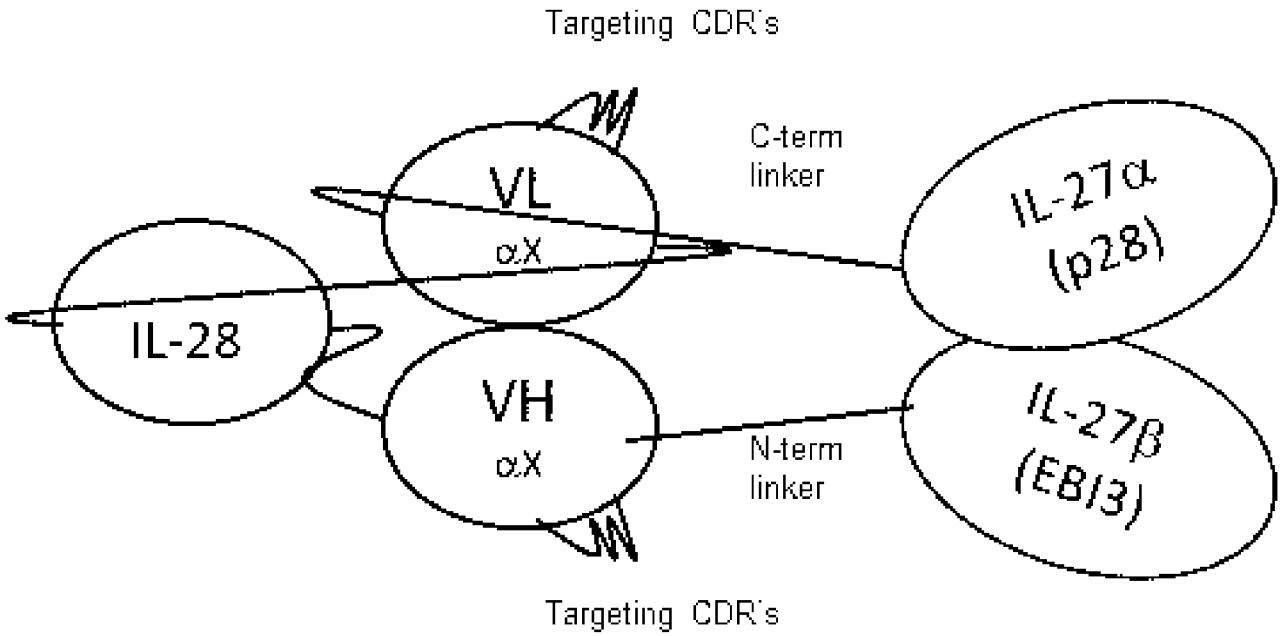

Targeting CDR's
VL
αX
C-term
linker
IL-27α
(p28)
IL-28
VH
αX
N-term
linker
IL-27β
(EBI3)
Targeting CDR's

FIG. 4

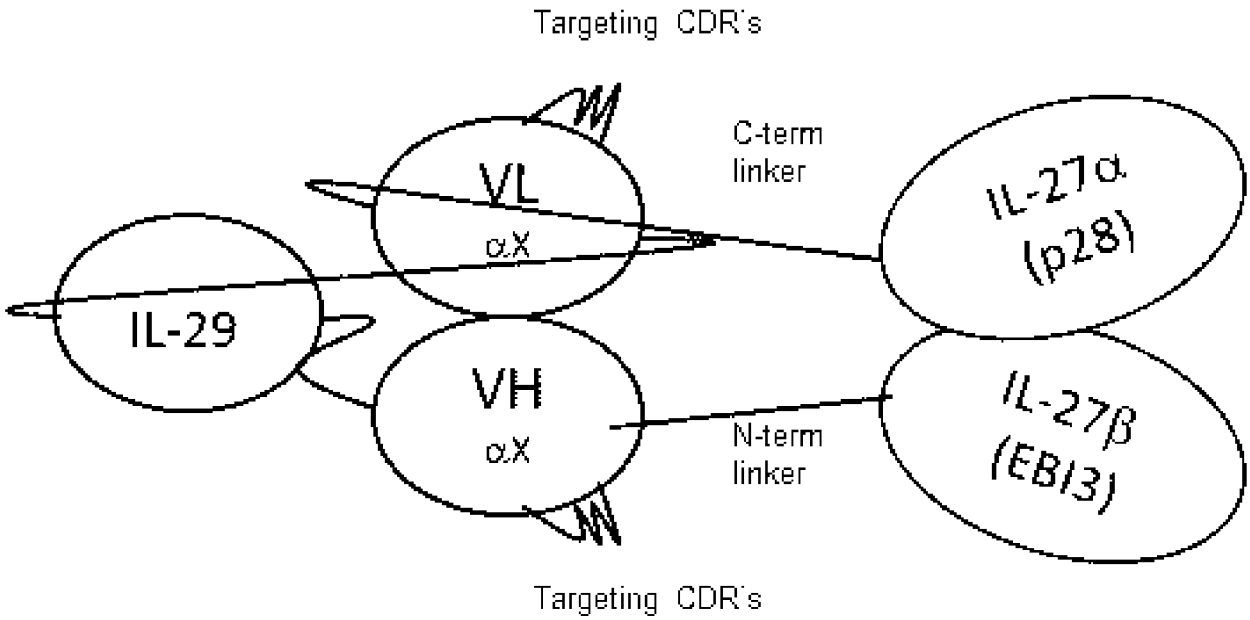

Targeting CDR's
C-term
linker
VL
αX
IL-27α
(p28)
IL-29
VH
αX
IL-27β
(EBI3)
N-term
linker
Targeting CDR's

FIG. 5

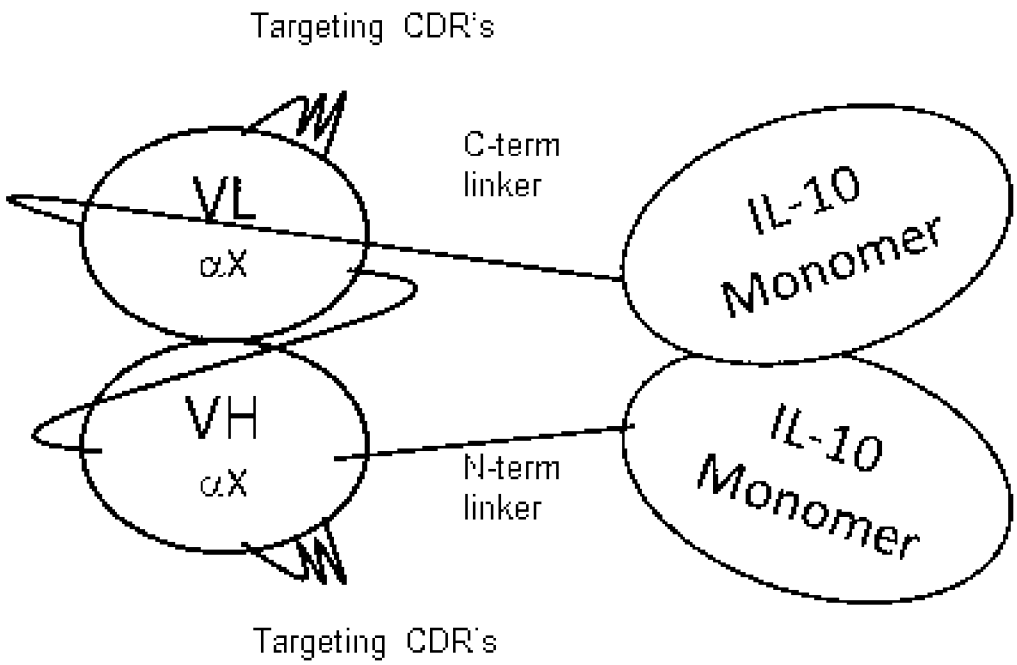

Targeting CDR's
VL
αX
C-term
linker
IL-10
Monomer
VH
αX
N-term
linker
IL-10
Monomer
Targeting CDR's

FIG. 6

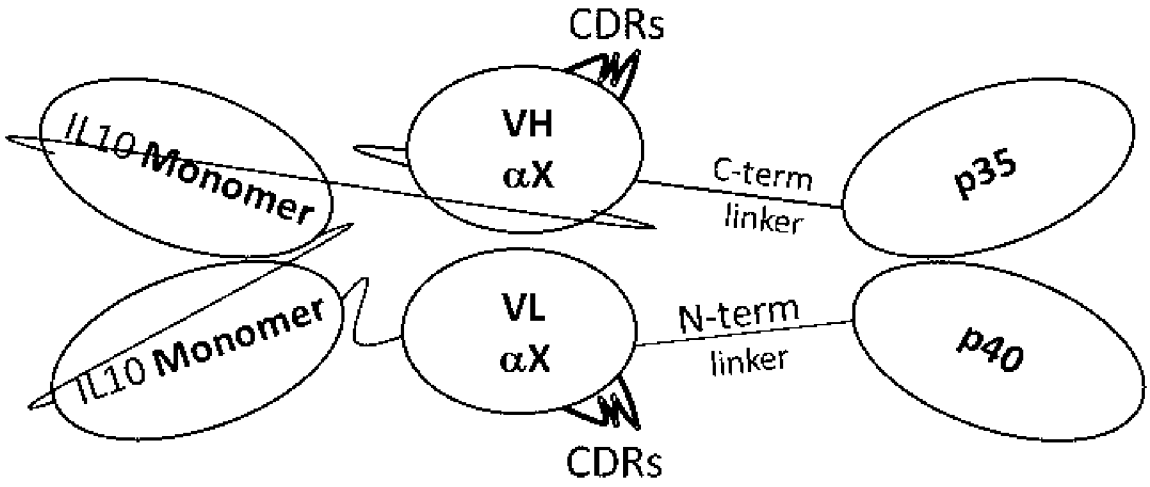

CDRs
IL10 Monomer
VH
αX
C-term
linker
p35
IL10 Monomer
VL
αX
N-term
linker
p40
CDRs

FIG. 7

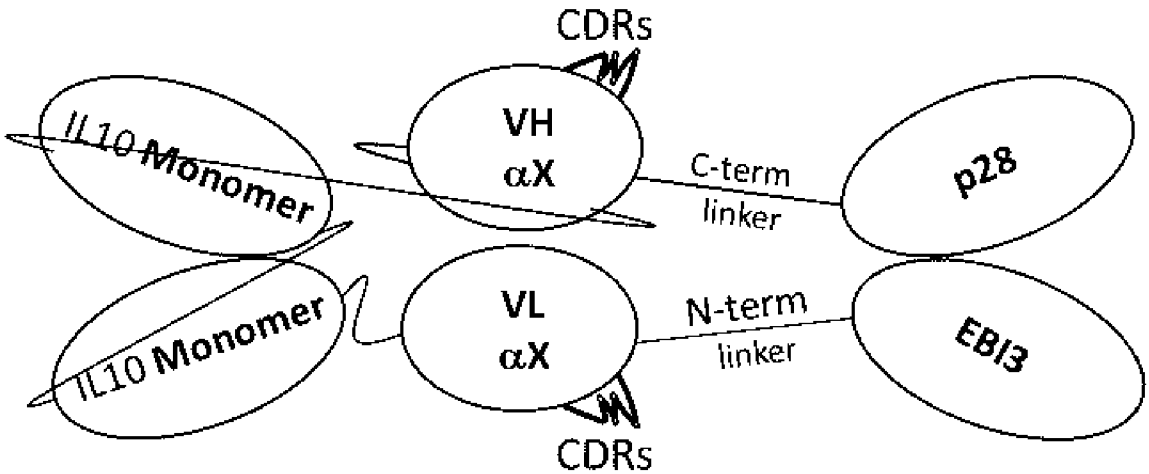

CDRs
IL10 Monomer
VH
αX
C-term
linker
p28
IL10 Monomer
VL
αX
N-term
linker
EBI3
CDRs

FIG. 8

Fig. 9

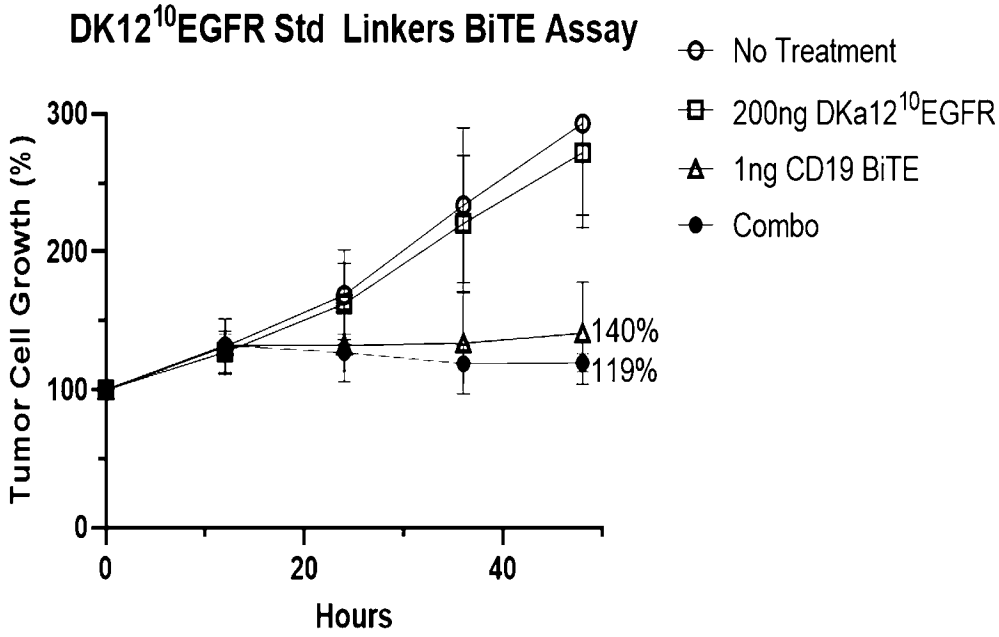
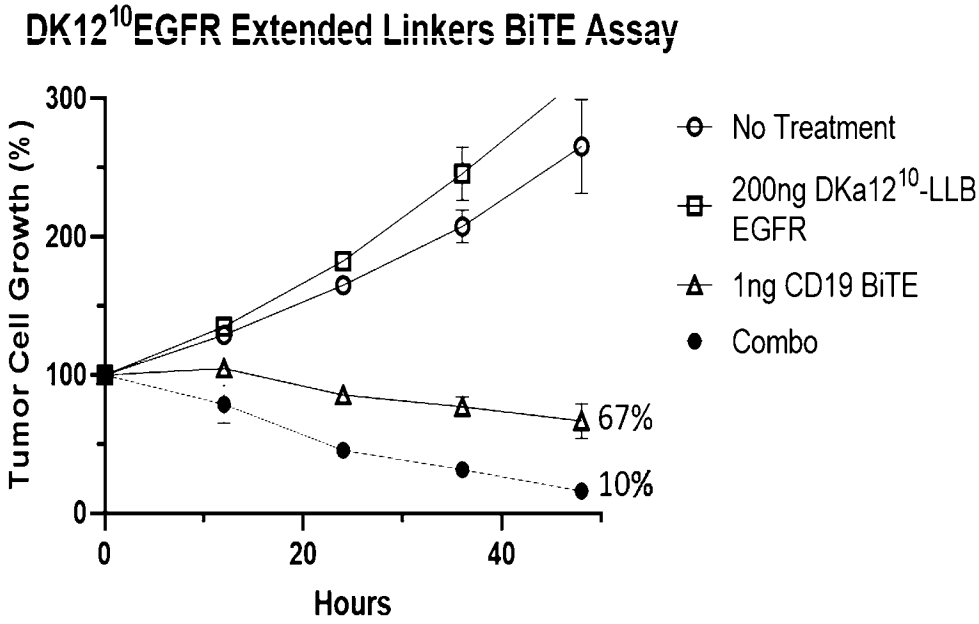

DK12^{10}EGFR Std Linkers BiTE Assay
No Treatment
200ng DKa12^{10}EGFR
1ng CD19 BiTE
Combo
Tumor Cell Growth (%)
300
200
100
0
0
20
40
Hours
140%
119%

Fig. 10


DK12^{10}EGFR Extended Linkers BiTE Assay
No Treatment
200ng DKa12^{10}-LLB EGFR
1ng CD19 BiTE
Combo
Tumor Cell Growth (%)
300
200
100
0
0
20
40
Hours
67%
10%