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(54) Titre : POLYPEPTIDES D'INTERLEUKINE 12 ACTIVABLES ET PROCEDES D'UTILISATION ASSOCIES
(54) Title: ACTIVATABLE INTERLEUKIN 12 POLYPEPTIDES AND METHODS OF USE THEREOF

(57) Abrégé/Abstract:
The disclosure features fusion proteins that are conditionally active variants of a cytokine of interest. In one aspect, the full-length polypeptides of the invention have reduced or minimal cytokine-receptor activating activity even though they contain a functional cytokine polypeptide. Upon activation, e.g., by cleavage of a linker that joins a blocking moiety, e.g. a steric blocking polypeptide, in sequence to the active cytokine, the cytokine can bind its receptor and effect signaling.

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(54) Title: ACTIVATABLE INTERLEUKIN 12 POLYPEPTIDES AND METHODS OF USE THEREOF

(57) Abstract: The disclosure features fusion proteins that are conditionally active variants of a cytokine of interest. In one aspect, the full-length polypeptides of the invention have reduced or minimal cytokine-receptor activating activity even though they contain a functional cytokine polypeptide. Upon activation, e.g., by cleavage of a linker that joins a blocking moiety, e.g. a steric blocking polypeptide, in sequence to the active cytokine, the cytokine can bind its receptor and effect signaling.

ACTIVATABLE INTERLEUKIN 12 POLYPEPTIDES AND METHODS OF USE THEREOF

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application 62/671,225, filed on May 14, 2018, U.S. Provisional Application No. 62/756,504, filed on November 6, 2018, and U.S. Provisional Application No. 62/756,515, filed on November 6, 2018. The entire teachings of the above applications are incorporated herein by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing, which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 14, 2019, is named 105365-0022_SL.txt and is 232,520 bytes in size.

BACKGROUND

[1] The development of mature immunocompetent lymphoid cells from less-committed precursors, their subsequent antigen-driven immune responses, and the suppression of these and unwanted autoreactive responses are highly dependent and regulated by cytokines (including interleukin-2 [IL-2], IL-4, IL-7, IL-9, IL-15, and IL-21) that utilize receptors in the common γ-chain (γc) family (Rochman et al., 2009) and family members including Il-12, 18 and 23. IL-2 is essential for thymic development of Treg cells and critically regulates several key aspects of mature peripheral Treg and antigen-activated conventional T cells. Because of its potent T cell growth factor activity in vitro, IL-2 has been extensively studied in part because this activity offered a potential means to directly boost immunity, e.g., in cancer and AIDS-HIV patients, or a target to antagonize unwanted responses, e.g., transplantation rejection and autoimmune diseases. Although *in vitro* studies with IL-2 provided a strong rationale for these studies, the function of IL-2 in vivo is clearly much more complex as first illustrated in IL-2-deficient mice, where a rapid lethal autoimmune syndrome, not lack of immunity, was observed (Sadlack et al., 1993, 1995). Similar observations were later made when the gene encoding IL-2Rα (Il2ra) and IL-2Rβ (Il2rb) were individually ablated (Suzuki et al., 1995; Willerford et al., 1995).

[2] The present invention refers to conditionally active and/or targeted cytokines for use in the treatment of cancer and other diseases dependent on immune up or down regulation. For example, the antitumoral activity of some cytokines is well known and described and some cytokines have already been used therapeutically in humans. Cytokines such as interleukin-2 (IL-2) and interferon α (IFNα) have shown positive antitumoral activity in patients with different types of tumors, such as kidney metastatic carcinoma, hairy cell leukemia, Kaposi sarcoma, melanoma, multiple myeloma, and the like. Other cytokines like IFNβ, the Tumor Necrosis Factor (TNF) α, TNFβ, IL-1, 4, 6, 12, 15 and the

CSFs have shown a certain antitumoral activity on some types of tumors and therefore are the object of further studies.

SUMMARY

[3] Provided herein are therapeutic proteins, nucleic acids that encode the proteins, and compositions and methods of using the proteins and nucleic acids for the treatment of a disease or disorder, such as proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, a viral disease, an allergic reaction, a parasitic reaction, graft-versus-host disease and the like.

[4] The invention features fusion proteins that are conditionally active variants of IL-12. In one aspect, the full-length polypeptides of the invention have reduced or minimal IL-12-receptor activating activity even though they contain a functional IL-12 polypeptide. Upon activation, e.g., by cleavage of a linker that joins a blocking moiety, e.g. a steric blocking polypeptide, in sequence to the active cytokine, IL-12, or a functional fragment or mutein thereof, can bind its receptor and effect signaling. If desired, the full-length polypeptides can include a blocking polypeptide moiety that also provides additional advantageous properties. For example, the full-length polypeptide can contain a blocking polypeptide moiety that also extends the serum half-life and/or targets the full-length polypeptide to a desired site of cytokine activity. Alternatively, the full-length fusion polypeptides can contain a serum half-life extension element and/or targeting domain that are distinct from the blocking polypeptide moiety. Preferably, the fusion protein contains at least one element or domain capable of extending *in vivo* circulating half-life. Preferably, this element is removed enzymatically in the desired body location (e.g. protease cleavage in the tumor microenvironment), restoring pharmacokinetic properties to the payload molecule (e.g. IL2 or IFNa) substantially similar to the naturally occurring payload molecule. The fusion proteins are targeted to a desired cell or tissue. As described herein targeting is accomplished through the action of a blocking polypeptide moiety that also binds to a desired target, or through a targeting domain. The domain that recognizes a target antigen on a preferred target (for example a tumor-specific antigen), may be attached to the cytokine via a cleavable or non-cleavable linker. If attached by a non-cleavable linker, the targeting domain may further aid in retaining the cytokine in the tumor, and may be considered a retention domain. The targeting domain does not necessarily need to be directly linked to the payload molecule, and may be linked directly to another element of the fusion protein. This is especially true if the targeting domain is attached via a cleavable linker.

[5] In one aspect is provided a fusion polypeptide comprising an IL-12 polypeptide, or functional fragment or mutein thereof, and a blocking moiety, e.g. a steric blocking domain. The blocking moiety is fused to the IL-12 polypeptide, directly or through a linker, and can be separated from the IL-12 polypeptide by cleavage (e.g, protease mediate cleavage) of the fusion polypeptide at or near the fusion site or linker or in the blocking moiety. For example, when the IL-12 polypeptide is fused

to a blocking moiety through a linker that contains a protease cleavage site, the IL-12 polypeptide is released from the blocking moiety and can bind its receptor, upon protease mediated cleavage of the linker. The linker is designed to be cleaved at the site of desired cytokine activity, for example in the tumor microenvironment, avoiding off-target cytokine activity and reducing overall toxicity of cytokine therapy.

[6] The blocking moiety can also function as a serum half-life extension element. In some embodiments, the fusion polypeptide further comprises a separate serum half-life extension element. In some embodiments, the fusion polypeptide further comprises a targeting domain. In various embodiments, the serum half-life extension element is a water-soluble polypeptide such as optionally branched or multi- armed polyethylene glycol (PEG), full length human serum albumin (HSA) or a fragment that preserves binding to FcRn, an Fc fragment, or a nanobody that binds to FcRn directly or to human serum albumin.

[7] In addition to serum half-life extension elements, the pharmaceutical compositions described herein preferably comprise at least one, or more targeting domains that bind to one or more target antigens or one or more regions on a single target antigen. It is contemplated herein that a polypeptide construct of the invention is cleaved, for example, in a disease-specific microenvironment or in the blood of a subject at the protease cleavage site and that the targeting domain(s) will bind to a target antigen on a target cell. At least one target antigen is involved in and/or associated with a disease, disorder or condition. Exemplary target antigens include those associated with a proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, a viral disease, an allergic reaction, a parasitic reaction, a graft-versus-host disease or a host-versus-graft disease.

[8] In some embodiments, a target antigen is a cell surface molecule such as a protein, lipid or polysaccharide. In some embodiments, a target antigen is a on a tumor cell, virally infected cell, bacterially infected cell, damaged red blood cell, arterial plaque cell, or fibrotic tissue cell.

[9] Target antigens, in some cases, are expressed on the surface of a diseased cell or tissue, for example a tumor or a cancer cell. Target antigens for tumors include but are not limited to Fibroblast activation protein alpha (FAPa), Trophoblast glycoprotein (5T4), Tumor-associated calcium signal transducer 2 (Trop2), Fibronectin EDB (EDB-FN), fibronectin EIIIB domain, CGS-2, EpCAM, EGFR, HER-2, HER-3, c-Met, FOLR1, and CEA. Pharmaceutical compositions disclosed herein, also include proteins comprising two antigen binding domains that bind to two different target antigens known to be expressed on a diseased cell or tissue. Exemplary pairs of antigen binding domains include but are not limited to EGFR/CEA, EpCAM/CEA, and HER-2/HER-3.

[10] In some embodiments, the targeting polypeptides independently comprise a scFv, a VH domain, a VL domain, a non-Ig domain, or a ligand that specifically binds to the target antigen. In some embodiments, the targeting polypeptides specifically bind to a cell surface molecule. In some embodiments, the targeting polypeptides specifically bind to a tumor antigen. In some embodiments,

the targeting polypeptides specifically and independently bind to a tumor antigen selected from at least one of EpCAM, EGFR, HER-2, HER-3, cMet, CEA, and FOLR1. In some embodiments, the targeting polypeptides specifically and independently bind to two different antigens, wherein at least one of the antigens is a tumor antigen selected from EpCAM, EGFR, HER-2, HER-3, cMet, CEA, and FOLR1. In some embodiments, the targeting polypeptide serves as a retention domain and is attached to the cytokine via a non-cleavable linker.

[11] As described herein, the cytokine blocking moiety can bind to the cytokine and thereby block activation of the cognate receptor of the cytokine.

[12] This disclosure also related to nucleic acids, e.g., DNA, RNA, mRNA, that encode the conditionally active proteins described herein, as well as vectors and host cells that contain such nucleic acids.

[13] This disclosure also relates to pharmaceutical compositions that contain a conditionally active protein, nucleic acid that encodes the conditionally active protein, and vectors and host cells that contain such nucleic acids. Typically, the pharmaceutical composition contains one or more physiologically acceptable carriers and/or excipients.

[14] The disclosure also relates to therapeutic methods that include administering to a subject in need thereof an effective amount of a conditionally active protein, nucleic acid that encodes the conditionally active protein, vector or host cells that contain such a nucleic acid, and pharmaceutical compositions of any of the foregoing. Typically, the subject has, or is at risk of developing, a proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, a viral disease, an allergic reaction, a parasitic reaction, a graft-versus-host disease or a host-versus-graft disease.

[15] The disclosure also relates to the use of a conditionally active protein, nucleic acid that encodes the conditionally active protein, vector or host cells that contain such a nucleic acid, and pharmaceutical compositions of any of the foregoing, for treating a subject in need thereof. Typically the subject has, or is at risk of developing, a proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, a viral disease, an allergic reaction, a parasitic reaction, a graft-versus-host disease or a host-versus-graft disease.

[16] The disclosure also relates to the use of a conditionally active protein, nucleic acid that encodes the conditionally active protein, vector or host cells that contain such a nucleic acid for the manufacture of a medicament for treating a disease, such as a proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, a viral disease, an allergic reaction, a parasitic reaction, a graft-versus-host disease or a host-versus-graft disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[17] **Figure 1a** is a schematic illustrating a protease-activated cytokine or chemokine that includes a blocking moiety. The blocking moiety may optionally function as a serum half-life extending domain. To the left of the arrow the drawing shows that a cytokine is connected to a blocking moiety via a protease-cleavable linker, thus blocking its ability to bind to its receptor. To the right of the arrow the drawing shows that in an inflammatory or tumor environment a protease cleaves at a protease-cleavage site on the linker, releasing the blocking moiety and allowing the cytokine to bind to its receptor.

[18] **Figure 1b** is a schematic illustrating a protease-activated cytokine or chemokine wherein HSA (blocking moiety) is directly bound to the cytokine or chemokine of interest, with a protease cleavage site between the HSA and a cytokine or chemokine of interest. To the left of the arrow the drawing shows that a cytokine is connected to a blocking moiety via a protease-cleavable linker, thus blocking its ability to bind to its receptor. To the right of the arrow the drawing shows that in an inflammatory or tumor environment, the protease cleaves at a protease-cleavage site on linker, releasing the blocking moiety and allowing the cytokine to bind to its receptor.

[19] **Figure 1c** is a schematic illustrating a protease-activated cytokine or chemokine wherein more than one HSA (blocking moiety) is bound directly to the molecule of interest. If desired, one or more of the HSA can be bonded to the cytokine or chemokine through a linker, such as a linker that contains a protease cleavage site. To the left of the arrow the drawing shows that a cytokine is connected to a blocking moiety via a protease-cleavable linker, thus blocking its ability to bind to its receptor. To the right of the arrow the drawing shows that in an inflammatory or tumor environment, protease cleaves at protease-cleavage site on linker, releasing the blocking moiety and allowing cytokine to bind receptor. The cytokine now has similar pK properties as compared to the native cytokine (e.g., has a short half-life).

[20] **Figure 1d** is a schematic illustrating a protease-activated cytokine or chemokine comprising more than one cytokine, of the same type or different type, each of which is bonded to a binding domain through a protease-cleavable linker. To the left of the arrow the drawing shows that a cytokine is connected to a blocking moiety via a protease-cleavable linker, thus blocking its ability to bind to its receptor. To the right of the arrow the drawing shows that in an inflammatory or tumor environment a protease cleaves at a protease cleavage site on linker, releasing the blocking moiety and allowing the cytokine to bind to its receptor.

[21] **Figure 2** is a schematic illustrating a protease-activated cytokine or chemokine comprising a cytokine or chemokine polypeptide, a blocking moiety, and a serum half-life extending domain connected by at least one protease-cleavable linker. To the left of the arrow the drawing shows that a cytokine is connected to a blocking moiety via protease-cleavable linkers, thus blocking its ability to bind to its receptor. It is also bound to a separate half-life extension element, which extends half-life in serum. To the right of the arrow the drawing shows that in an inflammatory or tumor environment a protease cleaves at a protease-cleavage site on linker, thus releasing the serum half-life extension

element and the blocking moiety and allowing the cytokine to bind to its receptor. The cytokine now has similar pK properties as compared to the native cytokine (e.g., a short half-life).

[22] **Figure 3** is a schematic illustrating a protease-activated cytokine or chemokine comprising a cytokine or chemokine polypeptide, a blocking moiety, and a targeting domain connected by at least one protease-cleavable linker. To the left of the arrow the drawing shows that a cytokine is connected to a blocking moiety and a targeting domain via a protease-cleavable linker, thus blocking its ability to bind to its receptor. To the right of the arrow the drawing shows that in an inflammatory or tumor microenvironment a protease cleaves at the protease cleavage site in the linker, releasing the targeting domain and the blocking moiety and allowing the cytokine to bind to its receptor.

[23] **Figure 4a** is a schematic illustrating a protease-activated cytokine or chemokine comprising a cytokine or chemokine polypeptide, a blocking moiety, a targeting domain, and a serum half-life extending domain connected by at least one protease-cleavable linker, wherein the cytokine polypeptide and the targeting domain are connected by a protease-cleavable linker. To the left of the arrow, the drawing shows that a is connected to targeting domain, blocking moiety, and half-life extension element via protease-cleavable linker(s), thus blocking its ability to bind to its receptor. To the right of the arrow the drawing shows that in an inflammatory or tumor environment, the protease cleaves at a protease-cleavage site on linker(s), releasing the half-life extension element, the targeting domain, and the blocking moiety, and allowing the cytokine to bind to its receptor. The cytokine now has similar pK properties as compared to the native cytokine (e.g., short half-life).

[24] **Figure 4b** is a schematic illustrating a protease-activated cytokine or chemokine comprising a cytokine or chemokine polypeptide, a blocking moiety, a targeting domain, and a serum half-life extending domain connected by at least one protease-cleavable linker. To the left of the arrow, the drawing shows that a cytokine is connected to targeting domain, a blocking moiety, and a half-life extension element via protease-cleavable linker(s), thus blocking its ability to bind to its receptor. To the right of the arrow the drawing shows that in an inflammatory or tumor environment, the protease cleaves at a protease-cleavage site on linker(s), releasing the half-life extension element and the blocking moiety and allowing the cytokine to bind to the receptor. The targeting moiety remains bound, keeping the cytokine in the tumor microenvironment. The cytokine now has similar pK properties as compared to the native cytokine (e.g., a short half-life).

[25] **Figure 5** is a schematic illustrating the structure of a variable domain of an immunoglobulin molecule. The variable domains of both light and heavy immunoglobulin chains contain three hypervariable loops, or complementarity-determining regions (CDRs). The three CDRs of a V domain (CDR1, CDR2, CDR3) cluster at one end of the beta barrel. The CDRs are the loops that connect beta strands B-C, C'-C", and F-G of the immunoglobulin fold, whereas the bottom loops that connect beta strands AB, CC', C" -D and E-F of the immunoglobulin fold, and the top loop that connects the D-E strands of the immunoglobulin fold are the non-CDR loops.

[26] **Figure 6** is a schematic illustrating a protease-activated cytokine or chemokine comprising a

cytokine or chemokine polypeptide, a blocking moiety that is a serum albumin binding domain (e.g., a dAb), and a protease cleavable linker. In the illustrated example, the non-CDR loops in a serum albumin binding domain (e.g., a sdAb) can form a binding site for the cytokine IL-12. In this example, the binding site for serum albumin can be formed by the CDRs of the serum albumin binding domain.

[27] **Figure 7** is a graph depicting results from a HEK-Blue IL-12 reporter assay performed on human p40/murine p35 IL12 fusion proteins before and after protease cleavage. Analysis was performed based on quantification of Secreted Alkaline Phosphatase (SEAP) activity using the reagent QUANTI-Blue® (InvivoGen). Results confirm that IL12 protein fusion proteins are active.

[28] **Figure 8** shows a series of graphs depicting the results of HEK-blue assay of four IL-12 fusion proteins, before and after cleavage by MMP9. Analysis was performed based on quantification of Secreted Alkaline Phosphatase (SEAP) activity using the reagent QUANTI-Blue (InvivoGen). The data show greater activity in the cleaved IL12 than in the full fusion protein. Constructs tested were ACP06 (Fig 8a), ACP07 (Fig 8b), ACP08 (Fig 8c), ACP09 (Fig 8d), ACP10 (8e), ACP11 (8f).

[29] **Figure 9** shows results of protein cleavage assay. Fusion protein ACP11 was run on an SDS-PAGE gel in both cleaved and uncleaved form. As can be seen in the gel, cleavage was complete.

[30] **Figure 10** is a schematic which depicts a non-limiting example of an inducible cytokine protein, wherein the construct is activated upon protease cleavage of a linker attached between two subunits of the cytokine.

[31] **Figures 11a-11d** are graphs depicting results from a HEK-Blue assay performed on human p40/murine p35 IL12 fusion proteins before and after protease cleavage. The assay was performed. Results confirm that IL12 protein fusion proteins are active. Each proliferation assay was performed with HSA or without HSA.

Figure 12 is a series of graphs showing activity of fusion proteins in an HEKBlue IL-12 reporter assay. **Figure 12a** depicts IL-12/STAT4 activation in a comparison of ACP11 (a human p40/murine p35 IL12 fusion protein) to ACP04 (negative control). **Figure 12b** is a graph showing analysis of ACP91 (a chimeric IL-12 fusion protein). Circles depict the activity of the control polypeptide ACP04, squares depict activity of the uncut ACP91 polypeptide, and triangles depict the activity of the cut polypeptide (ACP91 + MMP9). EC50 values for each are shown in the table. **Figure 12c** is a graph showing analysis of ACP136 (a chimeric IL-12 fusion protein). Circles depict the activity of the control polypeptide ACP04, squares depict activity of the uncut ACP136 polypeptide, and triangles depict the activity of the cut polypeptide (ACP136 + MMP9). EC50 values for each are shown in the table insert.

[32] **Figure 13** is a series of graphs showing that cleaved IL-12 polypeptides are active in an HEKBlue reporter assay. The top left panel shows that murine IL-12 is active in this assay (positive control). The bottom left panel and the bottom right panel show the activity of uncut (squares) and cut (triangles), respectively. The EC50 values for each are shown in the table insert, top right panel.

[33] **Figure 14** is two graphs showing results of analyzing ACP31 (mouse IFNα1 fusion protein) and ACP11 (a human p40/murine p35 IL12 fusion protein) in a tumor xenograft model. **Figure 14a** shows tumor volume over time in mice treated with 30μg ACP31 (circles), 110μg ACP31 (triangles), 330μg ACP31 (diamonds), and as controls 1μg murine wild type IFNa1 (dashed line, squares) and 10 μg mIFNa1 (dashed line, small circles). Vehicle alone is indicated by large open circles. The data show tumor volume decreasing over time in a dose-dependent manner in mice treated with ACP31. **Figure 14b** shows tumor volume over time in mice treated with 17.5μg ACP11 (squares), 175μg ACP31 (triangles), 525μg ACP31 (circles), and as controls 2μg ACP04 (dashed line, triangles) and 10 μg ACP04 (dashed line, diamonds). Vehicle alone is indicated by large open circles. The data show tumor volume decreasing over time in a dose-dependent manner in mice treated with both ACP11 and ACP04 (a human p40/murine p35 IL12 fusion protein).

[34] **Figure 15** is a series of spaghetti plots showing tumor volume over time in a mouse xenograft tumor model in mice each treated with vehicle alone (top left), 2μg ACP04 (top middle), 10μg ACP04 (top right), 17.5μg ACP11 (bottom left), 175μg ACP11 (bottom middle), and 525μgACP11 (bottom right). Each line represents a single mouse.

[35] **Figures 16-20** illustrate the properties of TriTac polypeptides, which serve as exemplary protease cleavable fusion proteins.

DETAILED DESCRIPTION

[36] Disclosed herein are methods and compositions to engineer and use constructs comprising inducible IL-12. IL-12 is a potent immune agonist, which lead to it being considered a promising therapeutic agent for oncology. However, IL-12 and other cytokines proved to have a very narrow therapeutic window. Cytokines, such as IL-12, have short serum half-lives and are also considered to be highly potent. Consequently, therapeutic administration of cytokines produced undesirable systemic effects and toxicities. These were exacerbated by the need to administer large quantities of cytokine in order to achieve the desired levels of cytokine at the intended site of cytokine action (e.g., a tumor). Unfortunately, due to the biology of cytokines and inability to effectively target and control their activity, cytokines did not achieve the hoped-for clinical advantages in the treatment of tumors.

[37] Disclosed herein are fusion proteins that overcome the toxicity and short half-life problems that have severely limited the clinical use of IL-12 in oncology. The fusion proteins contain IL-12 polypeptides that have receptor agonist activity. But in the context of the fusion protein, the IL-12 receptor agonist activity is attenuated and the circulating half-life is extended. The fusion proteins include protease cleave sites, which are cleaved by proteases that are associated with a desired site of IL-12 activity (e.g., a tumor), and are typically enriched or selectively present at the site of desired

activity. Thus, the fusion proteins are preferentially (or selectively) and efficiently cleaved at the desired site of activity to limit cytokine activity substantially to the desired site of activity, such as the tumor microenvironment. Protease cleavage at the desired site of activity, such as in a tumor microenvironment, releases a form of IL-12 from the fusion protein that is much more active as a IL-12 receptor agonist than the fusion protein (typically at least about 100X more active than the fusion protein). The form of IL-12 that is released upon cleavage of the fusion protein typically has a short half-life, which is often substantially similar to the half-life of naturally occurring IL-12, further restricting IL-12 cytokine activity to the tumor microenvironment. Even though the half-life of the fusion protein is extended, toxicity is dramatically reduced or eliminated because the circulating fusion protein is attenuated and active cytokine is targeted to the tumor microenvironment. The fusion proteins described herein, for the first time, enable the administration of an effective therapeutic dose of a cytokine to treat tumors with the activity of the cytokine substantially limited to the tumor microenvironment, and dramatically reduces or eliminates unwanted systemic effects and toxicity of the cytokine.

[38] Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a difference over what is generally understood in the art. The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodologies by those skilled in the art, such as, for example, the widely utilized molecular cloning methodologies described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* 4th ed. (2012) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. As appropriate, procedures involving the use of commercially available kits and reagents are generally carried out in accordance with manufacturer-defined protocols and conditions unless otherwise noted.

[39] "Cytokine" is a well-known term of art that refers to any of a class of immunoregulatory proteins (such as interleukin or interferon) that are secreted by cells especially of the immune system and that are modulators of the immune system. Cytokine polypeptides that can be used in the fusion proteins disclosed herein include, but are not limited to transforming growth factors, such as TGF-α and TGF-β (e.g., TGFbeta1, TGFbeta2, TGFbeta3); interferons, such as interferon-α, interferon-β, interferon-γ, interferon-kappa and interferon-omega; interleukins, such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-21 and IL-25; tumor necrosis factors, such as tumor necrosis factor alpha and lymphotoxin; chemokines (e.g., C-X-C motif chemokine 10 (CXCL10), CCL19, CCL20, CCL21), and granulocyte macrophage-colony stimulating factor (GM-CS), as well as fragments of such polypeptides that active the cognate receptors for the cytokine (i.e., functional fragments of the foregoing). "Chemokine" is a

term of art that refers to any of a family of small cytokines with the ability to induce directed chemotaxis in nearby responsive cells.

[40] Cytokines are well-known to have short serum half-lives that frequently are only a few minutes. Even forms of cytokines that have altered amino acid sequences intended to extend the serum half-life yet retain receptor agonist activity typically also have short serum half-lives. As used herein, a "short-half-life cytokine" refers to a cytokine that has a substantially brief half-life circulating in the serum of a subject, such as a serum half-life that is less than 10, less than 15, less than 30, less than 60, less than 90, less than 120, less than 240, or less than 480 minutes. As used herein, a short half-life cytokine includes cytokines which have not been modified in their sequence to achieve a longer than usual half-life in the body of a subject and polypeptides that have altered amino acid sequences intended to extend the serum half-life yet retain receptor agonist activity. This latter case is not meant to include the addition of heterologous protein domains, such as a bona fide half-life extension element, such as serum albumin. Typically a short half-life cytokine polypeptide, such as an IL-12 polypeptide has a serum half-life that is comparable to naturally occurring IL-12, e.g., within 5 fold, 4 fold, 3 fold or 2 fold of naturally occurring IL-12.

[41] "Sortases" are transpeptidases that modify proteins by recognizing and cleaving a carboxyl-terminal sorting signal embedded in or terminally attached to a target protein or peptide. Sortase A catalyzes the cleavage of the LPXTG motif (SEQ ID NO: 80) (where X is any standard amino acid) between the Thr and Gly residue on the target protein, with transient attachment of the Thr residue to the active site Cys residue on the enzyme, forming an enzyme-thioacyl intermediate. To complete transpeptidation and create the peptide-monomer conjugate, a biomolecule with an N-terminal nucleophilic group, typically an oligoglycine motif, attacks the intermediate, displacing Sortase A and joining the two molecules.

[42] As used herein, the term "steric blocker" refers to a polypeptide or polypeptide moiety that can be covalently bonded to a cytokine polypeptide directly or indirectly through other moieties such as linkers, for example in the form of a chimeric polypeptide (fusion protein), but otherwise does not covalently bond to the cytokine polypeptide. A steric blocker can non-covalently bond to the cytokine polypeptide, for example though electrostatic, hydrophobic, ionic or hydrogen bonding. A steric blocker typically inhibits or blocks the activity of the cytokine moiety due to its proximity to the cytokine moiety and comparative size.

[43] As used and described herein, a "half-life extension element" is a part of the chimeric polypeptide that increases the serum half-life and improve pK, for example, by altering its size (e.g., to be above the kidney filtration cutoff), shape, hydrodynamic radius, charge, or parameters of absorption, biodistribution, metabolism, and elimination.

[44] As used herein, the terms "activatable," "activate," "induce," and "inducible" refer to the ability of a protein, i.e. a cytokine, that is part of a fusion protein, to bind its receptor and effectuate activity upon cleavage of additional elements from the fusion protein.

[45] As used herein, "plasmids" or "viral vectors" are agents that transport the disclosed nucleic acids into the cell without degradation and include a promoter yielding expression of the nucleic acid molecule and/or polypeptide in the cells into which it is delivered.

[46] As used herein, the terms "peptide", "polypeptide", or "protein" are used broadly to mean two or more amino acids linked by a peptide bond. Protein, peptide, and polypeptide are also used herein interchangeably to refer to amino acid sequences. It should be recognized that the term polypeptide is not used herein to suggest a particular size or number of amino acids comprising the molecule and that a peptide of the invention can contain up to several amino acid residues or more.

[47] As used throughout, "subject" can be a vertebrate, more specifically a mammal (e.g. a human, horse, cat, dog, cow, pig, sheep, goat, mouse, rabbit, rat, and guinea pig), birds, reptiles, amphibians, fish, and any other animal. The term does not denote a particular age or sex. Thus, adult and newborn subjects, whether male or female, are intended to be covered.

[48] As used herein, "patient" or "subject" may be used interchangeably and can refer to a subject with a disease or disorder (e.g. cancer). The term patient or subject includes human and veterinary subjects.

[49] As used herein the terms "treatment", "treat", or "treating" refers to a method of reducing the effects of a disease or condition or symptom of the disease or condition. Thus, in the disclosed method, treatment can refer to at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or substantially complete reduction in the severity of an established disease or condition or symptom of the disease or condition. For example, a method for treating a disease is considered to be a treatment if there is a 10% reduction in one or more symptoms of the disease in a subject as compared to a control. Thus, the reduction can be a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or any percent reduction in between 10% and 100% as compared to native or control levels. It is understood that treatment does not necessarily refer to a cure or complete ablation of the disease, condition, or symptoms of the disease or condition.

[50] As used herein, the terms "prevent", "preventing", and "prevention" of a disease or disorder refers to an action, for example, administration of the chimeric polypeptide or nucleic acid sequence encoding the chimeric polypeptide, that occurs before or at about the same time a subject begins to show one or more symptoms of the disease or disorder, which inhibits or delays onset or exacerbation of one or more symptoms of the disease or disorder.

[51] As used herein, references to "decreasing", "reducing", or "inhibiting" include a change of at least about 10%, of at least about 20%, of at least about 30%, of at least about 40%, of at least about 50%, of at least about 60%, of at least about 70%, of at least about 80%, of at least about 90% or greater as compared to a suitable control level. Such terms can include but do not necessarily include complete elimination of a function or property, such as agonist activity.

[52] An "attenuated cytokine receptor agonist" is a cytokine receptor agonist that has decreased

receptor agonist activity as compared to the cytokine receptor's naturally occurring agonist. An attenuated cytokine agonist may have at least about 10X, at least about 50X, at least about 100X, at least about 250X, at least about 500X, at least about 1000X or less agonist activity as compared to the receptor's naturally occurring agonist. When a fusion protein that contains a cytokine polypeptide as described herein is described as "attenuated" or having "attenuated activity", it is meant that the fusion protein is an attenuated cytokine receptor agonist.

[53] An "intact fusion protein" is a fusion protein in which no domain has been removed, for example by protease cleavage. A domain may be removable by protease cleavage or other enzymatic activity, but when the fusion protein is "intact", this has not occurred.

[54] As used herein "moiety" refers to a portion of a molecule that has a distinct function within that molecule, and that function may be performed by that moiety in the context of another molecule. A moiety may be a chemical entity with a particular function, or a portion of a biological molecule with a particular function. For example, a "blocking moiety" within a fusion protein is a portion of the fusion protein which is capable of blocking the activity of some or all of the fusion polypeptide. This may be a protein domain, such as serum albumin.

[55] In general, the therapeutic use of cytokines is strongly limited by their systemic toxicity. TNF, for example, was originally discovered for its capacity of inducing the hemorrhagic necrosis of some tumors, and for its in vitro cytotoxic effect on different tumoral lines, but it subsequently proved to have strong pro-inflammatory activity, which can, in case of overproduction conditions, dangerously affect the human body. As the systemic toxicity is a fundamental problem with the use of pharmacologically active amounts of cytokines in humans, novel derivatives and therapeutic strategies are now under evaluation, aimed at reducing the toxic effects of this class of biological effectors while keeping their therapeutic efficacy.

[56] Interleukin-12 (IL-12) is a disulfide-linked heterodimer of two separately encoded subunits (p35 and p40), which are linked covalently to give rise to the so-called bioactive heterodimeric (p70) molecule (Lieschke et al., 1997; Jana et al., 2014). Apart from forming heterodimers (IL-12 and IL-23), the p40 subunit is also secreted as a monomer (p40) and a homodimer ($p40_2$). It is known in the art that synthesis of the heterodimer as a single chain with a linker connecting the p35 to the p40 subunit preserves the full biological activity of the heterodimer. IL-12 plays a critical role in the early inflammatory response to infection and in the generation of Th1 cells, which favor cell-mediated immunity. It has been found that overproduction of IL-12 can be dangerous to the host because it is involved in the pathogenesis of a number of autoimmune inflammatory diseases (e.g. MS, arthritis, type 1 diabetes).

[57] The IL-12 receptor (IL-12R) is a heterodimeric complex consisting of IL-12Rβ1 and IL-12Rβ2 chains expressed on the surface of activated T-cells and natural killer cells (Trinchieri et al., 2003). The IL-12Rβ1 chain binds to the IL-12p40 subunit, whereas IL-12p35 in association with IL-

12Rβ2 confers an intracellular signaling ability (Benson et al., 2011). Signal transduction through IL-12R induces phosphorylation of Janus kinase (Jak2) and tyrosine kinase (Tyk2), that phosphorylate and activate signal transducer and activator of transcription (STAT)1, STAT3, STAT4, and STAT5. The specific cellular effects of IL-12 are due mainly to activation of STAT4. IL-12 induces natural killer and T-cells to produce cytokines, in particular interferon (IFN)γ, that mediate many of the proinflammatory activities of IL-12, including CD4+ T-cell differentiation toward the Th1 phenotype (Montepaone et al., 2014).

[58] IL-12 is a pleiotropic cytokine, the actions of which create an interconnection between the innate and adaptive immunity. IL-12 was first described as a factor secreted from PMA-induced EBV-transformed B-cell lines. Based on its actions, IL-12 has been designated as cytotoxic lymphocyte maturation factor and natural killer cell stimulatory factor. Due to bridging the innate and adaptive immunity and potently stimulating the production of IFNγ, a cytokine coordinating natural mechanisms of anticancer defense, IL-12 seemed ideal candidate for tumor immunotherapy in humans. However, severe side effects associated with systemic administration of IL-12 in clinical investigations and the very narrow therapeutic index of this cytokine markedly tempered enthusiasm for the use of this cytokine in cancer patients (Lasek et. al., 2014). Approaches to IL-12 therapy in which delivery of the cytokine is tumor-targeted, which may diminish some of the previous issues with IL-12 therapy, are currently in clinical trials for cancers.

[59] The present invention is designed to address the shortcomings of direct IL-12 therapy and therapy using other cytokines, for example using cytokine blocking moieties, e.g. steric blocking polypeptides, serum half-life extending polypeptides, targeting polypeptides, linking polypeptides, including protease cleavable linkers, and combinations thereof. Cytokines, including interleukins (e.g., IL-2, IL-7, IL-12, IL-15, IL-18, IL-21 IL-23), interferons (IFNs, including IFNalpha, IFNbeta and IFNgamma), tumor necrosis factors (e.g., TNFalpha, lymphotoxin), transforming growth factors (e.g., TGFbeta1, TGFbeta2, TGFbeta3), chemokines (C-X-C motif chemokine 10 (CXCL10), CCL19, CCL20, CCL21), and granulocyte macrophage-colony stimulating factor (GM-CS) are highly potent when administered to patients. As used herein, "chemokine" means a family of small cytokines with the ability to induce directed chemotaxis in nearby responsive cells Cytokines can provide powerful therapy, but are accompanied by undesired effects that are difficult to control clinically and which have limited the clinical use of cytokines. This disclosure relates to new forms of cytokines that can be used in patients with reduced or eliminated undesired effects. In particular, this disclosure relates to pharmaceutical compositions including chimeric polypeptides (fusion proteins), nucleic acids encoding fusion proteins and pharmaceutical formulations of the foregoing that contain cytokines or active fragments or muteins of cytokines that have decreased cytokine receptor activating activity in comparison to the corresponding cytokine. However, under selected conditions or in a selected biological environment the chimeric polypeptides activate their cognate receptors, often with the same or higher potency as the corresponding naturally occurring cytokine. As described herein, this is

typically achieved using a cytokine blocking moiety that blocks or inhibits the receptor activating function of the cytokine, active fragment or mutein thereof under general conditions but not under selected conditions, such as those present at the desired site of cytokine activity (e.g., an inflammatory site or a tumor).

[60] The chimeric polypeptides and nucleic acids encoding the chimeric polypeptides can be made using any suitable method. For example, nucleic acids encoding a chimeric polypeptide can be made using recombinant DNA techniques, synthetic chemistry or combinations of these techniques, and expressed in a suitable expression system, such as in CHO cells. Chimeric polypeptides can similarly be made, for example by expression of a suitable nucleic acid, using synthetic or semi-synthetic chemical techniques, and the like. In some embodiments, the blocking moiety can be attached to the cytokine polypeptide via sortase-mediated conjugation. "Sortases" are transpeptidases that modify proteins by recognizing and cleaving a carboxyl-terminal sorting signal embedded in or terminally attached to a target protein or peptide. Sortase A catalyzes the cleavage of the LPXTG motif (SEQ ID NO: 80) (where X is any standard amino acid) between the Thr and Gly residue on the target protein, with transient attachment of the Thr residue to the active site Cys residue on the enzyme, forming an enzyme-thioacyl intermediate. To complete transpeptidation and create the peptide-monomer conjugate, a biomolecule with an N-terminal nucleophilic group, typically an oligoglycine motif, attacks the intermediate, displacing Sortase A and joining the two molecules.

[61] To form the cytokine-blocking moiety fusion protein, the cytokine polypeptide is first tagged at the N-terminus with a polyglycine sequence, or alternatively, with at the C-terminus with a LPXTG motif (SEQ ID NO: 80). The blocking moiety or other element has respective peptides attached that serve as acceptor sites for the tagged polypeptides. For conjugation to domains carrying a LPXTG (SEQ ID NO: 80) acceptor peptide attached via its N-terminus, the polypeptide will be tagged with an N-terminal poly-glycine stretch. For conjugation to domain carrying a poly-glycine peptide attached via its C-terminus, the polypeptide will be tagged at its C-terminus with a LPXTG (SEQ ID NO: 80) sortase recognition sequence. Recognizing poly-glycine and LPXTG (SEQ ID NO: 80) sequences, sortase will form a peptide bond between polymer-peptide and tagged polypeptides. The sortase reaction cleaves off glycine residues as intermediates and occurs at room temperature.

[62] A variety of mechanisms can be exploited to remove or reduce the inhibition caused by the blocking moiety. For example, the pharmaceutical compositions can include an IL-12 polypeptide and a blocking moiety, e.g. a steric blocking moiety, with a protease cleavable linker comprising a protease cleavage site located between the IL-12 polypeptide and cytokine blocking moiety or within the cytokine blocking moiety. When the protease cleavage site is cleaved, the blocking moiety can dissociate from cytokine, and the cytokine can then activate cytokine receptor.

[63] Any suitable linker can be used. For example, the linker can comprise glycine-glycine, a sortase-recognition motif, or a sortase-recognition motif and a peptide sequence $(Gly_4Ser)_n$ (SEQ ID NO: 81) or $(Gly_3Ser)_n$ (SEQ ID NO: 82), wherein n is 1, 2, 3, 4 or 5. Typically, the sortase-

recognition motif comprises a peptide sequence LPXTG (SEQ ID NO: 80), where X is any amino acid. In some embodiments, the covalent linkage is between a reactive lysine residue attached to the C-terminal of the cytokine polypeptide and a reactive aspartic acid attached to the N-terminal of the blocker or other domain. In other embodiments, the covalent linkage is between a reactive aspartic acid residue attached to the N-terminal of the cytokine polypeptide and a reactive lysine residue attached to the C-terminal of said blocker or other domain.

[64] Accordingly, as described in detail herein, the cytokine blocking moieties (IL-12 blocking moieties) used can be steric blockers. As used herein, a "steric blocker" refers to a polypeptide or polypeptide moiety that can be covalently bonded to a cytokine polypeptide directly or indirectly through other moieties such as linkers, for example in the form of a chimeric polypeptide (fusion protein), but otherwise does not covalently bond to the cytokine polypeptide. A steric blocker can non-covalently bond to the cytokine polypeptide, for example though electrostatic, hydrophobic, ionic or hydrogen bonding. A steric blocker typically inhibits or blocks the activity of the cytokine moiety due to its proximity to the cytokine moiety and comparative size. The steric inhibition of the cytokine moiety can be removed by spatially separating the cytokine moiety from the steric blocker, such as by enzymatically cleaving a fusion protein that contains a steric blocker and a cytokine polypeptide at a site between the steric blocker and the cytokine polypeptide.

[65] As described in greater detail herein, the blocking function can be combined with or due to the presence of additional functional components in the pharmaceutical composition, such as a targeting domain, a serum half-life extension element, and protease-cleavable linking polypeptides. For example, a serum half-life extending polypeptide can also be a steric blocker.

[66] Various elements ensure the delivery and activity of IL-12 preferentially at the site of desired IL-12 activity and to severely limit systemic exposure to the interleukin via a blocking and/or a targeting strategy preferentially linked to a serum half-life extension strategy. In this serum half-life extension strategy, the blocked version of interleukin circulates for extended times (preferentially 1-2 or more weeks) but the activated version has the typical serum half-life of the interleukin.

[67] In some embodiments of this invention, the half-life extension element is linked to the interleukin via a linker which is cleaved at the site of action (e.g. by inflammation-specific or tumor-specific proteases) releasing the interleukin's full activity at the desired site and also separating it from the half-life extension of the uncleaved version. In such embodiments, the fully active and free interleukin would have very different pharmacokinetic (pK) properties – a half-life of hours instead of weeks. In addition, exposure to active cytokine is limited to the site of desired cytokine activity (e.g., an inflammatory site or tumor) and systemic exposure to active cytokine, and associated toxicity and side effects, are reduced.

[68] Blocking moieties, described in further detail below, can also be used to favor binding to or activation of one or more receptors. This blocking may be relievable by removal of the blocking

moieties in a particular environment, for example by proteolytic cleavage of a linker linking one or more blocking moieties to the cytokine.

[69] In another aspect, a similar approach can be applied to improve other cytokines, particularly for use as immunostimulatory agents, for example for treating cancer. For example, in this aspect, the pharmacokinetics and/or pharmacodynamics of the cytokine (e.g., IL-2, IL-7, IL-12, IL-15, IL-18, IL-21 IL-23, IFNalpha, IFNbeta and IFNgamma, TNFalpha, lymphotoxin, TGFbeta1, TGFbeta2, TGFbeta3 GM-CSF, CXCL10, CCL19, CCL20, and CCL21 can be tailored to maximally activate effector cells (e.g., effect T cells, NK cells) and/or cytotoxic immune response promoting cells (e.g., induce dendritic cell maturation) at a site of desired activity, such as in a tumor, but preferably not systemically.

[70] Thus, provided herein are pharmaceutical compositions comprising at least one cytokine polypeptide, such as interleukins (e.g., IL-2, IL-7, IL-12, IL-15, IL-18, IL-21, IL-23), interferons (IFNs, including IFNalpha, IFNbeta and IFNgamma), tumor necrosis factors (e.g., TNFalpha, lymphotoxin), transforming growth factors (e.g., TGFbeta1, TGFbeta2, TGFbeta3), chemokines (e.g. CXCL10, CCL19, CCL20, CCL21) and granulocyte macrophage-colony stimulating factor (GM-CS) or a functional fragment or mutein of any of the foregoing. The polypeptide typically also includes at least one linker amino acid sequence, wherein the amino acid sequence is in certain embodiments capable of being cleaved by an endogenous protease. In one embodiment, the linker comprises an amino acid sequence comprising HSSKLQ (SEQ ID NO: 24), GPLGVRG (SEQ ID NO: 83), IPVSLRSG (SEQ ID NO: 84), VPLSLYSG (SEQ ID NO: 85), or SGESPAYYTA (SEQ ID NO: 86). In other embodiments, the chimeric polypeptide further contains a blocking moiety, e.g. a steric blocking polypeptide moiety, capable of blocking the activity of the interleukin polypeptide. The blocking moiety, for example, can comprise a human serum albumin (HSA) binding domain or an optionally branched or multi-armed polyethylene glycol (PEG). Alternatively, the pharmaceutical composition comprises a first cytokine polypeptide or a fragment thereof, and blocking moiety, e.g. a steric blocking polypeptide moiety, wherein the blocking moiety blocks the activity of the cytokine polypeptide on the cytokine receptor, and wherein the blocking moiety in certain embodiments comprises a protease cleavable domain. In some embodiments, blockade and reduction of cytokine activity is achieved simply by attaching additional domains with very short linkers to the N or C terminus of the interleukin domain. In such embodiments, it is anticipated the blockade is relieved by protease digestion of the blocking moiety or of the short linker that tethers the blocker to the interleukin. Once the domain is clipped or is released, it will no longer be able to achieve blockade of cytokine activity.

[71] The pharmaceutical composition e.g., chimeric polypeptide can comprise two or more cytokines, which can be the same cytokine polypeptide or different cytokine polypeptides. For example, the two or more different types of cytokines have complementary functions. In some examples, a first cytokine is IL-12 and a second cytokine is IL-2. In some embodiments, each of the

two or more different types of cytokine polypeptides have activities that modulate the activity of the other cytokine polypeptides. In some examples of chimeric polypeptides that contain two cytokine polypeptides, a first cytokine polypeptide is T-cell activating, and a second cytokine polypeptide is non-T-cell-activating. In some examples of chimeric polypeptides that contain two cytokine polypeptides, a first cytokine is a chemoattractant, e.g. CXCL10, and a second cytokine is an immune cell activator.

[72] Preferably, the IL-12 polypeptides (including functional fragments) that are included in the fusion proteins disclosed herein are not mutated or engineered to alter the properties of the naturally occurring cytokine, including receptor binding affinity and specificity or serum half-life. However, changes in amino acid sequence from naturally occurring (including wild type) cytokine are acceptable to facilitate cloning and to achieve desired expression levels, for example.

Blocking Moiety

[73] The blocking moiety can be any moiety that inhibits the ability of the cytokine to bind and/or activate its receptor. The blocking moiety can inhibit the ability of the cytokine to bind and/or activate its receptor sterically blocking and/or by noncovalently binding to the cytokine. Examples of suitable blocking moieties include the full length or a cytokine-binding fragment or mutein of the cognate receptor of the cytokine. Antibodies and fragments thereof including, a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody a single chain variable fragment (scFv), single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL) and a variable domain of camelid-type nanobody (VHH), a dAb and the like that bind the cytokine can also be used. Other suitable antigen-binding domain that bind the cytokine can also be used, include non-immunoglobulin proteins that mimic antibody binding and/or structure such as, anticalins, affilins, affibody molecules, affimers, affitins, alphabodies, avimers, DARPins, fynomers, kunitz domain peptides, monobodies, and binding domains based on other engineered scaffolds such as SpA, GroEL, fibronectin, lipocallin and CTLA4 scaffolds. Further examples of suitable blocking polypeptides include polypeptides that sterically inhibit or block binding of the cytokine to its cognate receptor. Advantageously, such moieties can also function as half-life extending elements. For example, a peptide that is modified by conjugation to a water-soluble polymer, such as PEG, can sterically inhibit or prevent binding of the cytokine to its receptor. Polypeptides, or fragments thereof, that have long serum half-lives can also be used, such as serum albumin (human serum albumin), immunoglobulin Fc, transferring and the like, as well as fragments and muteins of such polypeptides. Antibodies and antigen-binding domains that bind to, for example, a protein with a long serum half-life such as HSA, immunoglobulin or transferrin, or to a receptor that is recycled to the plasma membrane, such as FcRn or transferrin receptor, can also inhibit the cytokine, particularly when bound to their antigen. Examples of such antigen-binding polypeptides include a single chain variable fragment (scFv), single-domain antibody such as a heavy chain variable domain (VH), a light chain

variable domain (VL) and a variable domain of camelid-type nanobody (VHH), a dAb and the like. Other suitable antigen-binding domain that bind the cytokine can also be used, include non-immunoglobulin proteins that mimic antibody binding and/or structure such as, anticalins, affilins, affibody molecules, affimers, affitins, alphabodies, avimers, DARPins, fynomers, kunitz domain peptides, monobodies, and binding domains based on other engineered scaffolds such as SpA, GroEL, fibronectin, lipocallin and CTLA4 scaffolds.

[74] In illustrative examples, when IL-12 is the cytokine in the chimeric polypeptide, the blocking moiety can be the full length or fragment or mutein of the first molecule of IL-12 receptor (IL-12Rβ1) or beta (IL-12Rβ2), an anti-IL-2 single-domain antibody (dAb) or scFv, an anti- IL-12Rβ1 antibody or fragment thereof, an anti- IL-12Rβ2 antibody or fragment thereof, and anti-HSA dAb or scFv, and the like.

Additional aspects of the invention

1. A fusion protein comprising a cytokine moiety that is operably linked to a binding moiety, the binding moiety comprising a non-CDR loop and a cleavable linker, wherein the binding moiety is capable of masking the binding the cytokine to its receptor and/or the activation of the receptor by the cytokine.
2. The fusion protein of aspect 1, wherein the binding moiety is a natural peptide, a synthetic peptide, an engineered scaffold, or an engineered bulk serum protein.
3. The fusion protein of aspect 1 or 2, wherein the engineered scaffold comprises a sdAb, a scFv, a Fab, a VHH, a fibronectin type III domain, immunoglobulin-like scaffold, DARPin, cystine knot peptide, lipocalin, three-helix bundle scaffold, protein G-related albumin-binding module, or a DNA or RNA aptamer scaffold.
4. The fusion protein of any one of aspects 1-2, wherein the binding moiety is capable of binding to a bulk serum protein.
5. The fusion protein of any one of aspects 1-3, wherein the non-CDR loop is from a variable domain, a constant domain, a C1-set domain, a C2-set domain, an I-domain, or any combinations thereof.
6. The fusion protein of any one of aspects 1-4, wherein the binding moiety further comprises complementarity determining regions (CDRs).
7. The fusion protein of aspect 5, wherein the binding moiety is capable of binding to the bulk serum protein.
8. The fusion protein of aspect 6, wherein the bulk serum protein is a half-life extending protein.
9. The fusion protein of aspect 6 or 7, wherein the bulk serum protein is albumin, transferrin, Factor XIII, or Fibrinogen.
10. The fusion protein of any one of aspects5-8, wherein the CDR loop provides the binding site specific for the bulk serum protein or the immunoglobulin light chain, or any combinations thereof.

11. The fusion protein of any one of aspects 1-9, wherein the cleavable linker comprises a cleavage site.
12. The fusion protein of aspect 10, wherein the cleavage site is recognized by a protease.
13. The fusion protein of aspect 11, wherein the binding moiety is bound to the cytokine.
14. The fusion protein of aspect 11 or 11, wherein the binding moiety is covalently linked to the cytokine.
15. The fusion protein of aspect 11, 11, or 14, wherein the binding moiety is capable of masking the binding of the cytokine to its target via specific intermolecular interactions between the binding moiety and the cytokine.
16. The fusion protein of any one of aspects 11-14, wherein the non-CDR loop provides a binding site specific for binding of the moiety to the cytokine.
17. The fusion protein of any one of aspects 11-15, wherein upon cleavage of the cleavable linker, the binding moiety is separated from the cytokine and the cytokine binds to its target.
18. The fusion protein of any one of aspects 1-16, wherein the cytokine binds to a cytokine receptor.
19. The fusion protein of aspect 17, wherein the cytokine receptor comprises a type I cytokine receptor, a type I IL receptor, a type II IL receptor, a chemokine receptor, or a tumor necrosis receptor superfamily receptor.
20. The fusion protein of any one of aspects 1-18, wherein the cleavable linker comprises a cleavage site.
21. The fusion protein of aspect 20, wherein the cleavage site is recognized by a protease.
22. The fusion protein of aspect 21, wherein the protease cleavage site is recognized by a serine protease, a cysteine protease, an aspartate protease, a threonine protease, a glutamic acid protease, a metalloproteinase, a gelatinase, or a asparagine peptide lyase.
23. The fusion protein of aspect 21, wherein the protease cleavage site is recognized by a Cathepsin B, a Cathepsin C, a Cathepsin D, a Cathepsin E, a Cathepsin K, a Cathepsin L, a kallikrein, a hK1, a hK10, a hK15, a plasmin, a collagenase, a Type IV collagenase, a stromelysin, a Factor Xa, a chymotrypsin-like protease, a trypsin-like protease, a elastase-like protease, a subtilisin-like protease, an actinidain, a bromelain, a calpain, a caspase, a caspase-3, a Mir1-CP, a papain, a HIV-1 protease, a HSV protease, a CMV protease, a chymosin, a renin, a pepsin, a matriptase, a legumain, a plasmepsin, a nepenthesin, a metalloexopeptidase, a metalloendopeptidase, a matrix metalloprotease (MMP), a MMP1, a MMP2, a MMP3, a MMP8, a MMP9, a MMP10, a MMP11, a MMP12, a MMP13, a MMP14, an ADAM10, an ADAM17, an ADAM12, an urokinase plasminogen activator (uPA), an enterokinase, a prostate-specific target (PSA, hK3), an interleukin-1β converting enzyme, a thrombin, a FAP (FAP-α), a dipeptidyl peptidase, or dipeptidyl peptidase IV (DPPIV/CD26), a type II transmembrane serine protease (TTSP), a neutrophil elastase, a cathepsin G, a proteinase 3, a neutrophil serine protease 4, a mast cell

chymase, a mast cell tryptase, a dipeptidyl peptidase, and a dipeptidyl peptidase IV (DPPIV/CD26).

24. A conditionally active binding protein comprising a binding moiety (M) which comprises a non-CDR loop, a cytokine, and a cleavable linker (L), wherein the non-CDR loop is capable of binding to the cytokine, and wherein the binding moiety is capable of inhibiting the binding of the cytokine to its receptor and/or inhibiting activation of the receptor by the cytokine.
25. The conditionally active binding protein of aspect 24, wherein the binding moiety is capable of binding to a half-life extending protein.
26. The conditionally active binding protein of aspect 24 or 25, wherein the binding moiety is a natural peptide, a synthetic peptide, an engineered scaffold, or an engineered serum bulk protein.
27. The conditionally active binding protein of aspect 26, wherein the engineered scaffold comprises a sdAb, a scFv, a Fab, a VHH, a fibronectin type III domain, immunoglobulin-like scaffold, DARPin, cystine knot peptide, lipocalin, three-helix bundle scaffold, protein G-related albumin-binding module, or a DNA or RNA aptamer scaffold.
28. The conditionally active binding protein of any one of aspects 24-27, wherein the non-CDR-loop is from a variable domain, a constant domain, a C1-set domain, a C2-set domain, an I-domain, or any combinations thereof.
29. The conditionally active binding protein of any one of aspects 24-28, wherein the binding moiety further comprises complementarity determining regions (CDRs).
30. The conditionally active binding protein of any one of aspects 24-29, wherein the binding moiety comprises a binding site specific for a bulk serum protein.
31. The conditionally active binding protein of aspect 30, wherein the bulk serum protein is albumin, transferrin, Factor XIII, or Fibrinogen.
32. The conditionally active binding protein of any one of aspects 29-31, wherein the CDRs provide the binding site specific for the bulk serum protein or the immunoglobulin light chain, or any combinations thereof.
33. The conditionally active binding protein of any one of aspects29-32, wherein the binding moiety is capable of masking the binding of the cytokine to its target via specific intermolecular interactions between the binding moiety and the cytokine.
34. The conditionally active binding protein of any one of aspects 29-33, wherein the non-CDR loop provides a binding site specific for binding of the binding moiety to the cytokine.
35. The conditionally active binding protein of any one of aspects 24-34, wherein the cytokine binds to a cytokine receptor.
36. The conditionally active binding protein of aspect 35, wherein the cytokine receptor comprises a type I cytokine receptor, a type I IL receptor, a type II IL receptor, a chemokine receptor, or a tumor necrosis receptor superfamily receptor.

37. The conditionally active binding protein of aspect 24-36, wherein the cleavable linker comprises a cleavage site.
38. The conditionally active binding protein of aspect 37, wherein the cleavage site is recognized by a protease.
39. The conditionally active binding protein of aspect 38, wherein the protease cleavage site is recognized by a serine protease, a cysteine protease, an aspartate protease, a threonine protease, a glutamic acid protease, a metalloproteinase, a gelatinase, or a asparagine peptide lyase.
40. The conditionally active binding protein of aspect 38, wherein the protease cleavage site is recognized by a Cathepsin B, a Cathepsin C, a Cathepsin D, a Cathepsin E, a Cathepsin K, a Cathepsin L, a kallikrein, a hK1, a hK10, a hK15, a plasmin, a collagenase, a Type IV collagenase, a stromelysin, a Factor Xa, a chymotrypsin-like protease, a trypsin-like protease, a elastase-like protease, a subtilisin-like protease, an actinidain, a bromelain, a calpain, a caspase, a caspase-3, a Mir1-CP, a papain, a HIV-1 protease, a HSV protease, a CMV protease, a chymosin, a renin, a pepsin, a matriptase, a legumain, a plasmepsin, a nepenthesin, a metalloexopeptidase, a metalloendopeptidase, a matrix metalloprotease (MMP), a MMP1, a MMP2, a MMP3, a MMP8, a MMP9, a MMP10, a MMP11, a MMP12, a MMP13, a MMP14, an ADAM10, an ADAM17, an ADAM12, an urokinase plasminogen activator (uPA), an enterokinase, a prostate-specific target (PSA, hK3), an interleukin-1β converting enzyme, a thrombin, a FAP (FAP-α), a dipeptidyl peptidase, or dipeptidyl peptidase IV (DPPIV/CD26), a type II transmembrane serine protease (TTSP), a neutrophil elastase, a cathepsin G, a proteinase 3, a neutrophil serine protease 4, a mast cell chymase, a mast cell tryptase, a dipeptidyl peptidase, and a dipeptidyl peptidase IV (DPPIV/CD26).
41. The conditionally active binding protein of aspect 24, further comprising a half-life extension domain bound to the binding moiety, wherein the half-life extension domain provides the binding protein with a safety switch, and wherein upon cleavage of the linker the binding protein is activated by separation of the binding moiety and the half-life extension domain from the cytokine, and the binding protein is thereby separated from the safety switch.
42. The conditionally active binding protein of aspect 41, wherein the cleavage of the linker is in a tumor microenvironment.
43. A conditionally active binding protein, comprising a binding moiety that binds a cytokine via a non-CDR loop within the binding moiety, wherein the binding moiety is further linked to a half-life extension domain and comprises a cleavable linker, wherein the binding protein has an extended half-life prior to its activation by cleavage of the linker, and wherein upon activation the binding moiety and the half-life extension domain are separated from the cytokine, and wherein the binding protein, in its activated state, does not have an extended half-life.
44. The conditionally active binding protein of aspect 43, wherein the cleavage of the linker is in a tumor microenvironment.

In vivo Half-life Extension Elements

[75] Preferably, the chimeric polypeptides comprise an in vivo half-life extension element. Increasing the in vivo half-life of therapeutic molecules with naturally short half-lives allows for a more acceptable and manageable dosing regimen without sacrificing effectiveness. As used herein, a "half-life extension element" is a part of the chimeric polypeptide that increases the in vivo half-life and improve pK, for example, by altering its size (e.g., to be above the kidney filtration cutoff), shape, hydrodynamic radius, charge, or parameters of absorption, biodistribution, metabolism, and elimination. An exemplary way to improve the pK of a polypeptide is by expression of an element in the polypeptide chain that binds to receptors that are recycled to the plasma membrane of cells rather than degraded in the lysosomes, such as the FcRn receptor on endothelial cells and transferrin receptor. Three types of proteins, e.g., human IgGs, HSA (or fragments), and transferrin, persist for much longer in human serum than would be predicted just by their size, which is a function of their ability to bind to receptors that are recycled rather than degraded in the lysosome. These proteins, or fragments of them that retain the FcRn binding are routinely linked to other polypeptides to extend their serum half-life. In one embodiment, the half-life extension element is a human serum albumin (HSA) binding domain. HSA (SEQ ID NO: 1) may also be directly bound to the pharmaceutical compositions or bound via a short linker. Fragments of HSA may also be used. HSA and fragments thereof can function as both a blocking moiety and a half-life extension element. Human IgGs can also carry out a similar function.

[76] The serum half-life extension element can also be antigen-binding polypeptide that binds to a protein with a long serum half-life such as serum albumin, transferrin and the like. Examples of such polypeptides include antibodies and fragments thereof including, a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody a single chain variable fragment (scFv), single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL) and a variable domain of camelid-type nanobody (VHH), a dAb and the like. Other suitable antigen-binding domain include non-immunoglobulin proteins that mimic antibody binding and/or structure such as, anticalins, affilins, affibody molecules, affimers, affitins, alphabodies, avimers, DARPins, fynomers, kunitz domain peptides, monobodies, and binding domains based on other engineered scaffolds such as SpA, GroEL, fibronectin, lipocallin and CTLA4 scaffolds. Further examples of antigen-binding polypeptides include a ligand for a desired receptor, a ligand-binding portion of a receptor, a lectin, and peptides that binds to or associates with one or more target antigens.

[77] Some preferred serum half-life extension elements are polypeptides that comprise complementarity determining regions (CDRs), and optionally non-CDR loops. Advantageously, such serum half-life extension elements can extend the serum half-life of the cytokine, and also function as inhibitors of the cytokine (e.g., via steric blocking, non-covalent interaction or combination thereof) and/or as targeting

domains. In some instances, the serum half-life extension elements are domains derived from an immunoglobulin molecule (Ig molecule) or engineered protein scaffolds that mimic antibody structure and/or binding activity. The Ig may be of any class or subclass (IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM etc). A polypeptide chain of an Ig molecule folds into a series of parallel beta strands linked by loops. In the variable region, three of the loops constitute the "complementarity determining regions" (CDRs) which determine the antigen binding specificity of the molecule. An IgG molecule comprises at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding fragment thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs) with are hypervariable in sequence and/or involved in antigen recognition and/or usually form structurally defined loops, interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. In some embodiments of this disclosure, at least some or all of the amino acid sequences of FR1, FR2, FR3, and FR4 are part of the "non-CDR loop" of the binding moieties described herein. As shown in **Fig. 5**, a variable domain of an immunoglobulin molecule has several beta strands that are arranged in two sheets. The variable domains of both light and heavy immunoglobulin chains contain three hypervariable loops, or complementarity-determining regions (CDRs). The three CDRs of a V domain (CDR1, CDR2, CDR3) cluster at one end of the beta barrel. The CDRs are the loops that connect beta strands B-C, C'-C", and F-G of the immunoglobulin fold, whereas the bottom loops that connect beta strands AB, CC', C" -D and E-F of the immunoglobulin fold, and the top loop that connects the D-E strands of the immunoglobulin fold are the non-CDR loops. In some embodiments of this disclosure, at least some amino acid residues of a constant domain, CH1, CH2, or CH3, are part of the "non-CDR loop" of the binding moieties described herein. Non-CDR loops comprise, in some embodiments, one or more of AB, CD, EF, and DE loops of a C1-set domain of an Ig or an Ig-like molecule; AB, CC', EF, FG, BC, and EC' loops of a C2-set domain of an Ig or an Ig-like molecule; DE, BD, GF, A(A1A2)B, and EF loops of I(Intermediate)-set domain of an Ig or Ig-like molecule.

[78] Within the variable domain, the CDRs are believed to be responsible for antigen recognition and binding, while the FR residues are considered a scaffold for the CDRs. However, in certain cases, some of the FR residues play an important role in antigen recognition and binding. Framework region residues that affect Ag binding are divided into two categories. The first are FR residues that contact the antigen, thus are part of the binding-site, and some of these residues are close in sequence to the CDRs. Other residues are those that are far from the CDRs in sequence, but are in close proximity to it in the 3-D structure of the molecule, *e.g.*, a loop in heavy chain. The serum half-life extension domain (e.g., a domain that comprises

CDRs) can comprise at least one non-CDR loop. In some embodiments, a non-CDR loop provides a binding site for binding to a cytokine, bulk serum protein or other target antigen.

[79] The serum half-life extension element, in addition to or alternatively to containing CDRs, comprises a non-CDR loop. In some embodiments, the non-CDR loop is modified to generate an antigen binding site specific for a desired target antigen, such as a bulk serum protein, such as albumin, or for the cytokine moiety or other targeting antigen. It is contemplated that various techniques can be used for modifying the non-CDR loop, e.g., site-directed mutagenesis, random mutagenesis, insertion of at least one amino acid that is foreign to the non-CDR loop amino acid sequence, amino acid substitution. An antigen peptide is inserted into a non-CDR loop, in some examples. In some examples, an antigenic peptide is substituted for the non-CDR loop. The modification, to generate an antigen binding site, is in some cases in only one non-CDR loop. In other instances, more than one non-CDR loop are modified. For instance, the modification is in any one of the non-CDR loops shown in **Fig. 5**, *i.e.*, AB, CC', C" D, EF, and D-E. In some cases, the modification is in the DE loop. In other cases the modifications are in all four of AB, CC', C" –D, E-F loops.

[80] In some examples, the serum half-life extension element has dual binding specificity and contains CDRs that specifically bind a bulk serum proteins, such as serum albumin, and non-CDR loops that specifically bind and block the cytokine domain. In other examples, the serum half-life extension element contains CDRs that specifically bind a target antigen, such as the cytokine domain or other target antigen, and non-CDR loops that specifically bind a bulk serum protein, such as serum albumin. Preferably, the serum half-life extension element inhibits binding of the cytokine domain to the cognate cytokine receptor, e.g. via steric occlusion, via specific intermolecular interactions, or a combination of both.

[81] In some embodiments, the serum half-life extension element noncovalently binds directly to the cytokine and inhibit its activity.

[82] In certain examples, the binding moiety binds to a cytokine via one or more of AB, CC', C" D, and E-F loop and binds to a bulk-serum protein, such as albumin, via one or more of BC, C'C", and FG loop. In certain examples, the binding moiety binds to a bulk serum protein, such as albumin, via its AB, CC', C" D, or EF loop and binds to a cytokine via its BC, C'C", or FG loop. In certain examples, the binding moiety of the binds to a bulk serum protein, such as albumin, via its AB, CC', C" D, and EF loop and is bound to a cytokine via its BC, C'C", and FG loop. In certain examples, the binding moiety binds to a bulk serum protein, such as albumin, via one or more of AB, CC', C" D, and E-F loop and binds to a cytokine, via one or more of BC, C'C", and FG loop.

[83] The binding moieties are any kinds of polypeptides. For example, in certain instances the binding moieties are natural peptides, synthetic peptides, or fibronectin scaffolds, or engineered bulk serum proteins. The bulk serum protein comprises, for example, albumin, fibrinogen, or a globulin. In some embodiments, the binding moieties are an engineered scaffolds. Engineered scaffolds comprise, for example, sdAb, a scFv, a Fab, a VHH, a fibronectin type III domain, immunoglobulin-like scaffold (as suggested in Halaby et al., 1999. Prot Eng 12(7):563-571), DARPin, cystine knot peptide, lipocalin, three-helix bundle scaffold, protein G-related albumin-binding module, or a DNA or RNA aptamer scaffold.

[84] In some cases, the serum half-life extension element binds to the cytokine domain via its non-CDR loops and the cytokine domain is further connected to a targeting domain as described herein. In some cases, the serum half-life extending element comprises a binding site for a bulk serum protein. In some embodiments, the CDRs provide the binding site for the bulk serum protein. The bulk serum protein is, in some examples, a globulin, albumin, transferrin, IgG1, IgG2, IgG4, IgG3, IgA monomer, Factor XIII, Fibrinogen, IgE, or pentameric IgM. In some embodiments, the CDR form a binding site for an immunoglobulin light chain, such as an Igκ free light chain or an Igλ free light chain.

[85] One exemplary conditionally active protein is shown in **Fig. 6**. In the illustrated example, the non-CDR loops in a serum albumin binding domain (e.g. a dAb) can form a binding site for the cytokine IL-12. In this example, the binding site for serum albumin can be formed by the CDRs of the serum albumin binding domain.

[86] The serum half-life extension element can be any type of binding domain, including but not limited to, domains from a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody. In some embodiments, the binding moiety is a single chain variable fragment (scFv), single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL) and a variable domain (VHH) of camelid derived nanobody. In other embodiments, the binding moieties are non-Ig binding domains, i.e., antibody mimetic, such as anticalins, affilins, affibody molecules, affimers, affitins, alphabodies, avimers, DARPins, fynomers, kunitz domain peptides, and monobodies.

[87] In other embodiments, the serum half-life extension element can be a water-soluble polymer or a peptide that is conjugated to a water-soluble polymer, such as PEG. "PEG," "polyethylene glycol" and "poly(ethylene glycol)" as used herein, are interchangeable and encompass any nonpeptidic water-soluble poly(ethylene oxide). The term "PEG" also means a polymer that contains a majority, that is to say, greater than 50%, of —OCH_2CH_2— repeating subunits. With respect to specific forms, the PEG can take any number of a variety of molecular weights, as well as structures or geometries such as "branched," "linear," "forked," "multifunctional," and the like, to be described in greater detail below. The PEG is not limited to a particular structure and can be linear (e.g., an end capped, e.g., alkoxy PEG or a bifunctional PEG), branched or multi-armed (e.g., forked PEG or PEG attached to a polyol core), a dendritic (or star) architecture, each with or without one or more degradable linkages. Moreover, the internal structure of the PEG can be organized in any number of different repeat patterns and can be selected from the group consisting of homopolymer, alternating copolymer, random copolymer, block copolymer, alternating tripolymer, random tripolymer, and block tripolymer. PEGs can be conjugated to polypeptide and peptides through any suitable method. Typically a reactive PEG derivative, such as N-hydroxysuccinamidyl ester PEG, is reacted with a peptide or polypeptide that includes amino acids with a side chain that contains an amine, sulfhydryl, carboxylic acid or hydroxyl functional group, such as cysteine, lysine, asparagine, glutamine, theonine, tyrosine, serine, aspartic acid, and glutamic acid.

Targeting and Retention Domains

[88] For certain applications, it may be desirable to maximize the amount of time the construct is present in its desired location in the body. This can be achieved by including one further domain in the chimeric polypeptide (fusion protein) to influence its movements within the body. For example, the chimeric nucleic acids can encode a domain that directs the polypeptide to a location in the body, e.g., tumor cells or a site of inflammation; this domain is termed a "targeting domain" and/or encode a domain that retains the polypeptide in a location in the body, e.g., tumor cells or a site of inflammation; this domain is termed a "retention domain". In some embodiments a domain can function as both a targeting and a retention domain. In some embodiments, the targeting domain and/or retention domain are specific to a protease-rich environment. In some embodiments, the encoded targeting domain and/or retention domain are specific for regulatory T cells (Tregs), for example targeting the CCR4 or CD39 receptors. Other suitable targeting and/or retention domains comprise those that have a cognate ligand that is overexpressed in inflamed tissues, e.g., the IL-1 receptor, or the IL-6 receptor. In other embodiments, the suitable targeting and/or retention domains comprise those who have a cognate ligand that is overexpressed in tumor tissue, e.g., Epcam, CEA or mesothelin. In some embodiments, the targeting domain is linked to the interleukin via a linker which is cleaved at the site of action (e.g. by inflammation or cancer specific proteases) releasing the interleukin full activity at the desired site. In some embodiments, the targeting and/or retention domain is linked to the interleukin via a linker which is not cleaved at the site of action (e.g. by inflammation or cancer specific proteases), causing the cytokine to remain at the desired site.

[89] Antigens of choice, in some cases, are expressed on the surface of a diseased cell or tissue, for example a tumor or a cancer cell. Antigens useful for tumor targeting and retention include but are not limited to Fibroblast activation protein alpha (FAPa), Trophoblast glycoprotein (5T4), Tumor-associated calcium signal transducer 2 (Trop2), Fibronectin EDB (EDB-FN), FOLR1, fibronectin EIIIB domain, EpCAM, EGFR, HER-2, HER-3, c-Met, FOLR1, and CEA. Pharmaceutical compositions disclosed herein, also include proteins comprising two targeting and/or retention domains that bind to two different target antigens known to be expressed on a diseased cell or tissue. Exemplary pairs of antigen binding domains include but are not limited to EGFR/CEA, EpCAM/CEA, and HER-2/HER-3.

[90] Suitable targeting and/or retention domains include antigen-binding domains, such as antibodies and fragments thereof including, a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody a single chain variable fragment (scFv), single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL) and a variable domain of camelid-type nanobody (VHH), a dAb and the like. Other suitable antigen-binding domain include non-immunoglobulin proteins that mimic antibody binding and/or structure such as, anticalins, affilins, affibody molecules, affimers, affitins, alphabodies, avimers, DARPins, fynomers, kunitz domain peptides, monobodies, and binding domains based on other engineered scaffolds such as SpA,

GroEL, fibronectin, lipocallin and CTLA4 scaffolds. Further examples of antigen-binding polypeptides include a ligand for a desired receptor, a ligand-binding portion of a receptor, a lectin, and peptides that binds to or associates with one or more target antigens.

[91] In some embodiments, the targeting and/or retention domains specifically bind to a cell surface molecule. In some embodiments, the targeting and/or retention domains specifically bind to a tumor antigen. In some embodiments, the targeting polypeptides specifically and independently bind to a tumor antigen selected from at least one of Fibroblast activation protein alpha (FAPa), Trophoblast glycoprotein (5T4), Tumor-associated calcium signal transducer 2 (Trop2), Fibronectin EDB (EDB-FN), FOLR1, fibronectin EIIIB domain, EpCAM, EGFR, HER-2, HER-3, cMet, CEA, and FOLR1. In some embodiments, the targeting polypeptides specifically and independently bind to two different antigens, wherein at least one of the antigens is a tumor antigen selected from Fibroblast activation protein alpha (FAPa), Trophoblast glycoprotein (5T4), Tumor-associated calcium signal transducer 2 (Trop2), Fibronectin EDB (EDB-FN), FOLR1, fibronectin EIIIB domain, EpCAM, EGFR, HER-2, HER-3, cMet, CEA, and FOLR1.

[92] The targeting and/or retention antigen can be a tumor antigen expressed on a tumor cell. Tumor antigens are well known in the art and include, for example, Fibroblast activation protein alpha (FAPa), Trophoblast glycoprotein (5T4), Tumor-associated calcium signal transducer 2 (Trop2), Fibronectin EDB (EDB-FN), FOLR1, fibronectin EIIIB domain, EpCAM, EGFR, HER-2, HER-3, c-Met, FOLR1, PSMA, CD38, BCMA, and CEA. 5T4, AFP, B7-H3, Cadherin-6, CAIX, CD117, CD123, CD138, CD166, CD19, CD20, CD205, CD22, CD30, CD33, CD352, CD37, CD44, CD52, CD56, CD70, CD71, CD74, CD79b, DLL3, EphA2, FAP, FGFR2, FGFR3, GPC3, gpA33, FLT-3, gpNMB, HPV-16 E6, HPV-16 E7, ITGA2, ITGA3, SLC39A6, MAGE, mesothelin, Muc1, Muc16, NaPi2b, Nectin-4, P-cadherin, NY-ESO-1, PRLR, PSCA, PTK7, ROR1, SLC44A4, SLTRK5, SLTRK6, STEAP1, TIM1, Trop2, WT1.

[93] The targeting and/or retention antigen can be an immune checkpoint protein. Examples of immune checkpoint proteins include but are not limited to CD27, CD137, 2B4, TIGIT, CD155, ICOS, HVEM, CD40L, LIGHT, TIM-1, OX40, DNAM-1, PD-L1, PD1, PD-L2, CTLA-4, CD8, CD40, CEACAM1, CD48, CD70, A2AR, CD39, CD73, B7-H3, B7-H4, BTLA, IDO1, IDO2, TDO, KIR, LAG-3, TIM-3, or VISTA.

[94] The targeting and/or retention antigen can be a cell surface molecule such as a protein, lipid or polysaccharide. In some embodiments, a targeting and/or retention antigen is a on a tumor cell, virally infected cell, bacterially infected cell, damaged red blood cell, arterial plaque cell, inflamed or fibrotic tissue cell. The targeting and/or retention antigen can comprise an immune response modulator. Examples of immune response modulator include but are not limited to granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 12 (IL-12), interleukin 15 (IL-15), B7-1 (CD80), B7-2 (CD86), GITRL, CD3, or GITR.

[95] The targeting and/or retention antigen can be a cytokine receptor. Examples, of cytokine receptors include but are not limited to Type I cytokine receptors, such as GM-CSF receptor, G-CSF receptor, Type I

IL receptors, Epo receptor, LIF receptor, CNTF receptor, TPO receptor; Type II Cytokine receptors, such as IFN-alpha receptor (IFNAR1, IFNAR2), IFB-beta receptor, IFN-gamma receptor (IFNGR1, IFNGR2), Type II IL receptors; chemokine receptors, such as CC chemokine receptors, CXC chemokine receptors, CX3C chemokine receptors, XC chemokine receptors; tumor necrosis receptor superfamily receptors, such as TNFRSF5/CD40, TNFRSF8/CD30, TNFRSF7/CD27, TNFRSF1A/TNFR1/CD120a, TNFRSF1B / TNFR2 / CD120b; TGF-beta receptors, such as TGF-beta receptor 1, TGF-beta receptor 2; Ig super family receptors, such as IL-1 receptors, CSF-1R, PDGFR (PDGFRA, PDGFRB), SCFR.

Linkers

[96] As stated above, the pharmaceutical compositions comprise one or more linker sequences. A linker sequence serves to provide flexibility between polypeptides, such that, for example, the blocking moiety is capable of inhibiting the activity of the cytokine polypeptide. The linker sequence can be located between any or all of the cytokine polypeptide, the serum half-life extension element, and/or the blocking moiety. As described herein at least one of the linkers is protease cleavable, and contains a (one or more) cleavage site for a (one or more) desired protease. Preferably, the desired protease is enriched or selectively expressed at the desired site of cytokine activity (e.g., the tumor microenvironment). Thus, the fusion protein is preferentially or selectively cleaved at the site of desired cytokine activity.

[97] The orientation of the components of the pharmaceutical composition, are largely a matter of design choice and it is recognized that multiple orientations are possible and all are intended to be encompassed by this disclosure. For example, a blocking moiety can be located C-terminally or N-terminally to a cytokine polypeptide.

[98] Proteases known to be associated with diseased cells or tissues include but are not limited to serine proteases, cysteine proteases, aspartate proteases, threonine proteases, glutamic acid proteases, metalloproteases, asparagine peptide lyases, serum proteases, cathepsins, Cathepsin B, Cathepsin C, Cathepsin D, Cathepsin E, Cathepsin K, Cathepsin L, kallikreins, hKl, hK10, hK15, plasmin, collagenase, Type IV collagenase, stromelysin, Factor Xa, chymotrypsin-like protease, trypsin-like protease, elastase-like protease, subtili sin-like protease, actinidain, bromelain, calpain, caspases, caspase-3, Mirl-CP, papain, HIV-1 protease, HSV protease, CMV protease, chymosin, renin, pepsin, matriptase, legumain, plasmepsin, nepenthesin, metalloexopeptidases, metalloendopeptidases, matrix metalloproteases (MMP), MMP1, MMP2, MMP3, MMP8, MMP9, MMP13, MMP11, MMP14, urokinase plasminogen activator (uPA), enterokinase, prostate-specific antigen (PSA, hK3), interleukin-1β converting enzyme, thrombin, FAP (FAP-a), dipeptidyl peptidase, meprins, granzymes and dipeptidyl peptidase IV (DPPIV/CD26). Proteases capable of cleaving amino acid sequences encoded by the chimeric nucleic acid sequences provided herein can, for example, be selected from the group consisting of a prostate specific antigen (PSA), a matrix metalloproteinase (MMP), an A Disintigrin and a Metalloproteinase (ADAM), a plasminogen activator, a cathepsin, a caspase, a tumor

cell surface protease, and an elastase. The MMP can, for example, be matrix metalloproteinase 2 (MMP2) or matrix metalloproteinase 9 (MMP9).

[99] Proteases useful in the methods disclosed herein are presented in Table 1, and exemplary proteases and their cleavage site are presented in Table 1a:

Table 1. Proteases relevant to inflammation and cancer

Protease	Specificity	Other aspects
Secreted by killer T cells:		
Granzyme B (grB)	Cleaves after Asp residues (asp-ase)	Type of serine protease; strongly implicated in inducing perforin-dependent target cell apoptosis
Granzyme A (grA)	trypsin-like, cleaves after basic residues	Type of serine protease;
Granzyme H (grH)	Unknown substrate specificity	Type of serine protease; *Other granzymes are also secreted by killer T cells, but not all are present in humans*
Caspase-8	Cleaves after Asp residues	Type of cysteine protease; plays essential role in TCR-induced cellular expansion-exact molecular role unclear
Mucosa-associated lymphoid tissue (MALT1)	Cleaves after arginine residues	Type of cysteine protease; likely acts both as a scaffold and proteolytically active enzyme in the CBM-dependent signaling pathway
Tryptase	Targets: angiotensin I, fibrinogen, prourokinase, TGFβ; preferentially cleaves proteins after lysine or arginine residues	Type of mast cell-specific serine protease; trypsin-like; resistant to inhibition by macromolecular protease inhibitors expressed in mammals due to their tetrameric structure, with all sites facing narrow central pore; *also associated with inflammation*
Associated with inflammation:		
Thrombin	Targets: FGF-2, HB-EGF, Osteo-pontin, PDGF, VEGF	Type of serine protease; modulates activity of vascular growth factors, chemokines and extracellular proteins; strengthens VEGF-induced proliferation; induces cell migration; angiogenic factor; regulates hemostasis
Chymase	Exhibit chymotrypsin-like specificity, cleaving proteins after aromatic amino acid residues	Type of mast cell-specific serine protease
Carboxypeptidase A (MC-CPA)	Cleaves amino acid residues from C-terminal	Type of zinc-dependent metalloproteinase

	end of peptides and proteins	
Kallikreins	Targets: high molecular weight kininogen, pro-urokinase	Type of serine protease; modulate relaxation response; contribute to inflammatory response; fibrin degradation
Elastase	Targets: E-cadherin, GM-CSF, IL-1, IL-2, IL-6, IL8, $p38^{MAPK}$, TNFα, VE-cadherin	Type of neutrophil serine protease; degrades ECM components; regulates inflammatory response; activates pro-apoptotic signaling
Cathepsin G	Targets: EGF, ENA-78, IL-8, MCP-1, MMP-2, MT1-MMP, PAI-1, RANTES, TGFβ, TNFα	Type of serine protease; degrades ECM components; chemo-attractant of leukocytes; regulates inflammatory response; promotes apoptosis
PR-3	Targets: ENA-78, IL-8, IL-18, JNK, $p38^{MAPK}$, TNFα	Type of serine protease; promotes inflammatory response; activates pro-apoptotic signaling
Granzyme M (grM)	Cleaves after Met and other long, unbranched hydrophobic residues	Type of serine protease; only expressed in NK cells
Calpains	Cleave between Arg and Gly	Family of cysteine proteases; calcium-dependent; activation is involved in the process of numerous inflammation-associated diseases

Table 1a: Exemplary Proteases and Protease Recognition Sequences

Protease	Cleavage Domain Sequence	SEQ ID NO:
MMP7	**KRALGLPG**	**2**
MMP7	**$(DE)_8$RPLALWRS$(DR)_8$**	**3**
MMP9	**PR(S/T)(L/I)(S/T)**	**4**
MMP9	**LEATA**	**5**
MMP11	**GGAANLVRGG**	**6**

MMP14	SGRIGFLRTA	7
MMP	PLGLAG	8
MMP	PLGLAX	9
MMP	PLGC(me)AG	10
MMP	ESPAYYTA	11
MMP	RLQLKL	12
MMP	RLQLKAC	13
MMP2, MMP9, MMP14	EP(Cit)G(Hof)YL	14
Urokinase plasminogen activator (uPA)	SGRSA	15
Urokinase plasminogen activator (uPA)	DAFK	16
Urokinase plasminogen activator (uPA)	GGGRR	17
Lysosomal Enzyme	GFLG	18
Lysosomal Enzyme	ALAL	19
Lysosomal Enzyme	FK	20
Cathepsin B	NLL	21
Cathepsin D	PIC(Et)FF	22

Cathepsin K	GGPRGLPG	23
Prostate Specific Antigen	HSSKLQ	24
Prostate Specific Antigen	HSSKLQL	25
Prostate Specific Antigen	HSSKLQEDA	26
Herpes Simplex Virus Protease	LVLASSSFGY	27
HIV Protease	GVSQNYPIVG	28
CMV Protease	GVVQASCRLA	29
Thrombin	F(Pip)RS	30
Thrombin	DPRSFL	31
Thrombin	PPRSFL	32
Caspase-3	DEVD	33
Caspase-3	DEVDP	34
Caspase-3	KGSGDVEG	35
Interleukin 1β converting enzyme	GWEHDG	36
Enterokinase	EDDDDKA	37
FAP	KQEQNPGST	38

Kallikrein 2	**GKAFRR**	**39**
Plasmin	**DAFK**	**40**
Plasmin	**DVLK**	**41**
Plasmin	**DAFK**	**42**
TOP	**ALLLALL**	**43**

[100] Provided herein are pharmaceutical compositions comprising polypeptide sequences. As with all peptides, polypeptides, and proteins, including fragments thereof, it is understood that additional modifications in the amino acid sequence of the chimeric polypeptides (amino acid sequence variants) can occur that do not alter the nature or function of the peptides, polypeptides, or proteins. Such modifications include conservative amino acid substitutions and are discussed in greater detail below.

[101] The compositions provided herein have a desired function. The compositions are comprised of at least an IL-12 cytokine polypeptide, a blocking moiety, e.g. a steric blocking polypeptide, and an optional serum half-life extension element, and an optional targeting polypeptide, with one or more linkers connecting each polypeptide in the composition. The first polypeptide, e.g., an IL-12 polypeptide, is provided to be an active agent. The blocking moiety is provided to block the activity of the interleukin. The linker polypeptide, e.g., a protease cleavable polypeptide, is provided to be cleaved by a protease that is specifically expressed at the intended target of the active agent. Optionally, the blocking moiety blocks the activity of the first polypeptide by binding the interleukin polypeptide. In some embodiments, the blocking moiety, e.g. a steric blocking peptide, is linked to the interleukin via a protease-cleavable linker which is cleaved at the site of action (e.g. by inflammation specific proteases) releasing the cytokine full activity at the desired site.

[102] The protease cleavage site may be a naturally occurring protease cleavage site or an artificially engineered protease cleavage site. The artificially engineered protease cleavage site can be cleaved by more than one protease specific to the desired environment in which cleavage will occur, e.g. a tumor. The protease cleavage site may be cleavable by at least one protease, at least two proteases, at least three proteases, or at least four proteases.

[103] In some embodiments, the linker comprises glycine-glycine, a sortase-recognition motif, or a sortase-recognition motif and a peptide sequence $(Gly_4Ser)_n$ (SEQ ID NO: 81) or $(Gly_3Ser)_n$ (SEQ ID NO: 82), wherein n is 1, 2, 3, 4 or 5. In one embodiment, the sortase-recognition motif comprises a

peptide sequence LPXTG (SEQ ID NO: 80), where X is any amino acid. In one embodiment, the covalent linkage is between a reactive lysine residue attached to the C-terminal of the cytokine polypeptide and a reactive aspartic acid attached to the N-terminal of the blocking or other moiety. In one embodiment, the covalent linkage is between a reactive aspartic acid residue attached to the N-terminal of the cytokine polypeptide and a reactive lysine residue attached to the C-terminal of the blocking or other moiety.

Cleavage and Inducibility

[104] As described herein, the activity of the cytokine polypeptide the context of the fusion protein is attenuated, and protease cleavage at the desired site of activity, such as in a tumor microenvironment, releases a form of the cytokine from the fusion protein that is much more active as a cytokine receptor agonist than the fusion protein. For example, the cytokine-receptor activating (agonist) activity of the fusion polypeptide can be at least about 10X, at least about 50X, at least about 100X, at least about 250X, at least about 500X, or at least about 1000x less than the cytokine receptor activating activity of the cytokine polypeptide as a separate molecular entity. The cytokine polypeptide that is part of the fusion protein exists as a separate molecular entity when it contains an amino acid that is substantially identical to the cytokine polypeptide and does not substantially include additional amino acids and is not associated (by covalent or non-covalent bonds) with other molecules. If necessary, a cytokine polypeptide as a separate molecular entity may include some additional amino acid sequences, such as a tag or short sequence to aid in expression and/or purification.

[105] In other examples, the cytokine-receptor activating (agonist) activity of the fusion polypeptide is at least about 10X, at least about 50X, at least about 100X, at least about 250X, at least about 500X, or about 1000x less than the cytokine receptor activating activity of the polypeptide that contains the cytokine polypeptide that is produced by cleavage of the protease cleavable linker in the fusion protein. In other words, the cytokine receptor activating (agonist) activity of the polypeptide that contains the cytokine polypeptide that is produced by cleavage of the protease cleavable linker in the fusion protein is at least about 10X, at least about 50X, at least about 100X, at least about 250X, at least about 500X, or at least about 1000x greater than the cytokine receptor activating activity of the fusion protein.

Polypeptide substitutions

[106] The polypeptides described herein can include components (e.g., the cytokine, the blocking moiety) that have the same amino acid sequence of the corresponding naturally occurring protein (e.g., IL-2, IL-15, HSA) or can have an amino acid sequence that differs from the naturally occurring protein so long as the desired function is maintained. It is understood that one way to define any known modifications and derivatives or those that might arise, of the disclosed proteins and nucleic

acids that encode them is through defining the sequence variants in terms of identity to specific known reference sequences. Specifically disclosed are polypeptides and nucleic acids which have at least, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 percent identity to the chimeric polypeptides provided herein. For example, provided are polypeptides or nucleic acids that have at least, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 percent identity to the sequence of any of the nucleic acids or polypeptides described herein. Those of skill in the art readily understand how to determine the identity of two polypeptides or two nucleic acids. For example, the identity can be calculated after aligning the two sequences so that the identity is at its highest level.

[107] Another way of calculating identity can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman Adv. Appl. Math. 2:482 (1981), by the identity alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection.

[108] The same types of identity can be obtained for nucleic acids by, for example, the algorithms disclosed in Zuker, Science 244:48-52 (1989); Jaeger et al., Proc. Natl. Acad. Sci. USA 86:7706-7710 (1989); Jaeger et al., Methods Enzymol. 183:281-306 (1989), which are herein incorporated by reference for at least material related to nucleic acid alignment. It is understood that any of the methods typically can be used and that in certain instances the results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity, and be disclosed herein.

[109] Protein modifications include amino acid sequence modifications. Modifications in amino acid sequence may arise naturally as allelic variations (e.g., due to genetic polymorphism), may arise due to environmental influence (e.g., by exposure to ultraviolet light), or may be produced by human intervention (e.g., by mutagenesis of cloned DNA sequences), such as induced point, deletion, insertion and substitution mutants. These modifications can result in changes in the amino acid sequence, provide silent mutations, modify a restriction site, or provide other specific mutations. Amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional modifications. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; insertions usually will be on the order of about from 1 to 10

amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e. a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional modifications are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 2 and are referred to as conservative substitutions.

Table 2. Exemplary amino acid substitutions

Amino Acid	**Exemplary Substitutions**
Ala	Ser, Gly, Cys
Arg	Lys, Gln, Met, Ile
Asn	Gln, His, Glu, Asp
Asp	Glu, Asn, Gln
Cys	Ser, Met, Thr
Gln	Asn, Lys, Glu, Asp
Glu	Asp, Asn, Gln
Gly	Pro, Ala
His	Asn, Gln
Ile	Leu, Val, Met
Leu	Ile, Val, Met
Lys	Arg, Gln, Met, Ile
Met	Leu, Ile, Val
Phe	Met, Leu, Tyr, Trp, His

Ser	Thr, Met, Cys
Thr	Ser, Met, Val
Trp	Tyr, Phe
Tyr	Trp, Phe, His
Val	Ile, Leu, Met

[110] Modifications, including the specific amino acid substitutions, are made by known methods. For example, modifications are made by site specific mutagenesis of nucleotides in the DNA encoding the polypeptide, thereby producing DNA encoding the modification, and thereafter expressing the DNA in recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis.

[111] Modifications can be selected to optimize binding. For example, affinity maturation techniques can be used to alter binding of the scFv by introducing random mutations inside the complementarity determining regions (CDRs). Such random mutations can be introduced using a variety of techniques, including radiation, chemical mutagens or error-prone PCR. Multiple rounds of mutation and selection can be performed using, for example, phage display.

[112] The disclosure also relates to nucleic acids that encode the chimeric polypeptides described herein, and to the use of such nucleic acids to produce the chimeric polypeptides and for therapeutic purposes. For example, the invention includes DNA and RNA molecules (e.g., mRNA, self-replicating RNA) that encode a chimeric polypeptide and to the therapeutic use of such DNA and RNA molecules.

Exemplary Compositions

[113] Exemplary fusion proteins of the invention combine the above described elements in a variety of orientations. The orientations described in this section are meant as examples and are not to be considered limiting.

[114] In some embodiments, the fusion protein comprises an IL-12 polypeptide, a blocking moiety and a half-life extension element. In some embodiments, the IL-12 polypeptide, is positioned between the half-life extension element and the blocking moiety. In some embodiments, the IL-12 polypeptide, is N-terminal to the blocking moiety and the half-life extension element. In some such embodiments, IL-12 polypeptide, is proximal to the blocking moiety; in some such embodiments, the IL-12 polypeptide, is proximal to the half-life extension element. At least one protease-cleavable linker must be included in all embodiments, such that the IL-12 polypeptide, may be active upon cleavage. In some embodiments, the IL-12 polypeptide, is C-terminal to the blocking moiety and the

half-life extension element. Additional elements may be attached to one another by a cleavable linker, a non-cleavable linker, or by direct fusion.

[115] In some embodiments, the blocking domains used are capable of extending half-life, and the IL-12 polypeptide, is positioned between two such blocking domains. In some embodiments, the IL-12 polypeptide, is positioned between two blocking domains, one of which is capable of extending half-life.

[116] In some embodiments, two cytokines are included in the same construct. In some embodiments, the cytokines are connected to two blocking domains each (three in total in one molecule), with a blocking domain between the two cytokine domains. In some embodiments, one or more additional half-life extension domains may be included to optimize pharmacokinetic properties.

[117] In some embodiments, three cytokines are included in the same construct. In some embodiments, the third cytokine may function to block the other two in place of a blocking domain between the two cytokines.

[118] Preferred half-life extension elements for use in the fusion proteins are human serum albumin (HSA), an antibody or antibody fragment (e.g., scFV, dAb) which binds serum albumin, a human or humanized IgG, or a fragment of any of the foregoing. In some preferred embodiments, the blocking moiety is human serum albumin (HSA), or an antibody or antibody fragment which binds serum albumin, an antibody which binds the cytokine and prevents activation of binding or activation of the cytokine receptor, another cytokine, or a fragment of any of the foregoing. In preferred embodiments comprising an additional targeting domain, the targeting domain is an antibody which binds a cell surface protein which is enriched on the surface of cancer cells, such as EpCAM, FOLR1, and Fibronectin.

Methods of treatment and Pharmaceutical Compositions

[119] Further provided are methods of treating a subject with or at risk of developing an of a disease or disorder, such as proliferative disease, a tumorous disease, an inflammatory disease, an immunological disorder, an autoimmune disease, an infectious disease, a viral disease, an allergic reaction, a parasitic reaction, or graft-versus-host disease. The methods administering to a subject in need thereof an effective amount of a fusion protein as disclosed herein that is typically administered as a pharmaceutical composition. In some embodiments, the method further comprises selecting a subject with or at risk of developing such a disease or disorder. The pharmaceutical composition preferably comprises a blocked IL-12 polypeptide, fragment or mutein thereof that is activated at a site of inflammation. In one embodiment, the chimeric polypeptide comprises an IL-12 polypeptide, fragment or mutein thereof and a serum half-life extension element. In another embodiment, the chimeric polypeptide comprises an IL-12 polypeptide, fragment or mutein thereof and a blocking moiety, e.g. a steric blocking polypeptide, wherein the steric blocking polypeptide is capable of sterically blocking the activity of the IL-12 polypeptide, fragment or mutein thereof. In another embodiment, the chimeric polypeptide comprises an IL-12 polypeptide, fragment or mutein thereof, a

blocking moiety, and a serum half-life extension element.

[120] Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants, and is a protective response involving immune cells, blood vessels, and molecular mediators. The function of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair. Inflammation can occur from infection, as a symptom or a disease, e.g., cancer, atherosclerosis, allergies, myopathies, HIV, obesity, or an autoimmune disease. An autoimmune disease is a chronic condition arising from an abnormal immune response to a self-antigen. Autoimmune diseases that may be treated with the polypeptides disclosed herein include but are not limited to lupus, celiac disease, diabetes mellitus type 1, Graves disease, inflammatory bowel disease, multiple sclerosis, psoriasis, rheumatoid arthritis, and systemic lupus erythematosus.

[121] The pharmaceutical composition can comprise one or more protease-cleavable linker sequences. The linker sequence serves to provide flexibility between polypeptides, such that each polypeptide is capable of inhibiting the activity of the first polypeptide. The linker sequence can be located between any or all of the cytokine polypeptide, fragment or mutein thereof, the blocking moiety, and serum half-life extension element. Optionally, the composition comprises, two, three, four, or five linker sequences. The linker sequence, two, three, or four linker sequences can be the same or different linker sequences. In one embodiment, the linker sequence comprises GGGGS (SEQ ID NO: 87), GSGSGS (SEQ ID NO: 88), or $G(SGGG)_2SGGT$ (SEQ ID NO: 89). In another embodiment, the linker comprises a protease-cleavable sequence selected from group consisting of HSSKLQ (SEQ ID NO: 24), GPLGVRG (SEQ ID NO: 83), IPVSLRSG (SEQ ID NO: 84), VPLSLYSG (SEQ ID NO: 85), and SGESPAYYTA (SEQ ID NO: 86).

[122] Suitable linkers can be of different lengths, such as from 1 amino acid (e.g., Gly) to 20 amino acids, from 2 amino acids to 15 amino acids, from 3 amino acids to 12 amino acids, including 4 amino acids to 10 amino acids, amino acids to 9 amino acids, 6 amino acids to 8 amino acids, or 7 amino acids to 8 amino acids, and may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 amino acids.

[123] In some embodiments, the linker is cleaved by a protease selected from the group consisting of a kallikrein, thrombin, chymase, carboxypeptidase A, cathepsin G, an elastase, PR-3, granzyme M, a calpain, a matrix metalloproteinase (MMP), a plasminogen activator, a cathepsin, a caspase, a tryptase, or a tumor cell surface protease.

[124] Further provided are methods of treating a subject with or at risk of developing cancer. The methods comprise administering to the subject in need thereof an effective amount of a chimeric polypeptide (a fusion protein) as disclosed herein that is typically administered as a pharmaceutical composition. In some embodiments, the method further comprises selecting a subject with or at risk of

developing cancer. The pharmaceutical composition preferably comprises a blocked cytokine, fragment or mutein thereof that is activated at a tumor site. Preferably, the tumor is a solid tumor. The cancer may be a colon cancer, a lung cancer, a melanoma, a sarcoma, a renal cell carcinoma, a breast cancer…

[125] The method can further involve the administration of one or more additional agents to treat cancer, such as chemotherapeutic agents (e.g., Adriamycin, Cerubidine, Bleomycin, Alkeran, Velban, Oncovin, Fluorouracil, Thiotepa, Methotrexate, Bisantrene, Noantrone, Thiguanine, Cytaribine, Procarabizine), immuno-oncology agents (e.g., anti-PD-L1, anti-CTLA4, anti-PD-1, anti-CD47, anti-GD2), cellular therapies (e.g, CAR-T, T-cell therapy), oncolytic viruses and the like.

[126] Provided herein are pharmaceutical formulations or compositions containing the chimeric polypeptides and a pharmaceutically acceptable carrier. The herein provided compositions are suitable for administration *in vitro* or *in vivo*. By pharmaceutically acceptable carrier is meant a material that is not biologically or otherwise undesirable, i.e., the material is administered to a subject without causing undesirable biological effects or interacting in a deleterious manner with the other components of the pharmaceutical formulation or composition in which it is contained. The carrier is selected to minimize degradation of the active ingredient and to minimize adverse side effects in the subject.

[127] Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy,* 21st Edition, David B. Troy, ed., Lippicott Williams & Wilkins (2005). Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic, although the formulate can be hypertonic or hypotonic if desired. Examples of the pharmaceutically-acceptable carriers include, but are not limited to, sterile water, saline, buffered solutions like Ringer's solution, and dextrose solution. The pH of the solution is generally about 5 to about 8 or from about 7 to 7.5. Other carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the immunogenic polypeptides. Matrices are in the form of shaped articles, e.g., films, liposomes, or microparticles. Certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered. Carriers are those suitable for administration of the chimeric polypeptides or nucleic acid sequences encoding the chimeric polypeptides to humans or other subjects.

[128] The pharmaceutical formulations or compositions are administered in a number of ways depending on whether local or systemic treatment is desired and on the area to be treated. The compositions are administered via any of several routes of administration, including topically, orally, parenterally, intravenously, intra-articularly, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, intrahepatically, intracranially, nebulization/inhalation, or by installation via bronchoscopy. In some embodiments, the compositions are administered locally (non-systemically), including intratumorally, intra-articularly, intrathecally, etc.

[129] Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives are optionally present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

[130] Formulations for topical administration include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids, and powders. Conventional pharmaceutical carriers, aqueous, powder, or oily bases, thickeners and the like are optionally necessary or desirable.

[131] Compositions for oral administration include powders or granules, suspension or solutions in water or non-aqueous media, capsules, sachets, or tables. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders are optionally desirable.

[132] Optionally, the chimeric polypeptides or nucleic acid sequences encoding the chimeric polypeptides are administered by a vector. There are a number of compositions and methods which can be used to deliver the nucleic acid molecules and/or polypeptides to cells, either *in vitro* or *in vivo* via, for example, expression vectors. These methods and compositions can largely be broken down into two classes: viral based delivery systems and non-viral based delivery systems. Such methods are well known in the art and readily adaptable for use with the compositions and methods described herein. Such compositions and methods can be used to transfect or transduce cells in vitro or in vivo, for example, to produce cell lines that express and preferably secrete the encoded chimeric polypeptide or to therapeutically deliver nucleic acids to a subject. The components of the chimeric nucleic acids disclosed herein typically are operably linked in frame to encode a fusion protein.

[133] As used herein, plasmid or viral vectors are agents that transport the disclosed nucleic acids into the cell without degradation and include a promoter yielding expression of the nucleic acid molecule and/or polypeptide in the cells into which it is delivered. Viral vectors are, for example, Adenovirus, Adeno-associated virus, herpes virus, Vaccinia virus, Polio virus, Sindbis, and other RNA viruses, including these viruses with the HIV backbone. Also preferred are any viral families which share the properties of these viruses which make them suitable for use as vectors. Retroviral vectors, in general are described by Coffin et al., Retroviruses, Cold Spring Harbor Laboratory Press (1997), which is incorporated by reference herein for the vectors and methods of making them. The construction of replication-defective adenoviruses has been described (Berkner et al., J. Virol. 61:1213-20 (1987); Massie et al., Mol. Cell. Biol. 6:2872-83 (1986); Haj-Ahmad et al., J. Virol. 57:267-74 (1986); Davidson et al., J. Virol. 61:1226-39 (1987); Zhang et al., BioTechniques 15:868-72 (1993)). The benefit and the use of these viruses as vectors is that they are limited in the extent to

which they can spread to other cell types, since they can replicate within an initial infected cell, but are unable to form new infectious viral particles. Recombinant adenoviruses have been shown to achieve high efficiency after direct, in vivo delivery to airway epithelium, hepatocytes, vascular endothelium, CNS parenchyma, and a number of other tissue sites. Other useful systems include, for example, replicating and host-restricted non-replicating vaccinia virus vectors.

[134] The provided polypeptides and/or nucleic acid molecules can be delivered via virus like particles. Virus like particles (VLPs) consist of viral protein(s) derived from the structural proteins of a virus. Methods for making and using virus like particles are described in, for example, Garcea and Gissmann, Current Opinion in Biotechnology 15:513-7 (2004).

[135] The provided polypeptides can be delivered by subviral dense bodies (DBs). DBs transport proteins into target cells by membrane fusion. Methods for making and using DBs are described in, for example, Pepperl-Klindworth et al., Gene Therapy 10:278-84 (2003).

[136] The provided polypeptides can be delivered by tegument aggregates. Methods for making and using tegument aggregates are described in International Publication No. WO 2006/110728.

[137] Non-viral based delivery methods, can include expression vectors comprising nucleic acid molecules and nucleic acid sequences encoding polypeptides, wherein the nucleic acids are operably linked to an expression control sequence. Suitable vector backbones include, for example, those routinely used in the art such as plasmids, artificial chromosomes, BACs, YACs, or PACs. Numerous vectors and expression systems are commercially available from such corporations as Novagen (Madison, Wis.), Clonetech (Pal Alto, Calif.), Stratagene (La Jolla, Calif.), and Invitrogen/Life Technologies (Carlsbad, Calif.). Vectors typically contain one or more regulatory regions. Regulatory regions include, without limitation, promoter sequences, enhancer sequences, response elements, protein recognition sites, inducible elements, protein binding sequences, 5′ and 3′ untranslated regions (UTRs), transcriptional start sites, termination sequences, polyadenylation sequences, and introns. Such vectors can also be used to make the chimeric polypeptides by expression is a suitable host cell, such as CHO cells.

[138] Preferred promoters controlling transcription from vectors in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis B virus, and most preferably cytomegalovirus (CMV), or from heterologous mammalian promoters, e.g. β-actin promoter or EF1α promoter, or from hybrid or chimeric promoters (e.g., CMV promoter fused to the β-actin promoter). Of course, promoters from the host cell or related species are also useful herein.

[139] Enhancer generally refers to a sequence of DNA that functions at no fixed distance from the transcription start site and can be either 5′ or 3′ to the transcription unit. Furthermore, enhancers can be within an intron as well as within the coding sequence itself. They are usually between 10 and 300 base pairs (bp) in length, and they function in cis. Enhancers usually function to increase transcription from nearby promoters. Enhancers can also contain response elements that mediate the regulation of

transcription. While many enhancer sequences are known from mammalian genes (globin, elastase, albumin, fetoprotein, and insulin), typically one will use an enhancer from a eukaryotic cell virus for general expression. Preferred examples are the SV40 enhancer on the late side of the replication origin, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

[140] The promoter and/or the enhancer can be inducible (e.g. chemically or physically regulated). A chemically regulated promoter and/or enhancer can, for example, be regulated by the presence of alcohol, tetracycline, a steroid, or a metal. A physically regulated promoter and/or enhancer can, for example, be regulated by environmental factors, such as temperature and light. Optionally, the promoter and/or enhancer region can act as a constitutive promoter and/or enhancer to maximize the expression of the region of the transcription unit to be transcribed. In certain vectors, the promoter and/or enhancer region can be active in a cell type specific manner. Optionally, in certain vectors, the promoter and/or enhancer region can be active in all eukaryotic cells, independent of cell type. Preferred promoters of this type are the CMV promoter, the SV40 promoter, the β-actin promoter, the EF1α promoter, and the retroviral long terminal repeat (LTR).

[141] The vectors also can include, for example, origins of replication and/or markers. A marker gene can confer a selectable phenotype, e.g., antibiotic resistance, on a cell. The marker product is used to determine if the vector has been delivered to the cell and once delivered is being expressed. Examples of selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase, neomycin, neomycin analog G418, hygromycin, puromycin, and blasticidin. When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective pressure. Examples of other markers include, for example, the *E. coli* lacZ gene, green fluorescent protein (GFP), and luciferase. In addition, an expression vector can include a tag sequence designed to facilitate manipulation or detection (e.g., purification or localization) of the expressed polypeptide. Tag sequences, such as GFP, glutathione S-transferase (GST), polyhistidine, c-myc, hemagglutinin, or FLAG™ tag (Kodak; New Haven, Conn.) sequences typically are expressed as a fusion with the encoded polypeptide. Such tags can be inserted anywhere within the polypeptide including at either the carboxyl or amino terminus.

[142] As used herein, the terms peptide, polypeptide, or protein are used broadly to mean two or more amino acids linked by a peptide bond. Protein, peptide, and polypeptide are also used herein interchangeably to refer to amino acid sequences. It should be recognized that the term polypeptide is not used herein to suggest a particular size or number of amino acids comprising the molecule and that a peptide of the invention can contain up to several amino acid residues or more. As used throughout, subject can be a vertebrate, more specifically a mammal (e.g. a human, horse, cat, dog, cow, pig, sheep, goat, mouse, rabbit, rat, and guinea pig), birds, reptiles, amphibians, fish, and any other animal. The term does not denote a particular age or sex. Thus, adult and newborn subjects, whether male or female, are intended to be covered. As used herein, patient or subject may be used

interchangeably and can refer to a subject with a disease or disorder (e.g. cancer). The term patient or subject includes human and veterinary subjects.

[143] A subject at risk of developing a disease or disorder can be genetically predisposed to the disease or disorder, e.g., have a family history or have a mutation in a gene that causes the disease or disorder, or show early signs or symptoms of the disease or disorder. A subject currently with a disease or disorder has one or more than one symptom of the disease or disorder and may have been diagnosed with the disease or disorder.

[144] The methods and agents as described herein are useful for both prophylactic and therapeutic treatment. For prophylactic use, a therapeutically effective amount of the chimeric polypeptides or chimeric nucleic acid sequences encoding the chimeric polypeptides described herein are administered to a subject prior to onset (e.g., before obvious signs of cancer or inflammation) or during early onset (e.g., upon initial signs and symptoms of cancer or inflammation). Prophylactic administration can occur for several days to years prior to the manifestation of symptoms of cancer or inflammation. Prophylactic administration can be used, for example, in the preventative treatment of subjects diagnosed with a genetic predisposition to cancer. Therapeutic treatment involves administering to a subject a therapeutically effective amount of the chimeric polypeptides or nucleic acid sequences encoding the chimeric polypeptides described herein after diagnosis or development of cancer or inflammation (e.g., an autoimmune disease). Prophylactic use may also apply when a patient is undergoing a treatment, e.g., a chemotherapy, in which inflammation is expected.

[145] According to the methods taught herein, the subject is administered an effective amount of the agent (e.g., a chimeric polypeptide). The terms effective amount and effective dosage are used interchangeably. The term effective amount is defined as any amount necessary to produce a desired physiologic response. Effective amounts and schedules for administering the agent may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for administration are those large enough to produce the desired effect in which one or more symptoms of the disease or disorder are affected (e.g., reduced or delayed). The dosage should not be so large as to cause substantial adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, type of disease, the extent of the disease or disorder, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any contraindications. Dosages can vary and can be administered in one or more dose administrations daily, for one or several days. Guidance can be found in the literature for appropriate dosages for given classes of pharmaceutical products.

[146] As used herein the terms treatment, treat, or treating refers to a method of reducing the effects of a disease or condition or symptom of the disease or condition. Thus, in the disclosed method, treatment can refer to a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% reduction in the severity of an established disease or condition or symptom of the disease or condition. For example, a

method for treating a disease is considered to be a treatment if there is a 10% reduction in one or more symptoms of the disease in a subject as compared to a control. Thus, the reduction can be a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or any percent reduction in between 10% and 100% as compared to native or control levels. It is understood that treatment does not necessarily refer to a cure or complete ablation of the disease, condition, or symptoms of the disease or condition.

[147] As used herein, the terms prevent, preventing, and prevention of a disease or disorder refers to an action, for example, administration of the chimeric polypeptide or nucleic acid sequence encoding the chimeric polypeptide, that occurs before or at about the same time a subject begins to show one or more symptoms of the disease or disorder, which inhibits or delays onset or exacerbation of one or more symptoms of the disease or disorder. As used herein, references to decreasing, reducing, or inhibiting include a change of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater as compared to a control level. Such terms can include but do not necessarily include complete elimination.

[148] Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutations of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a method is disclosed and discussed and a number of modifications that can be made to a number of molecules including the method are discussed, each and every combination and permutation of the method, and the modifications that are possible are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed. This concept applies to all aspects of this disclosure including, but not limited to, steps in methods using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed, it is understood that each of these additional steps can be performed with any specific method steps or combination of method steps of the disclosed methods, and that each such combination or subset of combinations is specifically contemplated and should be considered disclosed.

[149] Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference in their entireties.

EXAMPLES

[150] The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided herein.

Example 1: Protease cleavage of fusion protein by MMP9 protease

[151] One of skill in the art would be familiar with methods of setting up protein cleavage assay. 100ug of protein in 1xPBS pH 7.4 were cleaved with 1 ug active MMP9 (Sigma catalog # SAE0078-50 or Enzo catalog BML-SE360) and incubated at room temperature for up to 16 hours. Digested protein can be subsequently used in functional assays or stored at -80°C prior to testing. Extent of cleavage was monitored by SDS PAGE using methods well known in the art.

Example 2: HEK Blue assay

[152] HEK-Blue IL12 cells (InvivoGen) were plated in suspension at a concentration of 250,000 cells/well in culture media with or without 40mg/ml human serum albumin (HSA) and stimulated with a dilution series of recombinant hIL12, chimeric IL12 (mouse p35/human p40) or activatable hIL12 for 24 hours at 37°C and 5% CO_2. Activity of uncleaved and cleaved activatable hIL12 was tested. Cleaved inducible hIL12 was generated by incubation with active MMP9. IL12 activity was assessed by quantification of Secreted Alkaline Phosphatase (SEAP) activity using the reagent QUANTI-Blue (InvivoGen), a colorimetric based assay.

Example 3: Splenocyte T-Blast assay

[153] T-Blasts were induced from murine splenocytes with a 6-day incubation with PHA and a 24hr incubation with recombinant hIL12. Tblasts were then plated in suspension at a concentration of 200,000 cells/well in culture media with or without 40mg/ml human serum albumin (HSA) and stimulated with a dilution series of recombinant hIL12 or chimeric IL12 (mouse p35/human p40) or mouse IL12 for 72 hours at 37°C and 5% CO_2. Activity of uncleaved and cleaved IL12 was tested. Cleaved inducible hIL12 was generated by incubation with active MMP9. IL12 activity was assessed by downstream quantification of IFNγ production using a mIFNγ alphaLISA.

Example 4: *In Vivo* Delivery of a Protease Activated Fusion Protein Results in Decreased Tumor Growth

[154] The chimeric polypeptide is examined to determine if it could have biological effects *in vivo*. For these experiments a system is used in which tumor cells injected intraperitoneally rapidly and preferentially attach and grow initially on the milky spots, a series of organized immune aggregates found on the omentum (Gerber et al., Am. J. Pathol. 169:1739-52 (2006)). This system offers a convenient way to examine the effects of fusion protein treatment on tumor growth since fusion proteins can be delivered intraperitoneally multiple times and tumor growth can be analyzed by examining the dissociated omental cells. For these experiments, the Colon 38 cell line, a rapidly growing tumor cell line that expresses both MMP2 and MMP9 *in vitro*, may be used. The omental tissue normally expresses a relatively small amount of MMP2 and MMP9, but, when Colon 38 tumor is present on the omentum, MMP levels increase. Using this tumor model, the ability of IL-2 mutein

fusion proteins to affect tumor growth is examined. Colon 38 cells are injected intraperitoneally, allowed to attach and grow for 1 day, and then treated daily with fusion protein interaperitoneally. At day 7, the animals are sacrificed and the omenta examined for tumor growth using flow cytometry and by a colony-forming assay.

Example 5: Construction of an Exemplary Activatable Interleukin Protein Targeting CD20

Generation of an activatable interleukin domain

[155] The human IL-12p35 chain canonical sequence is Uniprot Accession No. P29459. The human IL-12p40 chain canonical sequence is Uniprot Accession No. P29460. IL-12p35 and IL-12p40 are cloned into an expression construct. A protease cleavage site is included between the IL-12p35 and IL-12p40 domains. An IL-12 polypeptide capable of binding to CD20 polypeptide present in a tumor or on a tumor cell is produced as follows. A nucleic acid is produced that contains nucleic acid sequences: (1) encoding an IFNg polypeptide sequence and (2) one or more polypeptide linkers. Activatable interleukin plasmid constructs can have optional Flag, His or other affinity tags, and are electroporated into HEK293 or other suitable human or mammalian cell lines and purified. Validation assays include T cell activation assays using T cells responsive to IL-12 stimulation in the presence of a protease.

Generation of a scFv CD20 binding domain

[156] CD20 is one of the cell surface proteins present on B-lymphocytes. CD20 antigen is found in normal and malignant pre-B and mature B lymphocytes, including those in over 90% of B-cell non-Hodgkin's lymphomas (NHL). The antigen is absent in hematopoietic stem cells, activated B lymphocytes (plasma cells) and normal tissue. As such, several antibodies mostly of murine origin have been described: 1F5, 2B8/C2B8, 2H7, and 1H4.

[157] Human or humanized anti-CD20 antibodies are therefore used to generate scFv sequences for CD20 binding domains of an activatable interleukin protein. DNA sequences coding for human or humanized VL and VH domains are obtained, and the codons for the constructs are, optionally, optimized for expression in cells from Homo sapiens. The order in which the VL and VH domains appear in the scFv is varied (i.e., VL-VH, or VH-VL orientation), and three copies of the "G4S" (SEQ ID NO: 87) or "G_4S" (SEQ ID NO: 87) subunit $(G_4S)_3$ (SEQ ID NO: 90) connect the variable domains to create the scFv domain. Anti-CD20 scFv plasmid constructs can have optional Flag, His or other affinity tags, and are electroporated into HEK293 or other suitable human or mammalian cell lines and purified. Validation assays include binding analysis by FACS, kinetic analysis using Proteon, and staining of CD20-expressing cells.

Cloning of DNA expression constructs encoding the activatable interleukin protein

[158] The activatable interleukin construct with protease cleavage site domains are used to construct an activatable interleukin protein in combination with an anti-CD20 scFv domain and a serum half-life extension element (e.g., a HSA binding peptide or VH domain). For expression of an activatable interleukin protein in CHO cells, coding sequences of all protein domains are cloned into a mammalian expression vector system. In brief, gene sequences encoding the activatable interleukin domain, serum half-life extension element, and CD20 binding domain along with peptide linkers L1 and L2 are separately synthesized and subcloned. The resulting constructs are then ligated together in the order of CD20 binding domain – L1 – IL-12p35– L2 – protease cleavage domain – L3 – IL-12p40 – L4 – anti-CD20 scFv – L5 – serum half-life extension element to yield a final construct. All expression constructs are designed to contain coding sequences for an N-terminal signal peptide and a C-terminal hexahistidine (6xHis)-tag (SEQ ID NO: 91) to facilitate protein secretion and purification, respectively.

Expression of activatable interleukin proteins in stably transfected CHO cells

[159] A CHO cell expression system (Flp-In®, Life Technologies), a derivative of CHO-K1 Chinese Hamster ovary cells (ATCC, CCL-61) (Kao and Puck, Proc. Natl. Acad Sci USA 1968;60(4):1275-81), is used. Adherent cells are subcultured according to standard cell culture protocols provided by Life Technologies.

[160] For adaption to growth in suspension, cells are detached from tissue culture flasks and placed in serum-free medium. Suspension-adapted cells are cryopreserved in medium with 10% DMSO.

[161] Recombinant CHO cell lines stably expressing secreted activatable interleukin proteins are generated by transfection of suspension-adapted cells. During selection with the antibiotic Hygromycin B viable cell densities are measured twice a week, and cells are centrifuged and resuspended in fresh selection medium at a maximal density of $0.1x10^6$ viable cells/mL. Cell pools stably expressing activatable interleukin proteins are recovered after 2-3 weeks of selection at which point cells are transferred to standard culture medium in shake flasks. Expression of recombinant secreted proteins is confirmed by performing protein gel electrophoresis or flow cytometry. Stable cell pools are cryopreserved in DMSO containing medium.

[162] Activatable interleukin proteins are produced in 10-day fed-batch cultures of stably transfected CHO cell lines by secretion into the cell culture supernatant. Cell culture supernatants are harvested after 10 days at culture viabilities of typically >75%. Samples are collected from the production cultures every other day and cell density and viability are assessed. On day of harvest, cell culture supernatants are cleared by centrifugation and vacuum filtration before further use.

[163] Protein expression titers and product integrity in cell culture supernatants are analyzed by SDS-PAGE.

Purification of activatable interleukin proteins

[164] Activatable interleukin proteins are purified from CHO cell culture supernatants in a two-step procedure. The constructs are subjected to affinity chromatography in a first step followed by preparative size exclusion chromatography (SEC) on Superdex 200 in a second step. Samples are buffer-exchanged and concentrated by ultrafiltration to a typical concentration of >1 mg/mL. Purity and homogeneity (typically >90%) of final samples are assessed by SDS PAGE under reducing and non-reducing conditions, followed by immunoblotting using an anti-HSA or anti idiotype antibody as well as by analytical SEC, respectively. Purified proteins are stored at aliquots at -80°C until use.

Example 6: Determination of antigen affinity by flow cytometry

[165] The activatable interleukin proteins of the preceding examples are tested for their binding affinities to human $CD20^+$ cells and cynomolgus $CD20^+$ cells.

[166] $CD20^+$ cells are incubated with 100 µL of serial dilutions of the activatable interleukin proteins of the preceding examples and at least one protease. After washing three times with FACS buffer the cells are incubated with 0.1 mL of 10 µg/mL mouse monoclonal anti-idiotype antibody in the same buffer for 45 min on ice. After a second washing cycle, the cells are incubated with 0.1 mL of 15 µg/mL FITC-conjugated goat anti-mouse IgG antibodies under the same conditions as before. As a control, cells are incubated with the anti-His IgG followed by the FITC-conjugated goat anti-mouse IgG antibodies without the activatable interleukin proteins. The cells were then washed again and resuspended in 0.2 mL of FACS buffer containing 2 µg/mL propidium iodide (PI) in order to exclude dead cells. The fluorescence of $1x10^4$ living cells is measured using a Beckman-Coulter FC500 MPL flow cytometer using the MXP software (Beckman-Coulter, Krefeld, Germany) or a Millipore Guava EasyCyte flow cytometer using the Incyte software (Merck Millipore, Schwalbach, Germany). Mean fluorescence intensities of the cell samples are calculated using CXP software (Beckman-Coulter, Krefeld, Germany) or Incyte software (Merck Millipore, Schwalbach, Germany). After subtracting the fluorescence intensity values of the cells stained with the secondary and tertiary reagents alone the values are then used for calculation of the K_D values with the equation for one-site binding (hyperbola) of the GraphPad Prism (version 6.00 for Windows, GraphPad Software, La Jolla California USA).

[167] CD20 binding and crossreactivity are assessed on the human $CD20^+$ tumor cell lines. The K_D ratio of crossreactivity is calculated using the K_D values determined on the CHO cell lines expressing either recombinant human or recombinant cynomolgus antigens.

Example 7: Cytotoxicity Assay

[168] The activatable interleukin protein of the preceding examples is evaluated *in vitro* on its mediation of immune response to $CD20^+$ target cells.

[169] Fluorescence labeled $CD20^+$ REC-1 cells (a Mantle cell lymphoma cell line, ATCC CRL-3004) are incubated with isolated PBMC of random donors or CB15 T-cells (standardized T-cell line)

as effector cells in the presence of the activatable interleukin protein of the preceding examples and at least one protease. After incubation for 4 h at 37°C in a humidified incubator, the release of the fluorescent dye from the target cells into the supernatant is determined in a spectrofluorimeter. Target cells incubated without the activatable interleukin protein of the preceding examples and target cells totally lysed by the addition of saponin at the end of the incubation serve as negative and positive controls, respectively.

[170] Based on the measured remaining living target cells, the percentage of specific cell lysis is calculated according to the following formula: [1-(number of living $targets_{(sample)}$/number of living $targets_{(spontaneous)}$)] x 100%. Sigmoidal dose response curves and EC_{50} values are calculated by non-linear regression/4-parameter logistic fit using the GraphPad Software. The lysis values obtained for a given antibody concentration are used to calculate sigmoidal dose-response curves by 4 parameter logistic fit analysis using the Prism software.

Example 8: Pharmacokinetics of activatable interleukin proteins

[171] The activatable interleukin protein of the preceding examples is evaluated for half-time elimination in animal studies.

[172] The activatable interleukin protein is administered to cynomolgus monkeys as a 0.5 mg/kg bolus injection into the saphenous vein. Another cynomolgus monkey group receives a comparable cytokine in size, but lacking a serum half-life extension element. A third and fourth group receive a cytokine with serum half-life extension elements and a cytokine with CD20 and serum half-life extension elements respectively, and both comparable in size to the activatable interleukin protein. Each test group consists of 5 monkeys. Serum samples are taken at indicated time points, serially diluted, and the concentration of the proteins is determined using a binding ELISA to CD20.

[173] Pharmacokinetic analysis is performed using the test article plasma concentrations. Group mean plasma data for each test article conforms to a multi-exponential profile when plotted against the time post-dosing. The data are fit by a standard two-compartment model with bolus input and first-order rate constants for distribution and elimination phases. The general equation for the best fit of the data for i.v. administration is: $c(t)=Ae^{-\alpha t}+Be^{-\beta t}$, where c(t) is the plasma concentration at time t, A and B are intercepts on the Y-axis, and α and β are the apparent first-order rate constants for the distribution and elimination phases, respectively. The α-phase is the initial phase of the clearance and reflects distribution of the protein into all extracellular fluid of the animal, whereas the second or β-phase portion of the decay curve represents true plasma clearance. Methods for fitting such equations are well known in the art. For example, $A=D/V(\alpha-k21)/(\alpha-\beta)$, $B=D/V(\beta-k21)/(\alpha-\beta)$, and α and β (for $\alpha>\beta$) are roots of the quadratic equation: $r^2+(k12+k21+k10)r+k21k10=0$ using estimated parameters of V=volume of distribution, k10=elimination rate, k12=transfer rate from compartment 1 to compartment 2 and k21=transfer rate from compartment 2 to compartment 1, and D=the administered dose.

[174] Data analysis: Graphs of concentration versus time profiles are made using KaleidaGraph (KaleidaGraph™ V. 3.09 Copyright 1986-1997. Synergy Software. Reading, Pa.). Values reported as less than reportable (LTR) are not included in the PK analysis and are not represented graphically. Pharmacokinetic parameters are determined by compartmental analysis using WinNonlin software (WinNonlin® Professional V. 3.1 WinNonlin™ Copyright 1998-1999. Pharsight Corporation. Mountain View, Calif.). Pharmacokinetic parameters are computed as described in Ritschel W A and Kearns G L, 1999, IN: *Handbook Of Basic Pharmacokinetics Including Clinical Applications,* 5th edition, American Pharmaceutical Assoc., Washington, D.C.

[175] It is expected that the activatable interleukin protein of the preceding examples has improved pharmacokinetic parameters such as an increase in elimination half-time as compared to proteins lacking a serum half-life extension element.

Example 9: Xenograft Tumor Model

[176] The activatable interleukin protein of the preceding examples is evaluated in a xenograft model.

[177] Female immune-deficient NOD/scid mice are sub-lethally irradiated (2 Gy) and subcutaneously inoculated with $4x10^6$ Ramos RA1 cells into the right dorsal flank. When tumors reach 100 to 200 mm^3, animals are allocated into 3 treatment groups. Groups 2 and 3 (8 animals each) are intraperitoneally injected with $1.5x10^7$ activated human T-cells. Three days later, animals from Group 3 are subsequently treated with a total of 9 intravenous doses of 50 μg activatable interleukin protein of the preceding examples (qdx9d). Groups 1 and 2 are only treated with vehicle. Body weight and tumor volume are determined for 30 days.

[178] It is expected that animals treated with the activatable interleukin protein of the preceding examples have a statistically significant delay in tumor growth in comparison to the respective vehicle-treated control group.

[179] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

Example 10: MC38 experiments

The MC38 cell line, a rapidly growing colon adenocarcinoma cell line that expresses MMP9 *in vitro*, was used. Using this tumor model, the ability of fusion proteins to affect tumor growth was examined.

Example 10a: MC38 IFNa and IL-12

Agents and Treatment:

Gr.	N	Agent	Formulation dose	Route	Schedule
1#	12	Vehicle	-	ip	biwk x 3
2	8	ACP11	17.5 µg/animal	ip	biwk x 3
3	8	ACP11	175 µg/animal	ip	biwk x 3
4	8	ACP11	525 µg/animal	ip	biwk x 3
13	8	IL-12-HM-WTI	2 µg/animal	ip	bid x 5 then 2-day pause then bid x 5 then 2-day pause
14	8	IL-12-HM-WTI	10 µg/animal	ip	bid x 5 then 2-day pause then bid x 5 then 2-day pause

#- Control Group

Procedures:

Mice were anaesthetized with isoflurane for implant of cells to reduce the ulcerations. CR female C57BL/6 mice were set up with $5x10^5$ MC38 tumor cells in 0% Matrigel sc in flank. Cell Injection Volume was 0.1 mL/mouse. Mouse age at start date was 8 to 12 weeks. Pair matches were performed when tumors reach an average size of 100 - 150 mm^3 and begin treatment. Body weights were taken at initiation and then biweekly to the end. Caliper measurements were taken biweekly to the end. Any adverse reactions were to be reported immediately. Any individual animal with a single observation of > than 30% body weight loss or three consecutive measurements of >25% body weight loss was euthanized. Any group with a mean body weight loss of >20 % or >10% mortality stopped dosing; the group was not euthanized and recovery is allowed. Within a group with >20% weight loss, individuals hitting the individual body weight loss endpoint were euthanized. If the group treatment related body weight loss is recovered to within 10% of the original weights, dosing resumed at a lower dose or less frequent dosing schedule. Exceptions to non-treatment body weight % recovery were allowed on a case-by-case basis. Endpoint was tumor growth delay (TGD). Animals were monitored individually. The endpoint of the experiment was a tumor volume of 1500 mm^3 or 45 days, whichever comes first. Responders were followed longer. When the endpoint was reached, the animals are to be euthanized.

Example 11: Conditionally Active Fusion Proteins That Contain a Blocking Moiety that is a Serum Albumin Binding Domain

[180] This example describes the production and activity of fusion proteins, preferably cytokines,

that have inducible activity, i.e., they are inactive until induced, typically by separation of a blocking moiety from the active moiety upon cleavage of a linker between the blocking moiety and the active moiety. The fusion proteins contain a single antibody variable domain (a dAb) that binds serum albumin via the CDR loops, and binds to an active moiety (here an anti-CD3 scFV) via one or more non-CDR loops (e.g., the C loop). The serum albumin-binding blocking moiety is operably linked to the active moiety through a protease cleavable linker, and active moiety is operably linked to a targeting domain (here an anti-epidermal growth factor receptor (EGFR) dAb or anti-prostate-specific membrane antigen (PSMA) dAb) through a linker that is not protease cleavable. These fusion proteins can be administered as inactive proteins that become activated upon cleavage of the protease cleavable linker and subsequent release of the inhibitory albumin-binding domain. The anti-CD3 scFV in the fusion proteins is a surrogate for a desired cytokine in the fusion proteins described in this disclosure. Similar fusion proteins that contain a desired cytokine (e.g., IL-2, IL-12, an Interferon) or functional fragment or mutein thereof, a targeting domain and an albumin-binding dAb that also binds and inhibits the cytokine or functional fragment or mutein thereof can be prepared using the methods described and exemplified herein. Anti-serum albumin dAb that bind and inhibit the activity of a desired cytokine or functional fragment or mutein thereof can provide both steric masking of the cytokine (through the cytokines proximity to bound serum albumin) and specific masking of the cytokine (through binding to cytokine via the non-CDR loop (e.g., the C loop)). Anti-serum albumin dAb that bind and inhibit the activity of a desired cytokine or functional fragment or mutein thereof can be obtained using suitable methods, such as by introducing amino acid sequence diversity into the non-CDR loops (e.g., C loop) of an anti-serum albumin binding dAb and screening for binding to the desired cytokine. Any suitable methods can be used for the selection, such as phage display. For example, an exemplary anti-serum albumin dab that can be used has the following sequence, and the amino acid sequence in the C loop (Bold Underlined) can be diversified (e.g., randomized) and resulting dAbs screened for binding to serum albumin via CDR interaction and to cytokine via non-CDR loop interaction. If desired, the amino acid sequence of a known cytokine binding peptide can be grafted into the C loop.

EVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQ**GGGGGLDGNEEPGG**LEWVSSI SGSGRDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQGTLVTVS S (SEQ ID NO: 92)

A. Protease activation of ProTriTAC leads to significantly enhanced activity *in vitro*

[181] Purified ProTriTAC (prodrug), non-cleavable ProTriTAC [prodrug (non-cleavable)], and recombinant active drug fragment mimicking the protease-activated ProTriTAC (active drug) were tested for binding to recombinant human CD3 in an ELISA assay, binding to purified human primary T cells in a flow cytometry assay, and functional potency in a T cell-dependent cellular cytotoxicity

assay.

[182] For ELISA, soluble ProTriTAC proteins at the indication concentrations were incubated with immobilized recombinant human CD3e (R&D Systems) for 1 h at room temperature in PBS supplemented with 15 mg/ml human serum albumin. Plates were blocked using SuperBlock (Thermo Fisher), washed using PBS with 0.05% Tween-20, and detected using a non-competitive anti-CD3 idiotype monoclonal antibody 11D3 followed by peroxidase-labeled secondary antibody and TMB-ELISA substrate solution (Thermo Fisher).

[183] For flow cytometry, soluble ProTriTAC proteins at the indicated concentrations were incubated with purified human primary T cells for 1 h at 4°C in the presence of PBS with 2% fetal bovine serum and 15 mg/ml human serum albumin. Plates were washed with PBS with 2% fetal bovine serum, detected using AlexaFluor 647-labeled non-competitive anti-CD3 idiotype monoclonal antibody 11D3, and data was analyzed using FlowJo 10 (FlowJo, LLC).

[184] For functional potency in a T cell-dependent cellular cytotoxicity assays, soluble ProTriTAC proteins at the indicated concentrations were incubated with purified resting human T cells (effector cell) and HCT116 cancer cell (target cell) at 10:1 effector:target cell ratio for 48 h at 37°C. The HCT116 target cell line has been stably transfected with a luciferase reporter gene to allow specific T cell-mediated cell killing measurement by ONE-Glo (Promega).

B. ProTriTAC exhibits potent, protease-dependent, anti-tumor activity in a rodent tumor xenograft model

[185] ProTriTAC was evaluated for their anti-tumor activity in vivo in an HCT116 subcutaneous xenograft tumor admixed with expanded human T cells in immunocompromised NCG mice. Specifically, 5x106 HCT116 cells were admixed with 2.5x106 expanded T cells per mouse on day 0. Dosing of ProTriTACs were performed starting on the following day with a q.d. x 10 schedule via intraperitoneal injection. Tumor volume measurements were determined using caliper measurements and calculated using the formula V = (length x width x width) / 2 at the indicated times.

C. Expression, purification and stability of exemplary ProTriTAC trispecific molecules

Protein Production

[186] Sequences encoding inducible fusion protein molecules were cloned into mammalian expression vector pcDNA 3.4 (Invitrogen) preceded by a leader sequence and followed by a 6x Histidine Tag (SEQ ID NO: 91). Expi293F cells (Life Technologies A14527) were maintained in suspension in Optimum Growth Flasks (Thomson) between 0.2 to 8 x 1e6 cells/ml in Expi 293 media. Purified plasmid DNA was transfected into Expi293 cells in accordance with Expi293 Expression System Kit (Life Technologies, A14635) protocols, and maintained for 4-6 days post transfection. Alternatively sequences encoding the fusion protein molecules were cloned into mammalian expression vector pDEF38 (CMC ICOS) transfected into CHO-DG44 dhfr- cells, stable pools

generated, and cultured in production media for up to 12 days prior to purification. The amount of the exemplary fusion proteins in conditioned media was quantified using an Octet RED 96 instrument with Protein A tips (ForteBio / Pall) using a control fusion protein for a standard curve. Conditioned media from either host cell was filtered and partially purified by affinity and desalting chromatography. Fusion proteins were subsequently polished by ion exchange and upon fraction pooling formulated in a neutral buffer containing excipients. Final purity was assessed by SDS-PAGE and analytical SEC using an Acquity BEH SEC 200 1.7u 4.6 x 150mm column (Waters Corporation) resolved in an aqueous/organic mobile phase with excipients at neutral pH on a 1290 LC system and peaks integrated with Chemstation CDS software (Agilent). Fusion proteins purified from CHO host cells are shown in the SDS-PAGE depicted below.

Stability Assessment

[187] Purified fusion proteins in two formulations were sub-aliquoted into sterile tubes and stressed by five freeze-thaw cycles each comprising greater than 1 hour at -80°C and room temperature or by incubation at 37°C for 1 week. Stressed samples were evaluated for concentration and turbidity by UV spectrometry using UV transparent 96 well plates (Corning 3635) with a SpectraMax M2 and SoftMaxPro Software (Molecular Devices), SDS-PAGE, and analytical SEC and compared to the same analysis of control non-stressed samples. An overlay of chromatograms from analytical SEC of control and stressed samples for a single exemplary ProTriTAC molecule purified from 293 host cells is depicted below.

[188] The results show that ProTriTACs were produced in comparable yields to regular TriTACs from CHO stable pools; and that the proteins were stable after repeated freeze-thaws and 37°C for 1 week.

D. Demonstration of functional masking and stability of ProTriTAC in vivo in a three-week cynomolgus monkey pharmacokinetic study

[189] Single dose of PSMA-targeting ProTriTAC (SEQ ID NO: 74), non-cleavable ProTriTAC (SEQ ID NO: 75), non-masked/non-cleavable TriTAC (SEQ ID NO: 78), and active drug mimicking protease-activated ProTriTAC (SEQ ID NO: 76) was dosed into cynomolgus monkeys at 0.1 mg/kg via intravenous injection. Plasma samples were collected at the indicated time points. ProTriTAC concentrations were determined using ligand binding assays with biotinylated recombinant human PSMA (R&D systems) and sulfo-tagged anti-CD3 idiotype antibody cloned 11D3 in a MSD assay (Meso Scale Diagnostic, LLC). Pharmacokinetic parameters were estimated using Phoenix WinNonlin pharmacokinetic software using a non-compartmental approach consistent with the intravenous bolus route of administration.

[190] To calculate the rate of in vivo prodrug conversion, the concentration of active drug in circulation was estimated by solving the following system of differential equations where P is the concentration of prodrug, A is the concentration of active drug, k_a is the rate of prodrug activation in

circulation, $k_{c,P}$ is the clearance rate of the prodrug, and $k_{c,A}$ is the clearance rate of the active drug.

$$\frac{dP}{dt} = -k_{c,P}P$$

$$\frac{dA}{dt} = k_aP - k_{c,A}A$$

[191] The clearance rates of the prodrug, active drug, and a non-cleavable prodrug control ($k_{c,NCLV}$) were determined empirically in cynomolgus monkeys. To estimate the rate of prodrug activation in circulation, we assumed that the difference between the clearance rate of cleavable prodrug and non-cleavable prodrug arose solely from non-specific activation in circulation. Therefore, the rate of prodrug conversion to active drug in circulation was estimated by subtracting the clearance rate of the cleavable prodrug from the non-cleavable prodrug.

$$k_a = k_{c,NCLV} - k_{c,P}$$

[192] The initial concentration of prodrug in circulation was determined empirically and the initial concentration of active drug was assumed to be zero.

Results and Discussion

[193] The results of this Example 11 show that fusion proteins that contain a polypeptide with desired therapeutic activity, such as a cytokine or functional fragment or mutein thereof or anti-CD3 scFV, can be prepared in which the therapeutic activity is masked by a masking domain that binds to both serum albumin and to the active polypeptide. The masking domain is operably linked to the active domain through a protease cleavable linker. The results show that this type of fusion protein can be administered as an inactive protein that becomes activated upon protease cleavage at the desired location of therapeutic activity, such as, at a tumor.

[194] Amino acid sequences of fusion proteins used in Example 11 are given as SEQ ID NOs: 71-78.

[195] Sample fusion protein constructs are detailed in Table 3. In table 3, "L" is an abbreviation of "linker", and "cleav. link." is an abbreviation of "cleavable linker". Other abbreviations "mIFNg" indicates mouse interferon gamma (IFNg); "hAlbumin" indicates human serum albumin (HSA); "mAlbumin" indicates mouse serum albumin.

Table 3: CONSTRUCT PERMUTATION TABLE

Construct Name	Construct Description
ACP63	anti-FN CGS-2 scFv (Vh/Vl)-6xHis
ACP04	human p40-murine p35-6xHis
ACP05	human p40-human p35-6xHis
ACP34	mouse p35-(cleav. link.)-mouse p40-6xHis
ACP35	mouse p35-GS-(cleav. link.)-GS-mouse p40-6xHis
ACP36	(anti-HSA)-(cleav. link.)-mouse p40-mouse p35-(cleav. link.)-(anti-HSA)-6xHis
ACP37	(anti-EpCAM)-(anti-HSA)-(cleav. link.)-mouse p40-mouse p35-(cleav. link.)-(anti-HSA)-6xHis
ACP79	(anti-EpCAM)-Linker-(anti-HSA)-(cleav. link.r)-mIL12-(cleav. link.)-(Anti-HSA)-6xHis
ACP80	(anti-HSA)-(cleav. link.)-mIL12-(cleav. link.)-(anti-HSA)-Linker-(anti-EpCAM)-6xHis
ACP06	Blocker12-Linker-(cleav. link)-human p40-Linker-mouse p35-(cleav. link)-(anti-HSA)-6xHis
ACP07	Blocker12-Linker-(cleav. link)-human p40-Linker-mouse p35-(cleav. link.)-(anti-HSA)-Linker-(anti-FOLR1)-6xHis
ACP08	(anti-FOLR1)-Linker-Blocker12-Linker-(cleav. link.)-human p40-Linker-mouse p35-(cleav. link.)-(anti-HSA)-6xHis
ACP09	(anti-HSA)-Linker-Blocker12-Linker-(cleav. link.)-human p40-Linker-mouse p35-6xHis
ACP10	(anti-HSA)-(cleav. link.)-human p40-L-mouse p35-(cleav. link.)-Linker-Blocker12-6xHis
ACP11	hp40-Linker-mp35-(cleav. link.)-Linker-Blocker12-Linker-(anti-HSA)-6xHis
ACP91	human_p40-Linker-mouse_p35-Linker-Linker-Blocker-Linker-(anti-HSA)_(non-cleavable control)
ACP136	human p40-L-mouse p35-XL-Blocker
ACP138	human_p40-L-mouse_p35-XL-Blocker-L-HSA-L-FOLR1
ACP139	FOLR1-L-human_p40-L-mouse_p35-XL-Blocker-L-HSA
ACP140	FOLR1-(cleav. link.)-human_p40-L-mouse_p35-XL-Blocker-L-HSA
ACP117	anti-FN CGS-2 scFv (Vh/Vl)-6xHis

Sequence Table

SEQ ID NO.	Name	Sequence

1	Human serum albumin	MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA FAQYLQQCPF EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT VATLRETYGE MADCCAKQEP ERNECFLQHK DDNPNLPRLV RPEVDVMCTA FHDNEETFLK KYLYEIARRH PYFYAPELLF FAKRYKAAFT ECCQAADKAA CLLPKLDELR DEGKASSAKQ GLKCASLQKF GERAFKAWAV ARLSQRFPKA EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVGSKDVC KNYAEAKDVF LGMFLYEYAR RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE FKPLVEEPQN LIKQNCELFE QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV SRNLGKVGSK CCKHPEAKRM PCAEDCLSVF LNQLCVLHEK TPVSDRVTKC CTESLVNGRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALV ELVKHK PKATKEQLKAVMDDFAAFVEKCCKADDKET CFAEEGKKLVAASQAALGL
44	ACP04 (human p40/murine p35 IL12 fusion protein)	iwelkkdvyvveldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvkefgdagqytchkggevlshsllllhkk edgiwstdilkdqkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvtcgaatlsaervrgdnkeyeysvecq edsacpaaeeslpievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvevsweypdtwstphsyfsltfcvqvqg kskrekkdrvftdktsatvicrknasisvraqdryyssswsewasvpcsggggsggggsggggsrvipvsgparclsqsrnllktt ddmvktareklkhysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqktslmmtlclgsiyedlkmyqte fqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillhafstrvvtinrvmgylssaHH HHHH
45	ACP05 (human p40/murine p35 IL12 fusion protein)	iwelkkdvyvveldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvkefgdagqytchkggevlshsllllhkk edgiwstdilkdqkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvtcgaatlsaervrgdnkeyeysvecq edsacpaaeeslpievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvevsweypdtwstphsyfsltfcvqvqg kskrekkdrvftdktsatvicrknasisvraqdryyssswsewasvpcsggggsggggsggggsrnlpvatpdpgmfpclhhs qnllravsnmlqkarqtlefypctseeidheditkdktstveaclpleltknesclnsretsfitngsclasrktsfmmalclssiyedlk myqvefktmnakllmdpkrqifldqnmlavidelmqalnfnsetvpqkssleepdfyktkiklcillhafriravtidrvmsylna sHHHHHH
46	ACP06 (human p40/murine p35 IL12 fusion protein)	QSVLTQPPSVSGAPGQRVTISCSGSRSNIGSNTVKWYQQLPGTAPKLLIYYNDQRPSG VPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDRYTHPALLFGTGTKVTVLggggs ggggsggggsQVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWV AFIRYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCKTHGSHDN WGQGTMVTVSSggggsggggsggggsggggsggggsggggsSGGPGPAGMKGLPGSiwelkkdvyvv eldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvkefgdagqytchkggevlshsllllhkkedgiwstdilkd qkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvtcgaatlsaervrgdnkeyeysvecqedsacpaaeesl pievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvevsweypdtwstphsyfsltfcvqvqgkskrekkdrvft dktsatvicrknasisvraqdryyssswsewasvpcsggggsggggsggggsrvipvsgparclsqsrnllkttddmvktarekl

		khysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqktslmmtlclgsiyedlkmyqtefqainaalqnhn hqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillhafstrvvtinrvmgylssaSGGPGPAGM KGLPGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWVSS ISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQG TLVTVSSHHHHHHEPEA
47	ACP07 (human p40/murine p35 IL12 fusion protein)	QSVLTQPPSVSGAPGQRVTISCSGSRSNIGSNTVKWYQQLPGTAPKLLIYYNDQRPSG VPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDRYTHPALLFGTGTKVTVLggggs ggggsggggsQVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWV AFIRYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCKTHGSHDN WGQGTMVTVSSggggsggggsggggsggggsggggsggggsSGGPGPAGMKGLPGSiwelkkdvyvv eldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvkefgdagqytchkggevlshsllllhkkedgiwstdilkd qkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvtcgaatlsaervrgdnkeyeysvecqedsacpaaeesl pievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvevsweypdtwstphsyfsltfcvqvqgkskrekkdrvft dktsatvicrknasisvraqdryyssswsewasvpcsggggsggggsggggsrvipvsgparclsqsrnllkttddmvktarekl khysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqktslmmtlclgsiyedlkmyqtefqainaalqnhn hqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillhafstrvvtinrvmgylssaSGGPGPAGM KGLPGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWVSS ISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQG TLVTVSSggggsggggsggggsQVQLQESGGGLAQAGGSLSLSCAASGFTVSNSVMAWYR QTPGKQREFVAIINSVGSTNYADSVKGRFTISRDNAKNTVYLQMNNLKPEDTAVYV CNRNFDRIYWGQGTQVTVSSHHHHHHEPEA
48	ACP08 (human p40/murine p35 IL12 fusion protein)	QVQLQESGGGLAQAGGSLSLSCAASGFTVSNSVMAWYRQTPGKQREFVAIINSVGS TNYADSVKGRFTISRDNAKNTVYLQMNNLKPEDTAVYVCNRNFDRIYWGQGTQVT VSSggggsggggsggggsQSVLTQPPSVSGAPGQRVTISCSGSRSNIGSNTVKWYQQLPGTA PKLLIYYNDQRPSGVPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDRYTHPALL FGTGTKVTVLggggsggggsggggsQVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMH WVRQAPGKGLEWVAFIRYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAED TAVYYCKTHGSHDNWGQGTMVTVSSggggsggggsggggsggggsggggsggggsSGGPGPAG MKGLPGSiwelkkdvyvveldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvkefgdagqytchkgg evlshsllllhkkedgiwstdilkdqkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvtcgaatlsaervrgd nkeyeysvecqedsacpaaeeslpievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvevsweypdtwstph syfsltfcvqvqgkskrekkdrvftdktsatvicrknasisvraqdryyssswsewasvpcsggggsggggsggggsrvipvsgp arclsqsrnllkttddmvktareklkhysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqktslmmtlclgs iyedlkmyqtefqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillhafstrvvtinr vmgylssaSGGPGPAGMKGLPGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMS

		WVRQAPGKGLEWVSSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDT AVYYCTIGGSLSVSSQGTLVTVSSHHHHHHHEPEA
49	ACP09 (human p40/murine p35 IL12 fusion protein)	EVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWVSSISGSGR DTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQGTLVTV SSggggsggggsggggsQSVLTQPPSVSGAPGQRVTISCSGSRSNIGSNTVKWYQQLPGTAP KLLIYYNDQRPSGVPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDRYTHPALLF GTGTKVTVLggggsggggsggggsQVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHW VRQAPGKGLEWVAFIRYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTA VYYCKTHGSHDNWGQGTMVTVSSggggsggggsggggsggggsggggsggggsSGGPGPAGMK GLPGSiwelkkdvyvveldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvkefgdagqytchkggevls hsllllhkkedgiwstdilkdqkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvtcgaatlsaervrgdnkey eysvecqedsacpaaeeslpievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvevsweypdtwstphsyfslt fcvqvqgkskrekkdrvftdktsatvicrknasisvraqdryyssswsewasvpcsggggsggggsggggsrvipvsgparcls qsrnllkttddmvktareklkhysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqktslmmtlclgsiyed lkmyqtefqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillhafstrvvtinrvmg ylssaHHHHHHEPEA
50	ACP10 (human p40/murine p35 IL12 fusion protein)	EVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWVSSISGSGR DTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQGTLVTV SSSGGPGPAGMKGLPGSiwelkkdvyvveldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvke fgdagqytchkggevlshsllllhkkedgiwstdilkdqkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvt cgaatlsaervrgdnkeyeysvecqedsacpaaeeslpievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvev sweypdtwstphsyfsltfcvqvqgkskrekkdrvftdktsatvicrknasisvraqdryyssswsewasvpcsggggsggggs ggggsrvipvsgparclsqsrnllkttddmvktareklkhysctaedidheditrdqtstlktclplelhknesclatretssttrgsclp pqktslmmtlclgsiyedlkmyqtefqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmkl cillhafstrvvtinrvmgylssaSGGPGPAGMKGLPGSggggsggggsggggsggggsggggsggggsQSVLT QPPSVSGAPGQRVTISCSGSRSNIGSNTVKWYQQLPGTAPKLLIYYNDQRPSGVPDRF SGSKSGTSASLAITGLQAEDEADYYCQSYDRYTHPALLFGTGTKVTVLggggsggggsgg ggsQVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAFIRY DGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCKTHGSHDNWGQG TMVTVSSHHHHHHEPEA
51	ACP11 (human p40/murine p35 IL12 fusion protein)	iwelkkdvyvveldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvkefgdagqytchkggevlshsllllhkk edgiwstdilkdqkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvtcgaatlsaervrgdnkeyeysvecq edsacpaaeeslpievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvevsweypdtwstphsyfsltfcvqvqg kskrekkdrvftdktsatvicrknasisvraqdryyssswsewasvpcsggggsggggsggggsrvipvsgparclsqsrnllktt ddmvktareklkhysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqktslmmtlclgsiyedlkmyqte fqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillhafstrvvtinrvmgylssaSG

		GPGPAGMKGLPGSggggsggggsggggsggggsggggsggggsQSVLTQPPSVSGAPGQRVTISC SGSRSNIGSNTVKWYQQLPGTAPKLLIYYNDQRPSGVPDRFSGSKSGTSASLAITGLQ AEDEADYYCQSYDRYTHPALLFGTGTKVTVLggggsggggsggggsQVQLVESGGGVVQ PGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAFIRYDGSNKYYADSVKGRF TISRDNSKNTLYLQMNSLRAEDTAVYYCKTHGSHDNWGQGTMVTVSSggggsggggsg gggsEVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWVSSISGS GRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQGTLV TVSSHHHHHHHEPEA
52	IL12 p40 human (Uniprot Accession No. P29460)	10 20 30 40 50 MCHQQLVISW FSLVFLASPL VAIWELKKDV YVVELDWYPD APGEMVVLTC 60 70 80 90 100 DTPEEDGITW TLDQSSEVLG SGKTLTIQVK EFGDAGQYTC HKGGEVLSHS 110 120 130 140 150 LLLLHKKEDG IWSTDILKDQ KEPKNKTFLR CEAKNYSGRF TCWWLTTIST 160 170 180 190 200 DLTFSVKSSR GSSDPQGVTC GAATLSAERV RGDNKEYEYS VECQEDSACP 210 220 230 240 250 AAEESLPIEV MVDAVHKLKY ENYTSSFFIR DIIKPDPPKN LQLKPLKNSR 260 270 280 290 300 QVEVSWEYPD TWSTPHSYFS LTFCVQVQGK SKREKKDRVF TDKTSATVIC 310 320 RKNASISVRA QDRYYSSSWS EWASVPCS
53	IL12 p35 mouse (Uniprot Accession No. P43431)	10 20 30 40 50 MCQSRYLLFL ATLALLNHLS LARVIPVSGP ARCLSQSRNL LKTTDDMVKT 60 70 80 90 100 AREKLKHYSC TAEDIDHEDI TRDQTSTLKT CLPLELHKNE SCLATRETSS 110 120 130 140 150 TTRGSCLPPQ KTSLMMTLCL GSIYEDLKMY QTEFQAINAA LQNHNHQQII 160 170 180 190 200 LDKGMLVAID ELMQSLNHNG ETLRQKPPVG EADPYRVKMK LCILLHAFST 210 RVVTINRVMG YLSSA

54	IL12Rb-2	10 20 30 40 50 MRHIFRGCSL AENFLIIKLL IFSKLDGCRA GDVIKPFSNN IHLGSIVMII 60 70 80 90 100 CSLKPRGGCK HVSERNKLII IKFDARINFH HGHSLNSGVT GLRLGTILEV 110 120 130 140 150 CNLACINSDE IQICGAELEV GVAPEQGENL SCLGGSGGGS VACTVERGRD 160 170 180 190 200 THLKTEVTLQ LGGENLTNQ KGCNDIKCDV LDFGINLTPE KHEGNFIANV 210 220 230 240 250 TAVNSLGSSS SLPDTFTFLD IVRPLAPWDI RIKFQKASVS RCTLYWRDKG 260 270 280 290 300 LVLLNRLRKR RGNSRLNNSV MNIKKAGSHS LLELKKFTKK EKQIFSKSHL 310 320 330 340 350 YKGSNSDNSH SLRAQIPKEE PTGMLSVNVD KGHIDVSSQQ ISLFNKQLSV 360 370 380 390 400 SEARGKILNV QVTLQFLTCG KRNTGNITGN TKKTVIRRKI GNWAVSVSRA 410 420 430 440 450 NSKGSSLRTD INIMDLCEAG LIAPRGVSAN SRGNTNILVT KQPPRKDPSA 460 470 480 490 500 VQAYVVKNKE LKPGSEIQVT LNVLAPERKN VSALIEDYLK SYICKRIDVK 510 520 530 540 550 ALSGPQGSCS SILGNSKHKA PLSGPHIDAI IEEKGSILIS SNSIPVQEQM 560 570 580 590 600 GCLLHVRIKN KERDSNSQPQ LCEIEKPVSG VSHPINSLQP EVIVVLMNTA 610 620 630 640 650 LIAAGPSSHC SEAFPCLQGK ANNMLFVAPS ICIAIDMGI FSTEVFQGKV 660 670 680 690 700 FVLLAALSPQ WCSEEIPDPA NSTCAKKYPI SEEKTQLPLD RLLIDWPTPE 710 720 730 740 750 DPEPLVISEV LHQVTPVFRH PPCSNWPQRE KGIQGHQASE KDMMHSASSP 760 770 780 790 800 PPSPALQAES SQLVDLVDVL EGRGSEGKPS NPACPWIVLR AGDLPTHDGY 810 820 830 840 850 LPSNIDDLPS HEAPLADSLE ELEPQHISLS VFPSSSLHPL TFSCGDKLTL 860 DQLSMPCPSL ML
55	IL12Rb-1	10 20 30 40 50 MEPLVTWVVP LLFLFLLSPQ GAACRISECC FQDPPYPDAD SGSASGPRDL 60 70 80 90 100 RCYRISSDRY ECSWQYEGPT AGVSHFLRCC LSSGRCCYFA AGSATRLQFS 110 120 130 140 150 DQAGVSVLYT VTLWVESWAP NQTEKSPEVT LQLYNSVKYE PPLGDIKVSK 160 170 180 190 200 LACQLRNESE IPDNQVGAEV QFRHRTPGSP WWLGDCGPQD DDTESCLCPL 210 220 230 240 250 EMNVAQEFQL PRRQLGSQGS SWSKWSSPVC VPPENPPQPQ VRFSVEQLGQ 260 270 280 290 300 DGRARLTLKE QPTQLELPEG CQGLAPGTEV TYRLQLHMLS CPCKAKATRT 310 320 330 340 350 LHLGKMPYLS GAAYNVAVIS SNQFGPGLNQ TWHIPADTHT EPVALNISVG 360 370 380 390 400 TNGTTMYWPA RAQSMTYCIE WQPVGQDGGL ATCSLTAPQD PDPAGMATYS 410 420 430 440 450 WSRESGAMGQ EKCYYITIFA SAHPEKLTLW STVLSTYHFG GNASEAGTPH 460 470 480 490 500 HVSVKNHSLD SVSVDWAPSL LSTCPGVLKE YVVRCRDEDS KQVSEHPVQP 510 520 530 540 550 TETQVILSGL RAGVAYTVQV RADTAWLRGV WSQPQRFSIE VQVSDWLIFF 560 570 580 590 600 ASLGSFLSIL LVGVLGYLGL NRAARHLCPP LPTPCASSAI EFPGGKETWQ 610 620 630 640 650 WINPVDFQEE ASLQEALVVE MSWDKGERTE PLEKTELPEG APELALDTEL 660 SLEDGDRCKA KM

56	IL-12 p35 human (Uniprot accession no. P29459)	10 20 30 40 50 MCHQQLVISW FSLVFLASPL VAIWELKKDV YVVELDWYPD APGEMVVLTC 60 70 80 90 100 DTPEEDGITW TLDQSSEVLG SGKTLTIQVK EFGDAGQYTC HKGGEVLSHS 110 120 130 140 150 LLLLHKKEDG IWSTDILKDQ KEPKNKTFLR CEAKNYSGRF TCWWLTTIST 160 170 180 190 200 DLTFSVKSSR GSSDPQGVTC GAATLSAERV RGDNKEYEYS VECQEDSACP 210 220 230 240 250 AAEESLPIEV MVDAVHKLKY ENYTSSFFIR DIIKPDPPKN LQLKPLKNSR 260 270 280 290 300 QVEVSWEYPD TWSTPHSYFS LTFCVQVQGK SKREKKDRVF TDKTSATVIC 310 320 RKNASISVRA QDRYYSSSWS EWASVPCS
57	IL-12 p40 mouse (Uniprot accession no. P43432)	10 20 30 40 50 MCPQKLTISW FAIVLLVSPL MAMWELEKDV YVVEVDWTPD APGETVNLTC 60 70 80 90 100 DTPEEDDITW TSDQRHGVIG SGKTLTITVK EFLDAGQYTC HKGGETLSHS 110 120 130 140 150 HLLLHKKENG IWSTEILKNF KNKTFLKCEA PNYSGRFTCS WLVQRNMDLK 160 170 180 190 200 FNIKSSSSSP DSRAVTCGMA SLSAEKVTLD QRDYEKYSVS CQEDVTCPTA 210 220 230 240 250 EETLPIELAL EARQQNKYEN YSTSFFIRDI IKPDPPKNLQ MKPLKNSQVE 260 270 280 290 300 VSWEYPDSWS TPHSYFSLKF FVRIQRKKEK MKETEEGCNQ KGAFLVEKTS 310 320 330 TEVQCKGGNV CVQAQDRYYN SSCSKWACVP CRVRS
58	ACP63 Anti-FN CGS-2 scFv	mdmrvpaqllglllwlrgarcEVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAP GKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCA RGVGAFRPYRKHEWGQGTLVTVSRggggsggggsggggsSSELTQDPAVSVALGQTVRIT CQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTTTGAQ AEDEADYYCNSSPFEHNLVVFGGGTKLTVLHHHHHHEPEA
59	ACP34 Mouse IL-12 fusion protein	mdmrvpaqllglllwlrgarcrvipvsgparclsqsrnllkttddmvktareklkhysctaedidheditrdqtstlktclplelhkn esclatretssttrgsclppqktslmmtlclgsiyedlkmyqtefqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqk ppvgeadpyrvkmklcillhafstrvvtinrvmgylssaSGGPGPAGMKGLPGSmwelekdvyvvevdwtpdap getvnltcdtpeedditwtsdqrhgvigsgktltitvkefldagqytchkggetlshshlllhkkengiwsteilknfknktflkceap nysgrftcswlvqrnmdlkfnikssssspdsravtcgmaslsaekvtldqrdyekysvscqedvtcptaeetlpielalearqqnk yenystsffirdiikpdppknlqmkplknsqvevsweypdswstphsyfslkffvriqrkkekmketeegcnqkgaflvektst evqckggnvcvqaqdryynsscskwacvpcrvrsHHHHHH
60	ACP35	mdmrvpaqllglllwlrgarcrvipvsgparclsqsrnllkttddmvktareklkhysctaedidheditrdqtstlktclplelhkn esclatretssttrgsclppqktslmmtlclgsiyedlkmyqtefqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqk

	Mouse IL-12 fusion protein	ppvgeadpyrvkmklcillhafstrvvtinrvmgylssaggggsggggsggggsSGGPGPAGMKGLPGSggggsg gggsggggsmwelekdvyvvevdwtpdapgetvnltcdtpeedditwtsdqrhgvigsgktltitvkefldagqytchkggetl shshlllhkkengiwsteilknfknktflkceapnysgrftcswlvqrnmdlkfnikssssspdsravtcgmaslsaekvtldqrdy ekysvscqedvtcptaeetlpielalearqqnkyenystsffirdiikpdppknlqmkplknsqvevsweypdswstphsyfslk ffvriqrkkekmketeegcnqkgaflvektstevqckggnvcvqaqdryynsscskwacvpcrvrsHHHHHH
61	ACP36 Mouse IL-12 fusion protein	mdmrvpaqllglllwlrgarcEVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAP GKGLEWVSSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTI GGSLSVSSQGTLVTVSSSGGPGPAGMKGLPGSmwelekdvyvvevdwtpdapgetvnltcdtpeedd itwtsdqrhgvigsgktltitvkefldagqytchkggetlshshlllhkkengiwsteilknfknktflkceapnysgrftcswlvqrn mdlkfnikssssspdsravtcgmaslsaekvtldqrdyekysvscqedvtcptaeetlpielalearqqnkyenystsffirdiikpd ppknlqmkplknsqvevsweypdswstphsyfslkffvriqrkkekmketeegcnqkgaflvektstevqckggnvcvqaq dryynsscskwacvpcrvrsggggsggggsggggsrvipvsgparclsqsrnllkttddmvktareklkhysctaedidheditr dqtstlktclplelhknesclatretssttrgsclppqktslmmtlclgsiyedlkmyqtefqainaalqnhnhqqiildkgmlvaide lmqslnhngetlrqkppvgeadpyrvkmklcillhafstrvvtinrvmgylssaSGGPGPAGMKGLPGSEVQLV ESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWVSSISGSGRDTLYA ESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQGTLVTVSSHHH HHH
62	ACP37 Mouse IL-12 fusion protein	mdmrvpaqllglllwlrgarcQVQLQESGGGLVQAGGSLRLSCAASGRIFSIDIMSWYRQAPG KQRELVARITRGGTISYDDSVKGRFTISRDNAKNTVYLQMNSLKPEDTGVYYCNAL YGTDYWGKGTQVTVSSggggsggggsggggsEVQLVESGGGLVQPGNSLRLSCAASGFTF SKFGMSWVRQAPGKGLEWVSSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNS LRPEDTAVYYCTIGGSLSVSSQGTLVTVSSSGGPGPAGMKGLPGSmwelekdvyvvevdwt pdapgetvnltcdtpeedditwtsdqrhgvigsgktltitvkefldagqytchkggetlshshlllhkkengiwsteilknfknktflk ceapnysgrftcswlvqrnmdlkfnikssssspdsravtcgmaslsaekvtldqrdyekysvscqedvtcptaeetlpielalearq qnkyenystsffirdiikpdppknlqmkplknsqvevsweypdswstphsyfslkffvriqrkkekmketeegcnqkgaflve ktstevqckggnvcvqaqdryynsscskwacvpcrvrsggggsggggsggggsrvipvsgparclsqsrnllkttddmvktare klkhysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqktslmmtlclgsiyedlkmyqtefqainaalqn hnhqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillhafstrvvtinrvmgylssaSGGPGPAG MKGLPGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWV SSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSS QGTLVTVSSHHHHHH
63	ACP79 Mouse IL-12 fusion protein	mdmrvpaqllglllwlrgarcQVQLQESGGGLVQAGGSLRLSCAASGRIFSIDIMSWYRQAPG KQRELVARITRGGTISYDDSVKGRFTISRDNAKNTVYLQMNSLKPEDTGVYYCNAL YGTDYWGKGTQVTVSSggggsggggsggggsEVQLVESGGGLVQPGNSLRLSCAASGFTF SKFGMSWVRQAPGKGLEWVSSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNS LRPEDTAVYYCTIGGSLSVSSQGTLVTVSSSGGPGPAGMKGLPGSmwelekdvyvvevdwt pdapgetvnltcdtpeedditwtsdqrhgvigsgktltitvkefldagqytchkggetlshshlllhkkengiwsteilknfknktflk ceapnysgrftcswlvqrnmdlkfnikssssspdsravtcgmaslsaekvtldqrdyekysvscqedvtcptaeetlpielalearq qnkyenystsffirdiikpdppknlqmkplknsqvevsweypdswstphsyfslkffvriqrkkekmketeegcnqkgaflve ktstevqckggnvcvqaqdryynsscskwacvpcrvrsggggsggggsggggsrvipvsgparclsqsrnllkttddmvktare klkhysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqktslmmtlclgsiyedlkmyqtefqainaalqn hnhqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillhafstrvvtinrvmgylssaSGGPGPAG MKGLPGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWV SSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSS QGTLVTVSSHHHHHH
64	ACP80 Mouse IL-12 fusion protein	mdmrvpaqllglllwlrgarcEVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAP GKGLEWVSSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTI GGSLSVSSQGTLVTVSSSGGPGPAGMKGLPGSmwelekdvyvvevdwtpdapgetvnltcdtpeedd itwtsdqrhgvigsgktltitvkefldagqytchkggetlshshlllhkkengiwsteilknfknktflkceapnysgrftcswlvqrn mdlkfnikssssspdsravtcgmaslsaekvtldqrdyekysvscqedvtcptaeetlpielalearqqnkyenystsffirdiikpd ppknlqmkplknsqvevsweypdswstphsyfslkffvriqrkkekmketeegcnqkgaflvektstevqckggnvcvqaq dryynsscskwacvpcrvrsggggsggggsggggsrvipvsgparclsqsrnllkttddmvktareklkhysctaedidheditr dqtstlktclplelhknesclatretssttrgsclppqktslmmtlclgsiyedlkmyqtefqainaalqnhnhqqiildkgmlvaide

		lmqslnhngetlrqkppvgeadpyrvkmklcillhafstrvvtinrvmgylssaSGGPGPAGMKGLPGSEVQLV ESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWVSSISGSGRDTLYA ESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQGTLVTVSSggggs ggggsggggsQVQLQESGGGLVQAGGSLRLSCAASGRIFSIDIMSWYRQAPGKQRELVA RITRGGTISYDDSVKGRFTISRDNAKNTVYLQMNSLKPEDTGVYYCNALYGTDYWG KGTQVTVSSHHHHHH
65	ACP91 Chimeric IL-12 fusion protein	mdmrvpaqllgllllwlrgarciwelkkdvyvveldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvkefgd agqytchkggevlshsllllhkkedgiwstdilkdqkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvtcga atlsaervrgdnkeyeysvecqedsacpaaeeslpievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvevswe ypdtwstphsyfsltfcvqvqgkskrekkdrvftdktsatvicrknasisvraqdryyssswsewasvpcsggggsggggsggg gsrvipvsgparclsqsrnllkttddmvktareklkhysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqkt slmmtlclgsiyedlkmyqtefqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillh afstrvvtinrvmgylssaggggsggggsggggsggggsggggsggggsggggsggggsggggsQSVLTQPPSVSG APGQRVTISCSGSRSNIGSNTVKWYQQLPGTAPKLLIYYNDQRPSGVPDRFSGSKSGT SASLAITGLQAEDEADYYCQSYDRYTHPALLFGTGTKVTVLggggsggggsggggsQVQL VESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAFIRYDGSNKY YADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCKTHGSHDNWGQGTMVTVS Sggggsggggsggggs EVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKG LEWVSSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGS LSVSSQGTLVTVSSHHHHHHEPEA
66	ACP136 Chimeric IL-12 fusion protein	mdmrvpaqllgllllwlrgarciwelkkdvyvveldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvkefgd agqytchkggevlshsllllhkkedgiwstdilkdqkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvtcga atlsaervrgdnkeyeysvecqedsacpaaeeslpievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvevswe ypdtwstphsyfsltfcvqvqgkskrekkdrvftdktsatvicrknasisvraqdryyssswsewasvpcsggggsggggsggg gsrvipvsgparclsqsrnllkttddmvktareklkhysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqkt slmmtlclgsiyedlkmyqtefqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillh afstrvvtinrvmgylssaSGGPGPAGMKGLPGSggggsggggsggggsggggsggggsggggsQSVLTQPP SVSGAPGQRVTISCSGSRSNIGSNTVKWYQQLPGTAPKLLIYYNDQRPSGVPDRFSGS KSGTSASLAITGLQAEDEADYYCQSYDRYTHPALLFGTGTKVTVLggggsggggsggggsQ VQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAFIRYDGS NKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCKTHGSHDNWGQGTM VTVSSHHHHHHEPEA
67	ACP138 Chimeric IL-12 fusion protein	mdmrvpaqllgllllwlrgarciwelkkdvyvveldwypdapgemvvltcdtpeedgitwtldqssevlgsgktltiqvkefgd agqytchkggevlshsllllhkkedgiwstdilkdqkepknktflrceaknysgrftcwwlttistdltfsvkssrgssdpqgvtcga atlsaervrgdnkeyeysvecqedsacpaaeeslpievmvdavhklkyenytssffirdiikpdppknlqlkplknsrqvevswe ypdtwstphsyfsltfcvqvqgkskrekkdrvftdktsatvicrknasisvraqdryyssswsewasvpcsggggsggggsggg gsrvipvsgparclsqsrnllkttddmvktareklkhysctaedidheditrdqtstlktclplelhknesclatretssttrgsclppqkt slmmtlclgsiyedlkmyqtefqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqkppvgeadpyrvkmklcillh afstrvvtinrvmgylssaSGGPGPAGMKGLPGSggggsggggsggggsggggsggggsggggsQSVLTQPP SVSGAPGQRVTISCSGSRSNIGSNTVKWYQQLPGTAPKLLIYYNDQRPSGVPDRFSGS KSGTSASLAITGLQAEDEADYYCQSYDRYTHPALLFGTGTKVTVLggggsggggsggggsQ VQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAFIRYDGS NKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCKTHGSHDNWGQGTM VTVSSggggsggggsggggsEVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQA PGKGLEWVSSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCT IGGSLSVSSQGTLVTVSSggggsggggsggggsQVQLQESGGGLAQAGGSLSLSCAASGFT VSNSVMAWYRQTPGKQREFVAIINSVGSTNYADSVKGRFTISRDNAKNTVYLQMNN LKPEDTAVYVCNRNFDRIYWGQGTQVTVSSHHHHHHEPEA
68	ACP139 Chimeric IL-12	mdmrvpaqllgllllwlrgarcQVQLQESGGGLAQAGGSLSLSCAASGFTVSNSVMAWYRQTP GKQREFVAIINSVGSTNYADSVKGRFTISRDNAKNTVYLQMNNLKPEDTAVYVCNR NFDRIYWGQGTQVTVSSggggsggggsggggsiwelkkdvyvveldwypdapgemvvltcdtpeedgitwtld qssevlgsgktltiqvkefgdagqytchkggevlshsllllhkkedgiwstdilkdqkepknktflrceaknysgrftcwwlttistd ltfsvkssrgssdpqgvtcgaatlsaervrgdnkeyeysvecqedsacpaaeeslpievmvdavhklkyenytssffirdiikpdp pknlqlkplknsrqvevsweypdtwstphsyfsltfcvqvqgkskrekkdrvftdktsatvicrknasisvraqdryyssswsew

	fusion protein	asvpcsggggsggggsggggsrvipvsgparclsqsrnllkttddmvktareklkhysctaedidheditrdqtstlktclplelhkn esclatretssttrgsclppqktslmmtlclgsiyedlkmyqtefqainaalqnhnhqqiildkgmlvaidelmqslnhngetlrqk ppvgeadpyrvkmklcillhafstrvvtinrvmgylssaSGGPGPAGMKGLPGSggggsggggsggggsggggsg gggsggggsQSVLTQPPSVSGAPGQRVTISCSGSRSNIGSNTVKWYQQLPGTAPKLLIYY NDQRPSGVPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDRYTHPALLFGTGTKV TVLggggsggggsggggsQVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPG KGLEWVAFIRYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCK THGSHDNWGQGTMVTVSSggggsggggsggggsEVQLVESGGGLVQPGNSLRLSCAASGF TFSKFGMSWVRQAPGKGLEWVSSISGSGRDTLYAESVKGRFTISRDNAKTTLYLQM NSLRPEDTAVYYCTIGGSLSVSSQGTLVTVSSHHHHHHHEPEA
69	ACP140 Chimeric IL-12 fusion protein	mdmrvpaqllgllllwlrgarcQVQLQESGGGLAQAGGSLSLSCAASGFTVSNSVMAWYRQTP GKQREFVAIINSVGSTNYADSVKGRFTISRDNAKNTVYLQMNNLKPEDTAVYVCNR NFDRIYWGQGTQVTVSSSGGPGPAGMKGLPGSiwelkkdvyvveldwypdapgemvvltcdtpeed gitwtldqssevlgsgktltiqvkefgdagqytchkggevlshsllllhkkedgiwstdilkdqkepknktflrceaknysgrftcw wlttistdltfsvkssrgssdpqgvtcgaatlsaervrgdnkeyeysvecqedsacpaaeeslpievmvdavhklkyenytssffir diikpdppknlqlkplknsrqvevsweypdtwstphsyfsltfcvqvqgkskrekkdrvftdktsatvicrknasisvraqdryys sswsewasvpcsggggsggggsggggsrvipvsgparclsqsrnllkttddmvktareklkhysctaedidheditrdqtstlktc lplelhknesclatretssttrgsclppqktslmmtlclgsiyedlkmyqtefqainaalqnhnhqqiildkgmlvaidelmqslnh ngetlrqkppvgeadpyrvkmklcillhafstrvvtinrvmgylssaSGGPGPAGMKGLPGSggggsggggsgggg sggggsggggsggggsQSVLTQPPSVSGAPGQRVTISCSGSRSNIGSNTVKWYQQLPGTAPK LLIYYNDQRPSGVPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDRYTHPALLFG TGTKVTVLggggsggggsggggsQVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWV RQAPGKGLEWVAFIRYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAV YYCKTHGSHDNWGQGTMVTVSSggggsggggsggggsEVQLVESGGGLVQPGNSLRLSC AASGFTFSKFGMSWVRQAPGKGLEWVSSISGSGRDTLYAESVKGRFTISRDNAKTTL YLQMNSLRPEDTAVYYCTIGGSLSVSSQGTLVTVSSHHHHHHHEPEA
70	ACP117 Anti-FN CGS-2 scFv	mdmrvpaqllgllllwlrgarcEVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAP GKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCA RGVGAFRPYRKHEWGQGTLVTVSRggggsggggsggggsSSELTQDPAVSVALGQTVRIT CQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTTTGAQ AEDEADYYCNSSPFEHNLVVFGGGTKLTVLHHHHHHHEPEA
71	EGFR (G8) Prodrug C1486	EVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQGGGGGLDGNEEPGGLE WVSSISGSGRDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLS VSSQGTLVTVSSGGGGKPLGLQARVVGGGGTQTVVTQEPSLTVSPGGTVTLTCASST GAVTSGNYPNWVQQKPGQAPRGLIGGTKFLVPGTPARFSGSLLGGKAALTLSGVQP EDEAEYYCTLWYSNRWVFGGGTKLTVLGGGGSGGGGSGGGGSEVQLVESGGGLV QPGGSLKLSCAASGFTFNKYAINWVRQAPGKGLEWVARIRSKYNNYATYYADQVK DRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHANFGNSYISYWAYWGQGTLVT VSSGGGGSGGGS*EVQLVESGGGLVQPGGSLTLSCAASGRTFSSYAMGWFRQAPGKEREF VVAINWASGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAAGYQINSGNYN FKDYEYDYWGQGTLVTVSS*HHHHHH
72	EGFR (G8) Non-cleavable	EVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQGGGGGLDGNEEPGGLE WVSSISGSGRDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLS VSSQGTLVTVSSGGGGSGGGGSGGVVGGGGTQTVVTQEPSLTVSPGGTVTLTCASS TGAVTSGNYPNWVQQKPGQAPRGLIGGTKFLVPGTPARFSGSLLGGKAALTLSGVQ PEDEAEYYCTLWYSNRWVFGGGTKLTVLGGGGSGGGGSGGGGSEVQLVESGGGLV

	Prodrug C1756	QPGGSLKLSCAASGFTFNKYAINWVRQAPGKGLEWVARIRSKYNNYATYYADQVK DRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHANFGNSYISYWAYWGQGTLVT VSSGGGGSGGGS*EVQLVESGGGLVQPGGSLTLSCAASGRTFSSYAMGWFRQAPGKEREF VVAINWASGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAAGYQINSGNYN FKDYEYDYWGQGTLVTVSS*HHHHHH
73	EGFR (G8) Active Drug C1300	VVGGGGTQTVVTQEPSLTVSPGGTVTLTCASSTGAVTSGNYPNWVQQKPGQAPRGL IGGTKFLVPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCTLWYSNRWVFGGGTK LTVLGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAINWV RQAPGKGLEWVARIRSKYNNYATYYADQVKDRFTISRDDSKNTAYLQMNNLKTED TAVYYCVRHANFGNSYISYWAYWGQGTLVTVSSGGGGSGGGS*EVQLVESGGGLVQ PGGSLTLSCAASGRTFSSYAMGWFRQAPGKEREFVVAINWASGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCAAGYQINSGNYNFKDYEYDYWGQGTLVTVSS*HHHHHH
74	PSMA Prodrug C1872	EVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQGGGGGLDGNEEPGGLE WVSSISGSGRDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLS VSSQGTLVTVSSGGGGKPLGLQARVVGGGGTQTVVTQEPSLTVSPGGTVTLTCASST GAVTSGNYPNWVQQKPGQAPRGLIGGTKFLVPGTPARFSGSLLGGKAALTLSGVQP EDEAEYYCTLWYSNRWVFGGGTKLTVLGGGGSGGGGSGGGGSEVQLVESGGGLV QPGGSLKLSCAASGFTFNKYAINWVRQAPGKGLEWVARIRSKYNNYATYYADQVK DRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHANFGNSYISYWAYWGQGTLVT VSSGGGGSGGGS*EVQLVESGGGLVQPGGSLTLSCAASRFMISEYHMHWVRQAPGKGLE WVSTINPAGTTDYAESVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYCDSYGYRGQGTQ VTVSS*HHHHHH
75	PSMA Non-cleavable Prodrug C1873	EVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQGGGGGLDGNEEPGGLE WVSSISGSGRDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLS VSSQGTLVTVSSGGGGSGGGGSGGVVGGGGTQTVVTQEPSLTVSPGGTVTLTCASS TGAVTSGNYPNWVQQKPGQAPRGLIGGTKFLVPGTPARFSGSLLGGKAALTLSGVQ PEDEAEYYCTLWYSNRWVFGGGTKLTVLGGGGSGGGGSGGGGSEVQLVESGGGLV QPGGSLKLSCAASGFTFNKYAINWVRQAPGKGLEWVARIRSKYNNYATYYADQVK DRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHANFGNSYISYWAYWGQGTLVT VSSGGGGSGGGS*EVQLVESGGGLVQPGGSLTLSCAASRFMISEYHMHWVRQAPGKGLE WVSTINPAGTTDYAESVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYCDSYGYRGQGTQ VTVSS*HHHHHH
76	PSMA Active Drug C1875	VVGGGGTQTVVTQEPSLTVSPGGTVTLTCASSTGAVTSGNYPNWVQQKPGQAPRGL IGGTKFLVPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCTLWYSNRWVFGGGTK LTVLGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAINWV RQAPGKGLEWVARIRSKYNNYATYYADQVKDRFTISRDDSKNTAYLQMNNLKTED

		TAVYYCVRHANFGNSYISYWAYWGQGTLVTVSSGGGGSGGGS*EVQLVESGGGLVQ PGGSLTLSCAASRFMISEYHMHWVRQAPGKGLEWVSTINPAGTTDYAESVKGRFTISRDN AKNTLYLQMNSLKPEDTAVYYCDSYGYRGQGTQVTVSS*HHHHHH
77	GFP TriTAC C646	*QVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPGKEREWVAGMSSAGDRSS YEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEYWGQGTQVTVSS*GGG GSGGGSEVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWVSS ISGSGRDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQG TLVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAINWVRQA PGKGLEWVARIRSKYNNYATYYADQVKDRFTISRDDSKNTAYLQMNNLKTEDTAV YYCVRHANFGNSYISYWAYWGQGTLVTVSSGGGGSGGGGSGGGGSQTVVTQEPSL TVSPGGTVTLTCASSTGAVTSGNYPNWVQQKPGQAPRGLIGGTKFLVPGTPARFSGS LLGGKAALTLSGVQPEDEAEYYCTLWYSNRWVFGGGTKLTVLHHHHHH
78	non-masked/non-cleavable TriTAC C1874	EVQLVESGGGLVQPGNSLRLSCAASGFTFSKFGMSWVRQAPGKGLEWVSSISGSGR DTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLSVSSQGTLVTV SSGGGGSGGGGSGGVVGGGGTQTVVTQEPSLTVSPGGTVTLTCASSTGAVTSGNYP NWVQQKPGQAPRGLIGGTKFLVPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCT LWYSNRWVFGGGTKLTVLGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLKLSC AASGFTFNKYAINWVRQAPGKGLEWVARIRSKYNNYATYYADQVKDRFTISRDDS KNTAYLQMNNLKTEDTAVYYCVRHANFGNSYISYWAYWGQGTLVTVSSGGGGSG GGSEVQLVESGGGLVQPGGSLTLSCAASRFMISEYHMHWVRQAPGKGLEWVSTINP AGTTDYAESVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYYCDSYGYRGQGTQVT VSSHHHHHH
79	Blocker 12 (IL-12 blocker)	mdmrvpaqllgllllwlrgarcQSVLTQPPSVSGAPGQRVTISCSGSRSNIGSNTVKWYQQLPGTAPKLLIYYND QRPSGVPDRFSGSKSGTSASLAITGLQAEDEADYYCQSYDRYTHPALLFGTGTKVTVLggggsggggsggggs QVQLVESGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAFIRYDGSNKYYADSVKGR FTISRDNSKNTLYLQMNSLRAEDTAVYYCKTHGSHDNWGQGTMVTVSSHHHHHH

INCORPORATION BY REFERENCE

[196] The entire disclosures of all patent and non-patent publications cited herein are each incorporated by reference in their entireties for all purposes.

OTHER EMBODIMENTS

[197] The disclosure set forth above may encompass multiple distinct inventions with independent utility. Although each of these inventions has been disclosed in its preferred form(s), the specific embodiments thereof as disclosed and illustrated herein are not to be considered in a limiting sense,

because numerous variations are possible. The subject matter of the inventions includes all novel and nonobvious combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein. The following claims particularly point out certain combinations and subcombinations regarded as novel and nonobvious. Inventions embodied in other combinations and subcombinations of features, functions, elements, and/or properties may be claimed in this application, in applications claiming priority from this application, or in related applications. Such claims, whether directed to a different invention or to the same invention, and whether broader, narrower, equal, or different in scope in comparison to the original claims, also are regarded as included within the subject matter of the inventions of the present disclosure.

CLAIMS

1. A fusion polypeptide of the formula:

[A]-[L1]-[D] or [A]-[L1]-[D]-[L2]-[B] or [B]-[L1]-[A]-[L1]-[D]

wherein,

A is an interleukin 12 (IL-12) polypeptide;

B is a half-life extension element;

L1 and L2 are each independently a polypeptide linker, wherein L1 is a protease-cleavable polypeptide linker and L2 is polypeptide linker that is optionally protease cleavable;

D is an IL-12 blocking moiety; and

the fusion polypeptide is an attenuated IL-12 receptor agonist, but upon (i) cleavage of the L1 protease-cleavable polypeptide linker, or (ii) cleavage of both L1 and L2 when L2 is a protease cleavable polypeptide linker, the IL-12 polypeptide-containing fragment of the fusion polypeptide that is produced is an IL-12 receptor agonist of comparable potency and half-life to the naturally occurring IL-12 polypeptide.

2. A fusion polypeptide comprising at least one of each of:

a) an interleukin 12 (IL-12) polypeptide [A];

b) an IL-12 blocking moiety [D]; and

c) a protease-cleavable polypeptide linker [L]; and

wherein the IL-12 polypeptide and the IL-12 blocking moiety are operably linked by the protease-cleavable polypeptide linker and the fusion polypeptide has attenuated IL-12 receptor activating activity, wherein the IL-12-receptor activating activity of the fusion polypeptide is at least about 10X less than the IL-12 receptor activating activity of the polypeptide that contains the IL-12 polypeptide that is produced by cleavage of the protease cleavable polypeptide linker.

3. The fusion polypeptide of claim 2, wherein [A] is the N-terminus of the fusion polypeptide.

4. The fusion polypeptide of any one of claims 1-3, wherein [A] has the formula:

[A1]-[L3]-[A2] or [A2]-[L3]-[A1], wherein

A1 is an IL-12 p40 subunit polypeptide;

A2 is an IL-12 p35 subunit polypeptide; and

L3 is a polypeptide linker that is optionally protease cleavable.

5. The fusion polypeptide of claim 4, wherein [A] has the formula [A1]-[L3]-[A2], and L3 is not protease cleavable.

6. The fusion polypeptide of any one of claims 1-5, wherein the agonist activity of the IL-12 polypeptide containing fragment of the cleaved polypeptide is increased at least 10X compared to the agonist activity of the uncleaved fusion polypeptide.

7. The fusion polypeptide of claim 6, wherein the agonist activity is assessed using a HEK Blue reporter cell assay.

8. The fusion polypeptide of any one of claims 1-7, wherein each protease-cleavable polypeptide linker independently comprises a sequence that is capable of being cleaved by a protease selected from the group consisting of a kallikrein, thrombin, chymase, carboxypeptidase A, cathepsin G, cathepsin L, an elastase, PR-3, granzyme M, a calpain, a matrix metalloproteinase (MMP), a fibroblast activation protein (FAP), an ADAM metalloproteinase, a plasminogen activator, a cathepsin, a caspase, a tryptase, and a tumor cell surface protease.

9. The fusion polypeptide of claim 8 wherein each protease-cleavable polypeptide independently comprises two or more cleavage sites for the same protease, or two or more cleavage sites that are cleaved by different proteases or at least one of the protease-cleavable polypeptides comprises a cleavage site for two or more different proteases.

10. The fusion polypeptide of any one of claims 1-9, wherein IL-12 blocking moiety inhibits activation of the IL-12 receptor by the fusion polypeptide.

11. The fusion polypeptide of any one of claims 1-10, wherein the IL-12 blocking moiety noncovalently binds to the IL-12 polypeptide.

12. The fusion polypeptide of any one of claims 1-11, wherein the IL-12 blocking moiety binds the IL-12 p40 subunit polypeptide.

13. The fusion polypeptide of any one of claims 1-11, wherein the IL-12 blocking moiety binds the IL-12 p35 subunit polypeptide.

14. The fusion polypeptide of any one of claims 1-11, wherein the IL-12 blocking moiety binds IL-12 p70.

15. The fusion polypeptide of any one of claims 1-14, wherein the IL-12 blocking moiety comprises a ligand binding domain or fragment of a cognate receptor for the IL-12, a single domain antibody, Fab or scFv that binds the IL-12 polypeptide, or an antibody or antibody fragment selected from a single domain antibody, an Fab and an scFv that binds a receptor of the IL-12.

16. The fusion polypeptide of any one of claims 1-15, wherein the IL-12 blocking moiety is also an half-life extension element.

17. The fusion polypeptide of any one of claims 1-10 or 16, wherein the IL-12 blocking moiety sterically blocks agonist activity of the IL-12.

18. The fusion polypeptide of claim 17, wherein the IL-12 blocking moiety is human serum albumin, or an antigen binding polypeptide which recruits human serum albumin.

19. The fusion polypeptide of any one of claims 1-18, wherein the IL-12 is free to dissociate from the IL-12 blocking moiety after the protease-cleavable polypeptide linker is cleaved by a protease.

20. The fusion polypeptide of any of claims 1-19, wherein the fusion polypeptide binds to the IL-12 receptor.

21. The fusion polypeptide of any one of claims 1-20, further comprising at least one half-life extension element.

22. The fusion polypeptide of claim 21, wherein the half-life extension element is human serum albumin, or an antigen binding polypeptide which binds human serum albumin.

23. The fusion polypeptide of claim 21, wherein the half-life extension element is an immunoglobulin Fc.

24. The fusion polypeptide of claim 11, wherein the noncovalent association is pH-dependent.

25. The fusion polypeptide of any one of claims 1-24, wherein IL-12 receptor activation is determined using a standard *in vitro* receptor activation assay and equal amounts on a mole basis of the IL-12 polypeptide and the fusion polypeptide

26. The fusion polypeptide of any one of claims 1-25, wherein IL-12 is free to dissociate from the IL-12 blocking moiety and/or half-life extension element after the protease-cleavable sequence is cleaved by a protease.

27. The fusion polypeptide of any one of claims 2-23 further comprising a half-life extension element [B] and having the formula

[B]-[L1]-[A]-[L2]-[D]

wherein L1 and L2 are each independently a polypeptide linker, wherein L1 is a protease-cleavable polypeptide linker and L2 is polypeptide linker that is optionally protease cleavable.

28. The fusion polypeptide of claim 27, wherein L2 is a protease cleavable linker.

29. The fusion polypeptide of any one of claims 1 or 3-28, wherein L1 is a substrate for a first protease and L2 is a substrate for a second protease.

30. The fusion polypeptide of any of claims 1-29, further comprising a tumor specific antigen binding peptide.

31. The fusion polypeptide of claim 30, where the tumor specific antigen binding peptide is linked to the IL-12 polypeptide by a non-cleavable linker.

32. The fusion polypeptide of claim 30, where the tumor specific antigen binding peptide is linked to the IL-12 polypeptide, the half-life extension element or the IL-12 blocking moiety by a cleavable linker.

33. The fusion polypeptide of any one of claims 1-32, further comprising a linker between domains of IL-12.

34. The fusion polypeptide of any one of claims 3-32, wherein the serum half-life of the IL-12 polypeptide-containing fragment that is produced by protease cleavage of the protease cleavable polypeptide linker is comparable to the half-life of naturally occurring IL-12.

35. A nucleic acid encoding the polypeptide of any of claims 1-34.

36. A vector comprising the nucleic acid of claim 35.

37. A host cell comprising the vector of claim 36.

38. A method of making a pharmaceutical composition, comprising culturing the host cell of claim 37 under suitable conditions for expression and collection of desired polypeptides.

39. A pharmaceutical composition comprising i) an effective amount of the fusion polypeptide of any of claims 1-34, and ii) a pharmaceutically acceptable excipient.

40. A method for treating a tumor, comprising administering to a subject in need thereof an effective amount of a fusion polypeptide of any one of claims 1-34.

41. A fusion polypeptide of any one of claims 1-34 for use as a medicament.

42. A fusion polypeptide of any one of claims 1-34 for use in treating a tumor in a subject in need thereof.

43. A pharmaceutical composition for treating a tumor in a subject in need thereof comprising as an active ingredient a fusion polypeptide of any one of claims 1-34.

FIG. 1a

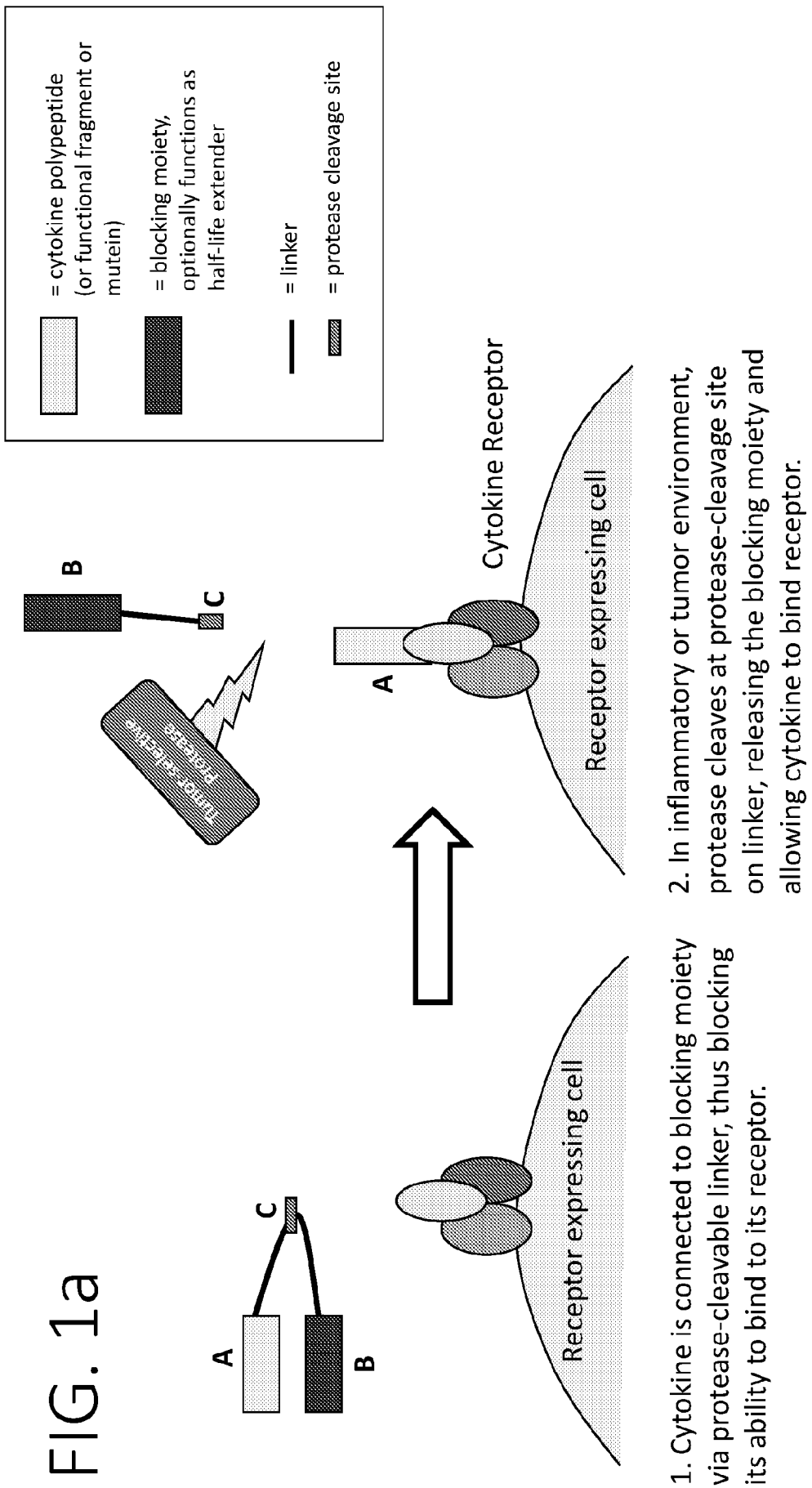
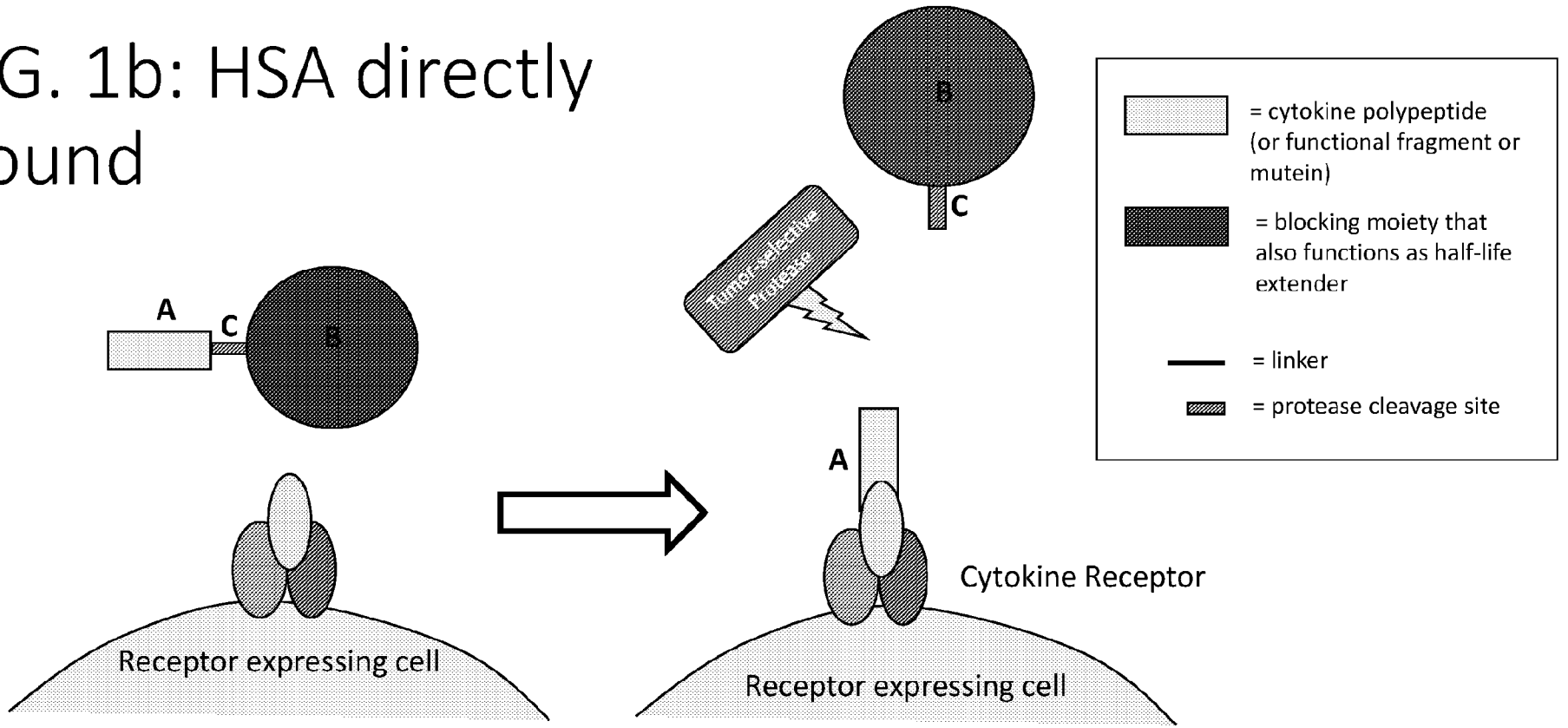

= cytokine polypeptide (or functional fragment or mutein)
= blocking moiety, optionally functions as half-life extender
= linker
= protease cleavage site
A
B
C
Tumor-selective protease
Cytokine Receptor
Receptor expressing cell
Receptor expressing cell
1. Cytokine is connected to blocking moiety via protease-cleavable linker, thus blocking its ability to bind to its receptor.
2. In inflammatory or tumor environment, protease cleaves at protease-cleavage site on linker, releasing the blocking moiety and allowing cytokine to bind receptor.

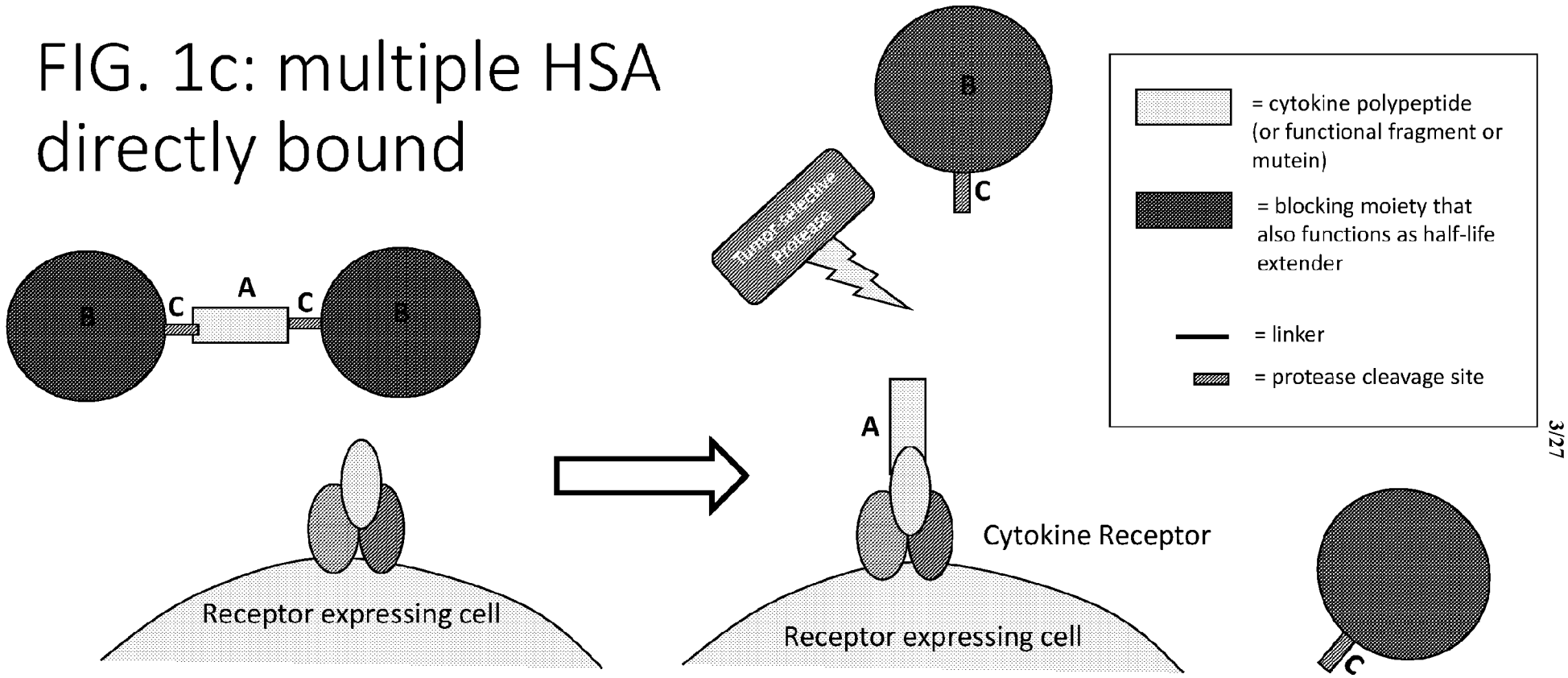
FIG. 1b: HSA directly bound



1. Cytokine is connected to blocking moiety via protease-cleavable linker, thus blocking its ability to bind to its receptor.

2. In inflammatory or tumor environment, protease cleaves at protease-cleavage site on linker, releasing the blocking moiety and allowing cytokine to bind receptor.

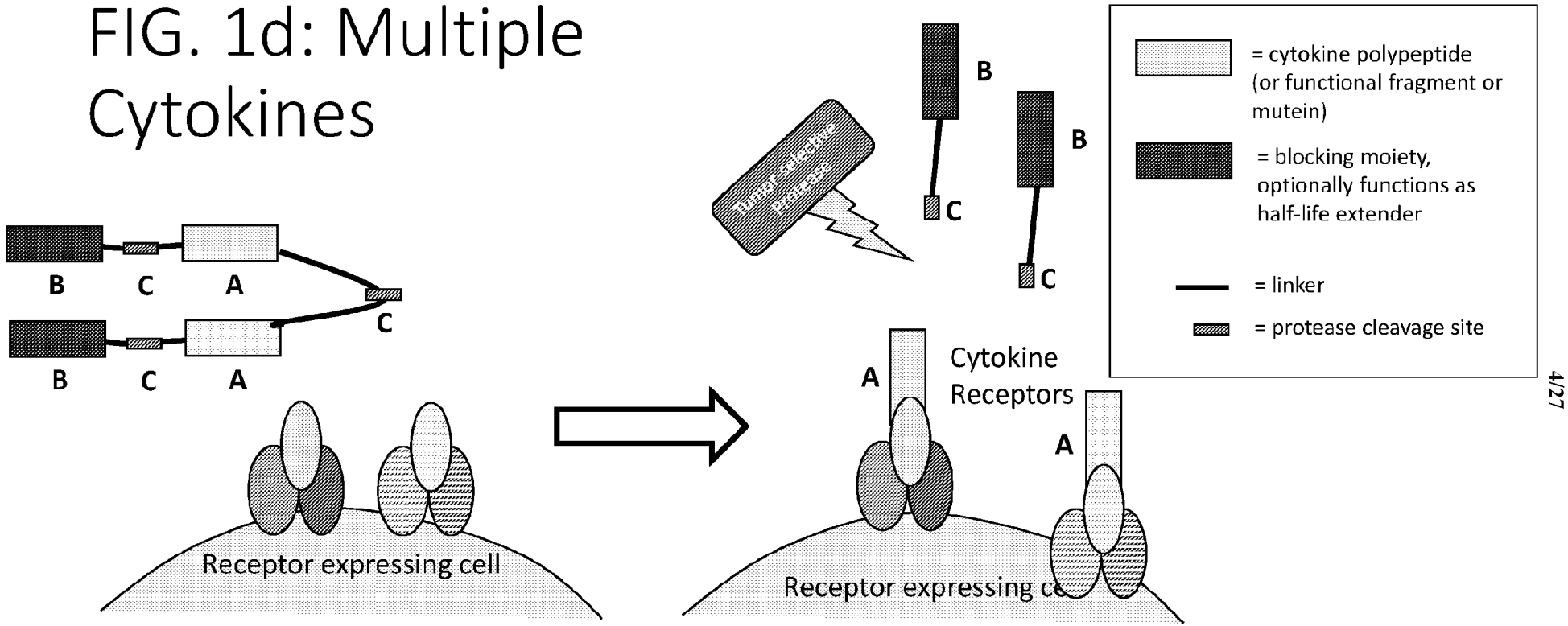
FIG. 1c: multiple HSA directly bound



1. Cytokine is connected to blocking moiety via protease-cleavable linker, thus blocking its ability to bind to its receptor.

2. In inflammatory or tumor environment, protease cleaves at protease-cleavage site on linker, releasing the blocking moiety and allowing cytokine to bind receptor. The cytokine now has similar pK properties as compared to the native cytokine (e.g. short half-life).

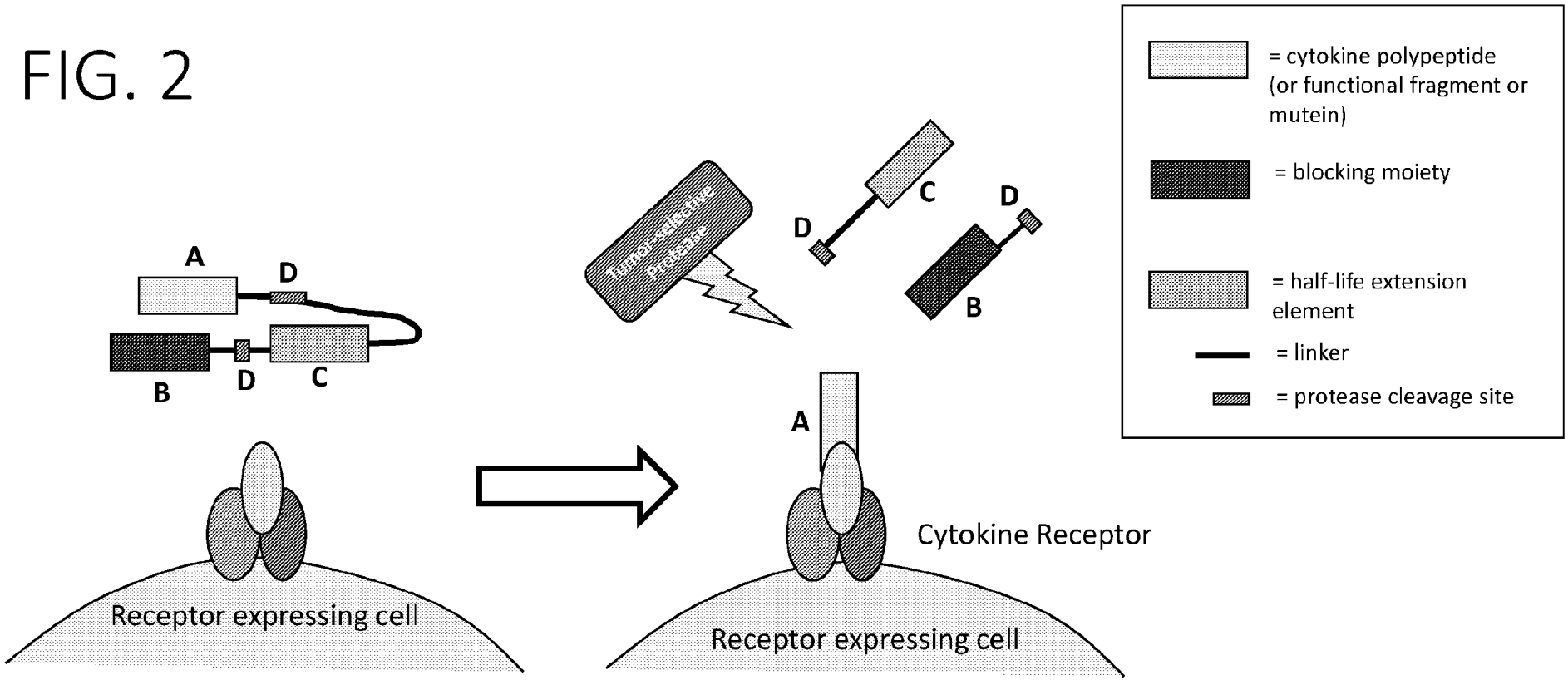
FIG. 1d: Multiple Cytokines



1. Cytokine is connected to blocking moiety via protease-cleavable linker, thus blocking its ability to bind to its receptor.

2. In inflammatory or tumor environment, protease cleaves at protease-cleavage site on linker, releasing the blocking moiety and allowing cytokine to bind receptor.

FIG. 2



1. Cytokine is connected to blocking moiety via protease-cleavable linker(s), thus blocking its ability to bind to its receptor. It is also bound to a separate half-life extension element, which extends half-life in serum.

2. In inflammatory or tumor environment, protease cleaves at protease-cleavage site on linker, releasing the half-life extension element and blocking moiety and allowing cytokine to bind receptor. The cytokine now has similar pK properties as compared to the native cytokine (e.g. short half-life).

FIG. 3

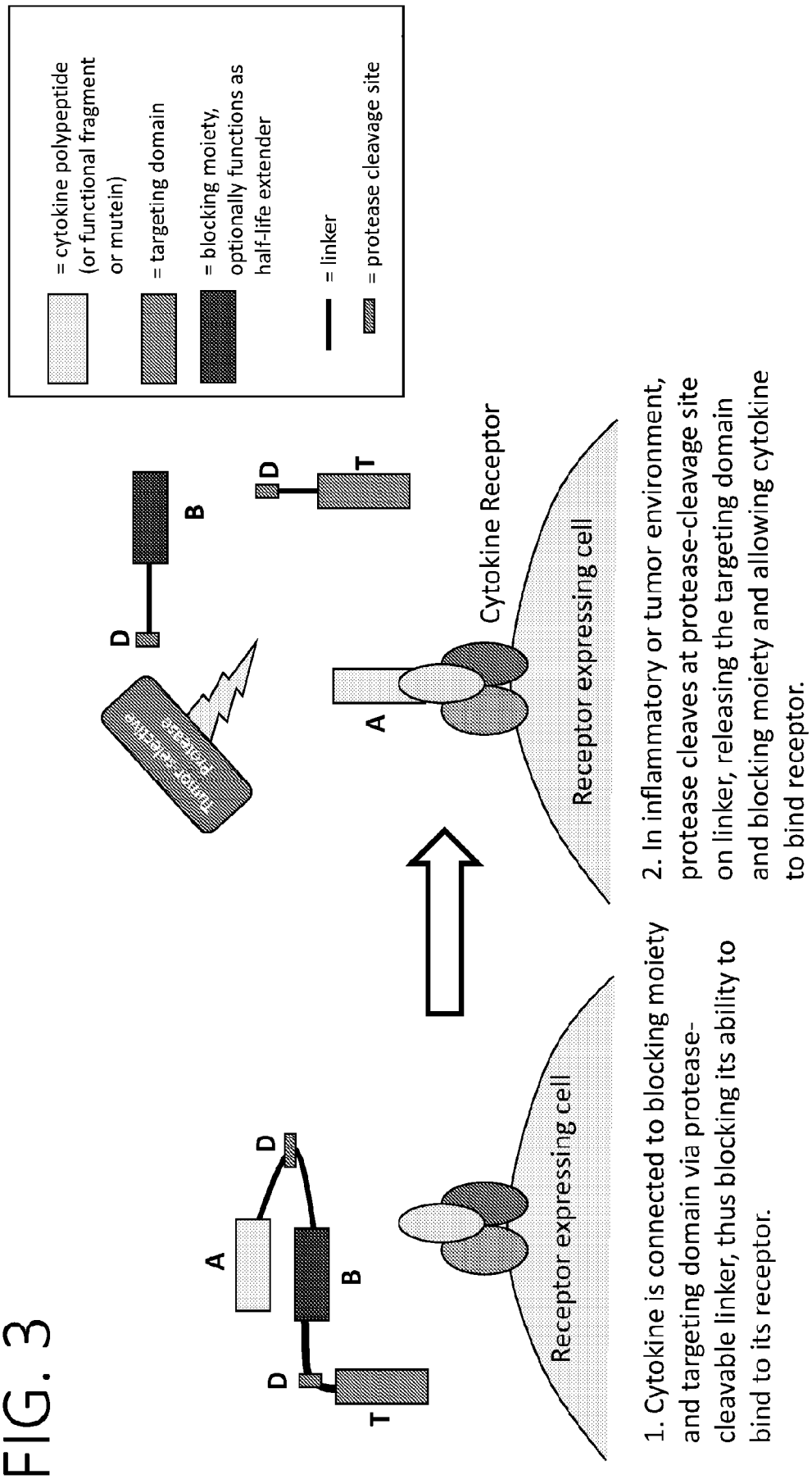
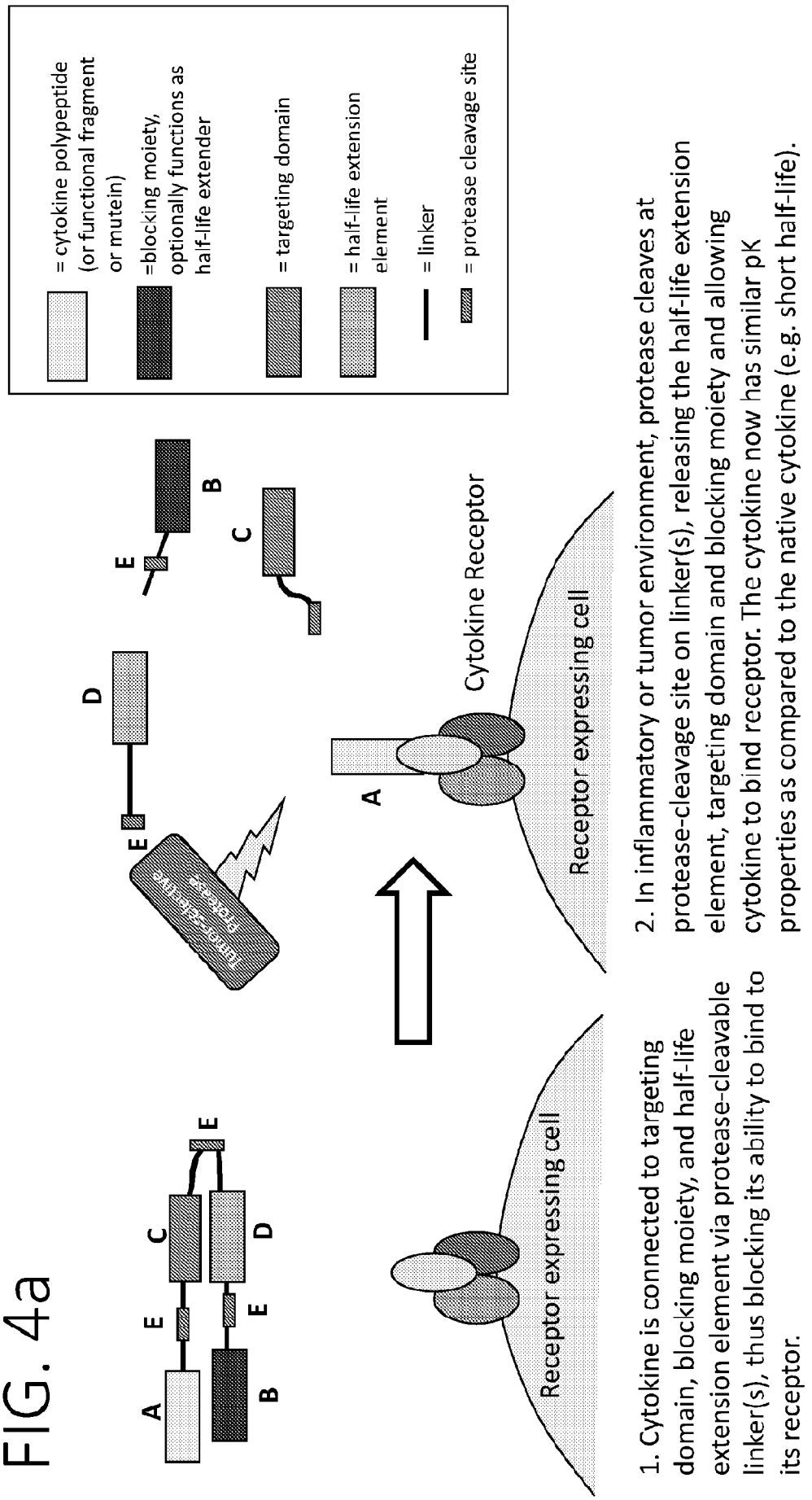

= cytokine polypeptide (or functional fragment or mutein)
= targeting domain
= blocking moiety, optionally functions as half-life extender
= linker
= protease cleavage site
A
B
D
T
Tumor-associated protease
Cytokine Receptor
Receptor expressing cell
Receptor expressing cell
1. Cytokine is connected to blocking moiety and targeting domain via protease-cleavable linker, thus blocking its ability to bind to its receptor.
2. In inflammatory or tumor environment, protease cleaves at protease-cleavage site on linker, releasing the targeting domain and blocking moiety and allowing cytokine to bind receptor.

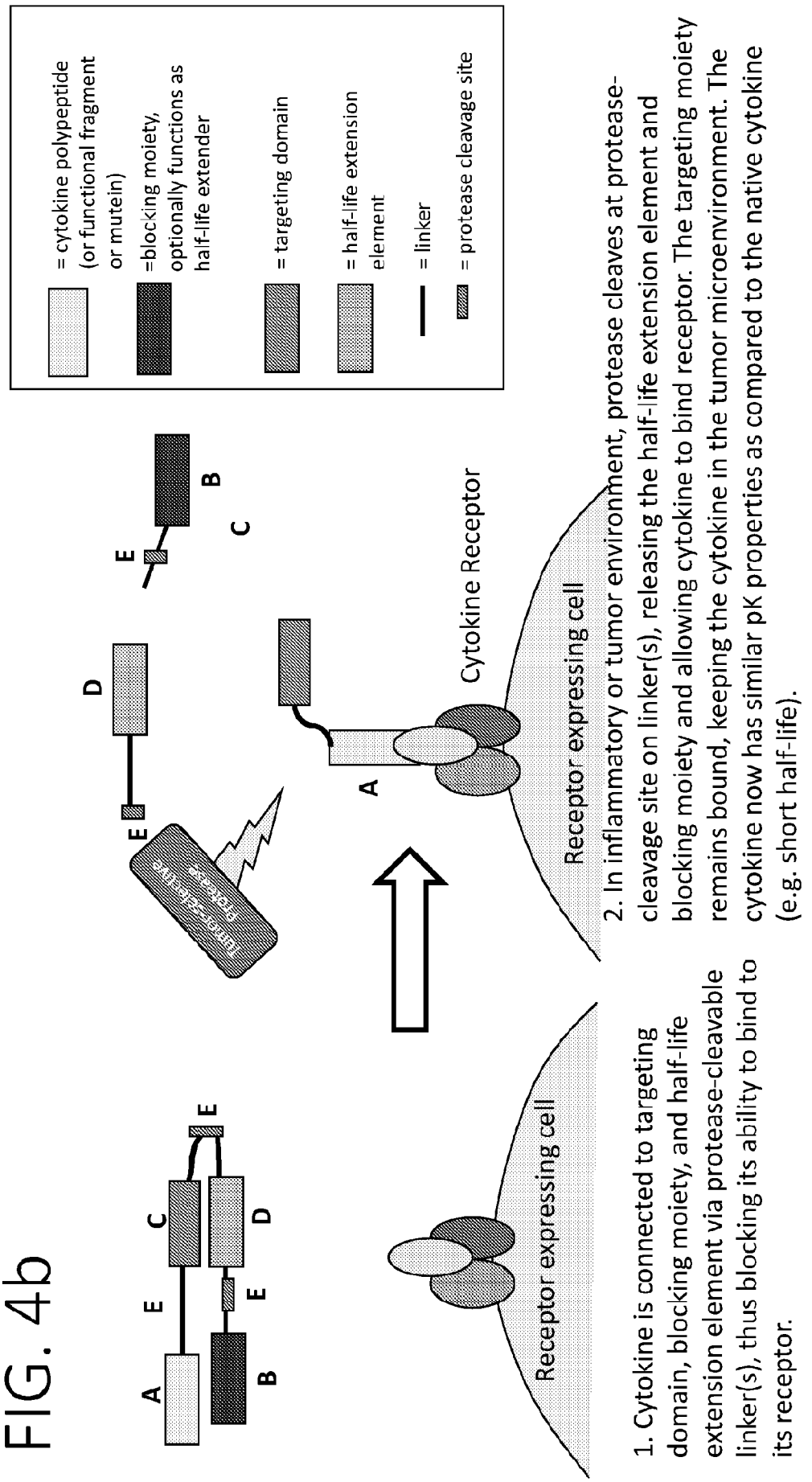
FIG. 4a



1. Cytokine is connected to targeting domain, blocking moiety, and half-life extension element via protease-cleavable linker(s), thus blocking its ability to bind to its receptor.

2. In inflammatory or tumor environment, protease cleaves at protease-cleavage site on linker(s), releasing the half-life extension element, targeting domain and blocking moiety and allowing cytokine to bind receptor. The cytokine now has similar pK properties as compared to the native cytokine (e.g. short half-life).

FIG. 4b


= cytokine polypeptide (or functional fragment or mutein)
=blocking moiety, optionally functions as half-life extender
= targeting domain
= half-life extension element
= linker
= protease cleavage site
A
B
C
D
E
Cytokine Receptor
Receptor expressing cell
Receptor expressing cell
1. Cytokine is connected to targeting domain, blocking moiety, and half-life extension element via protease-cleavable linker(s), thus blocking its ability to bind to its receptor.
2. In inflammatory or tumor environment, protease cleaves at protease-cleavage site on linker(s), releasing the half-life extension element and blocking moiety and allowing cytokine to bind receptor. The targeting moiety remains bound, keeping the cytokine in the tumor microenvironment. The cytokine now has similar pK properties as compared to the native cytokine (e.g. short half-life).

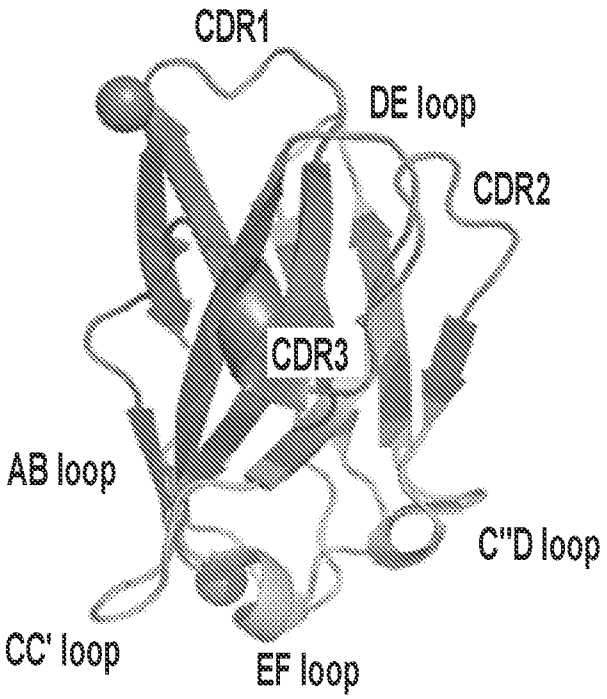


FIG. 5

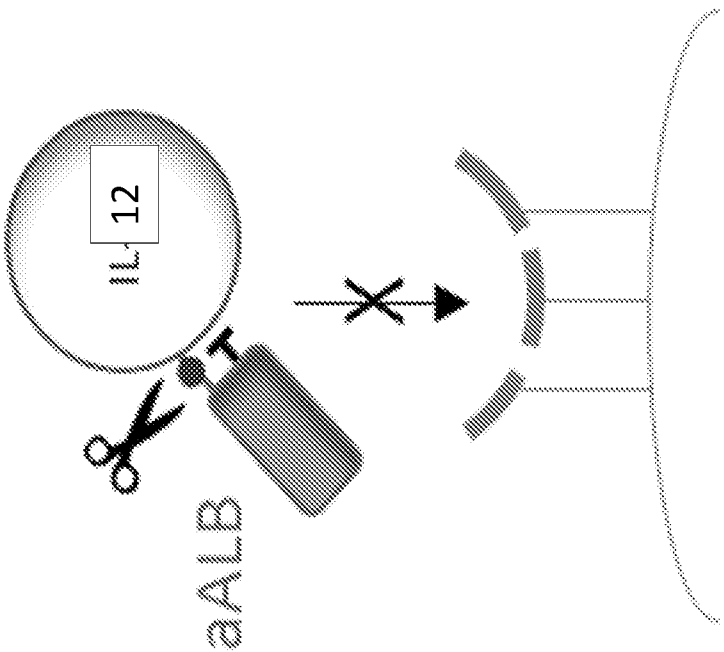


FIG. 6

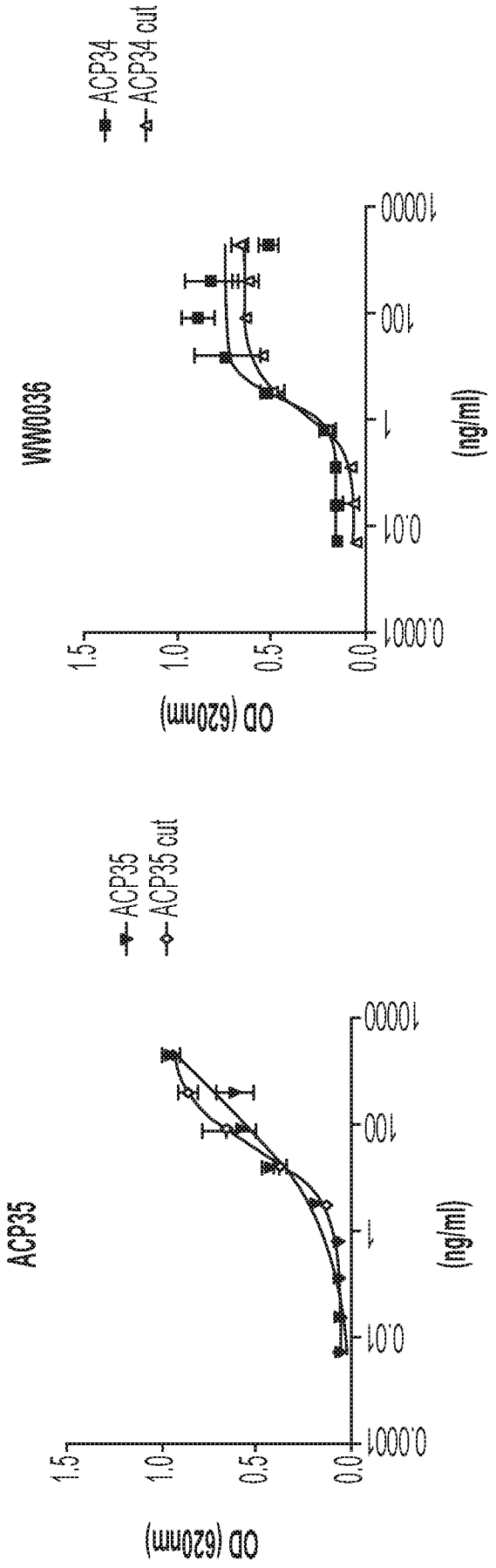

ACP34
ACP34 cut
WW0036
OD (620nm)
1.5
1.0
0.5
0.0
0.0001
0.01
1
100
10000
(ng/ml)
ACP35
ACP35 cut
ACP35
OD (620nm)
1.5
1.0
0.5
0.0
0.0001
0.01
1
100
10000
(ng/ml)

FIG. 7

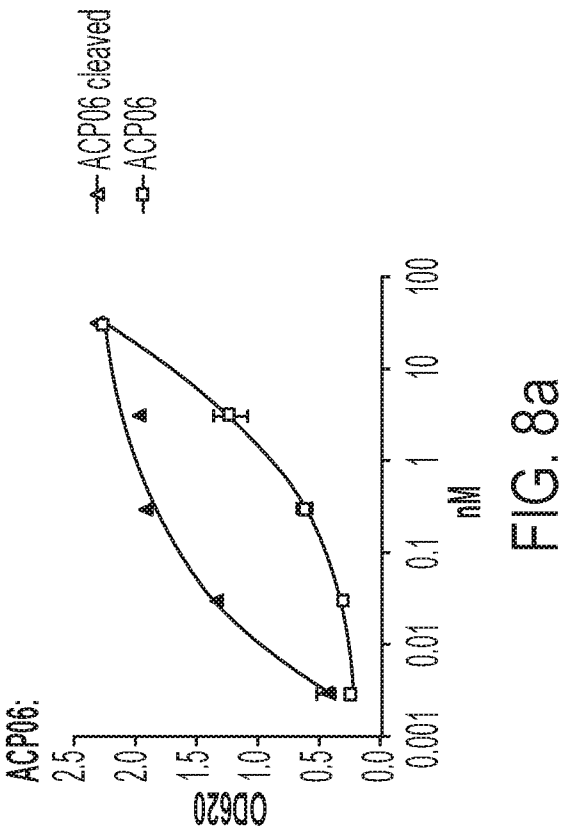


FIG. 8a

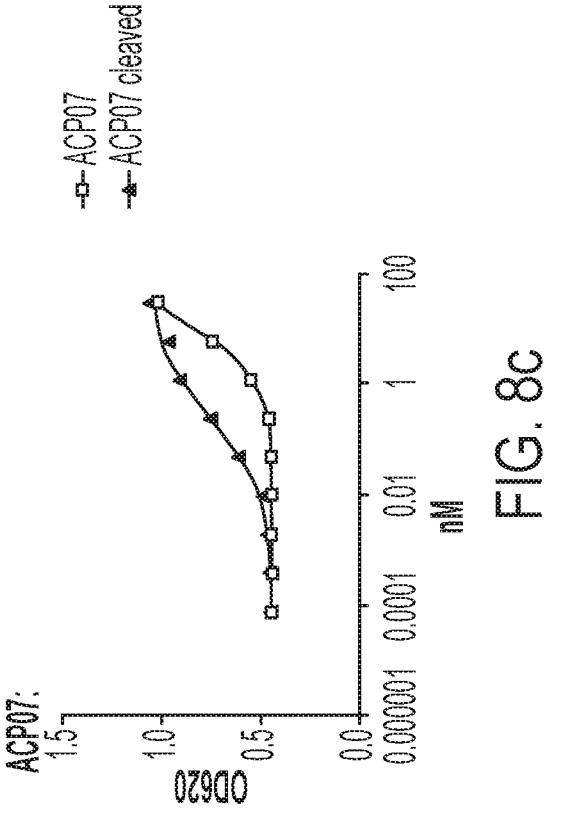


FIG. 8c

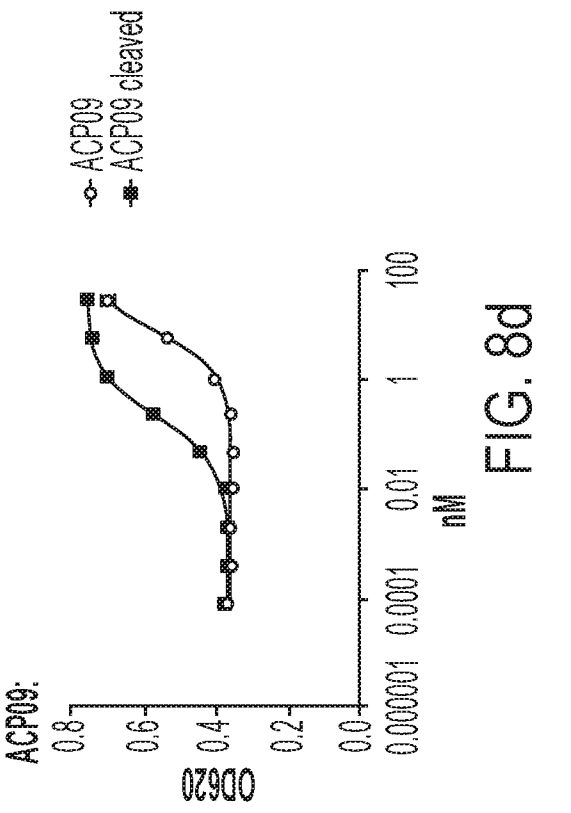


FIG. 8d

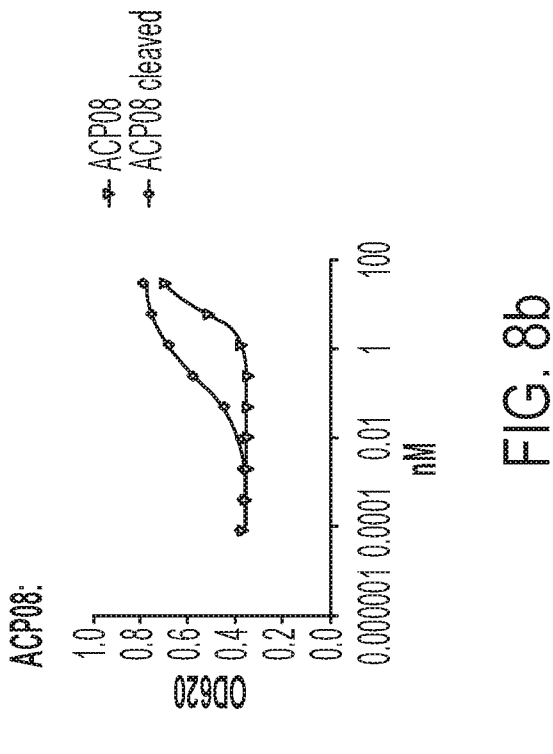


FIG. 8b

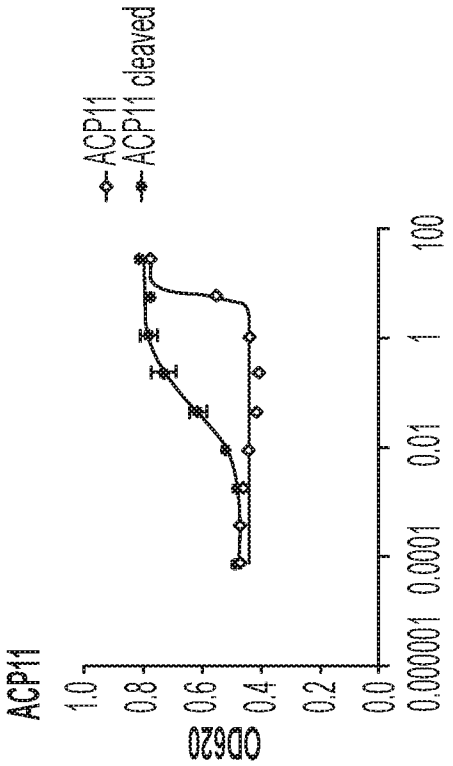

ACP11
ACP11
ACP11 cleaved
OD620
1.0
0.8
0.6
0.4
0.2
0.0
0.000001
0.0001
0.01
1
100
nM

FIG. 8f

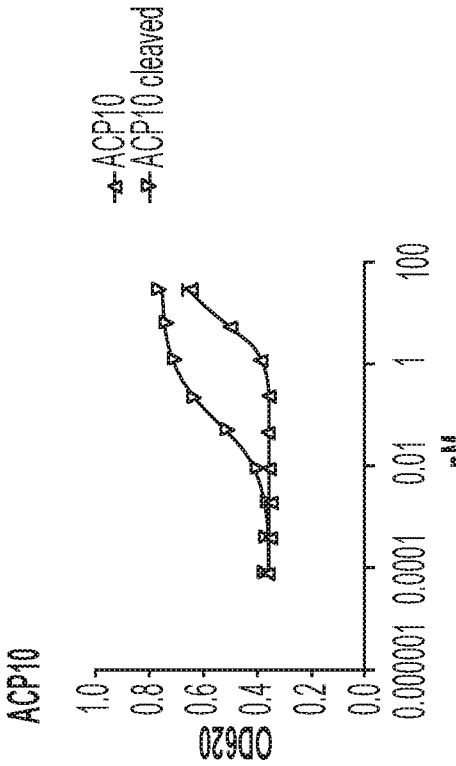

ACP10
ACP10
ACP10 cleaved
OD620
1.0
0.8
0.6
0.4
0.2
0.0
0.000001
0.0001
0.01
1
100
nM

FIG. 8e

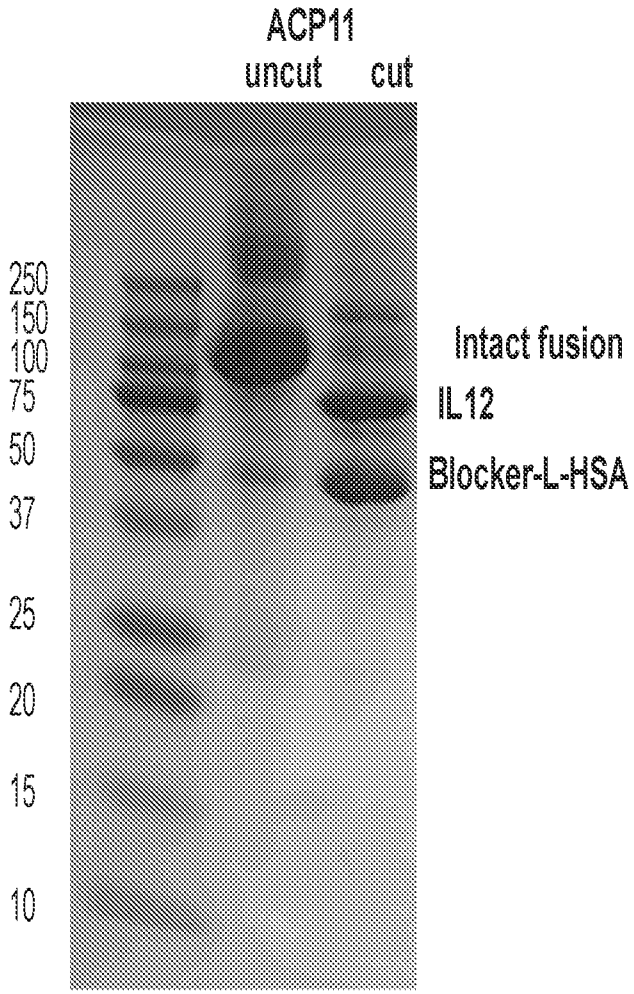

ACP11
uncut
cut
250
150
100
75
50
37
25
20
15
10
Intact fusion
IL12
Blocker-L-HSA

FIG. 9

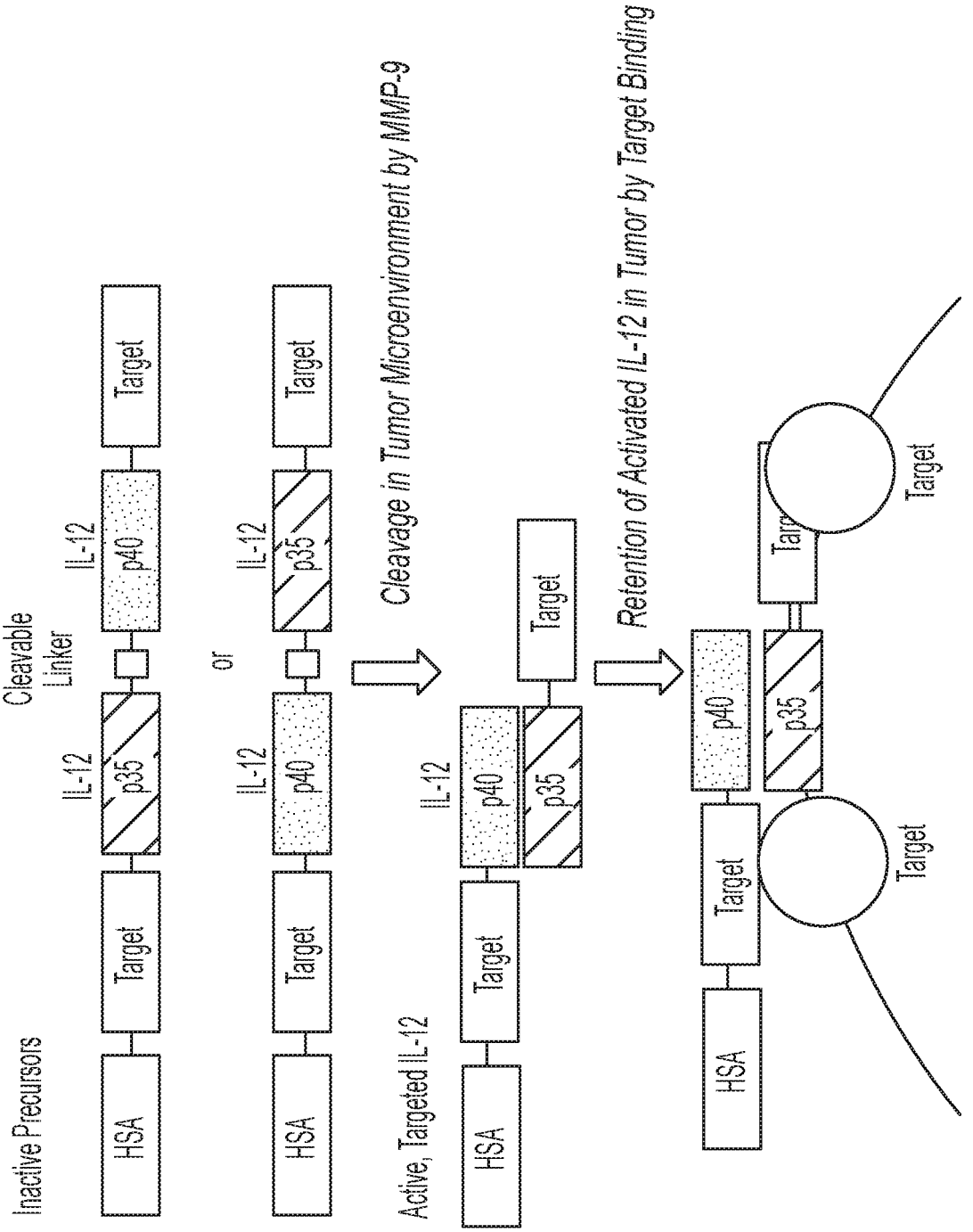

Inactive Precursors
HSA
Target
Cleavable Linker
IL-12
p35
p40
or
Cleavage in Tumor Microenvironment by MMP-9
Active, Targeted IL-12
Retention of Activated IL-12 in Tumor by Target Binding

FIG. 10

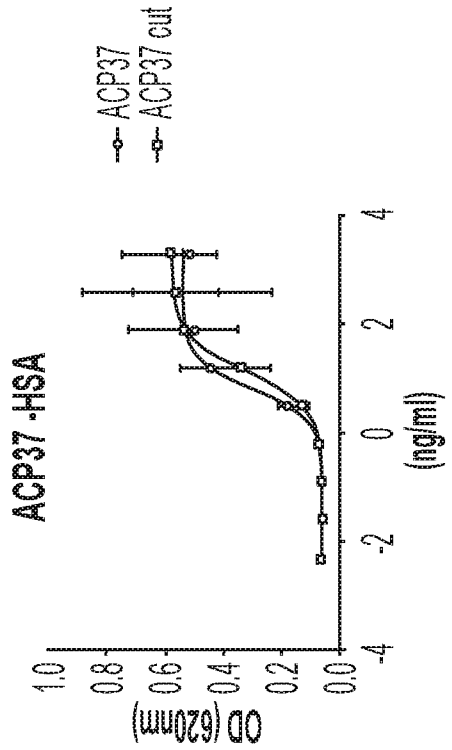


FIG. 11c

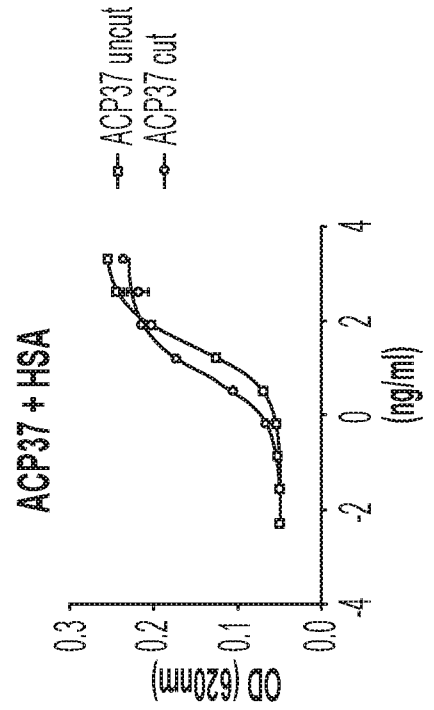


FIG. 11d

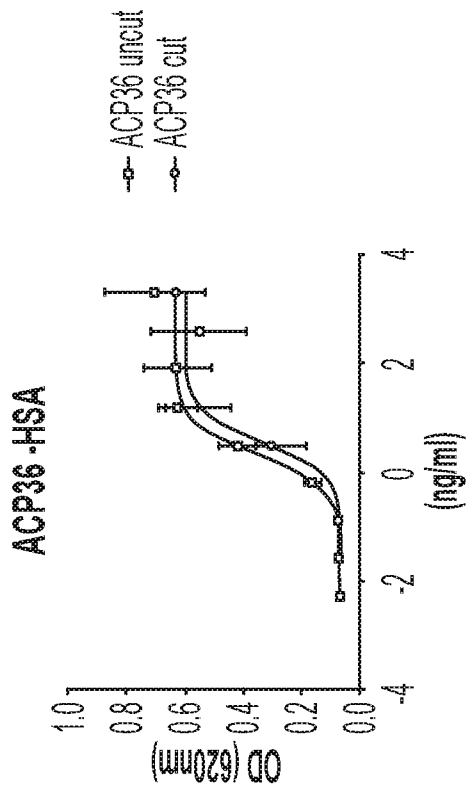


FIG. 11a

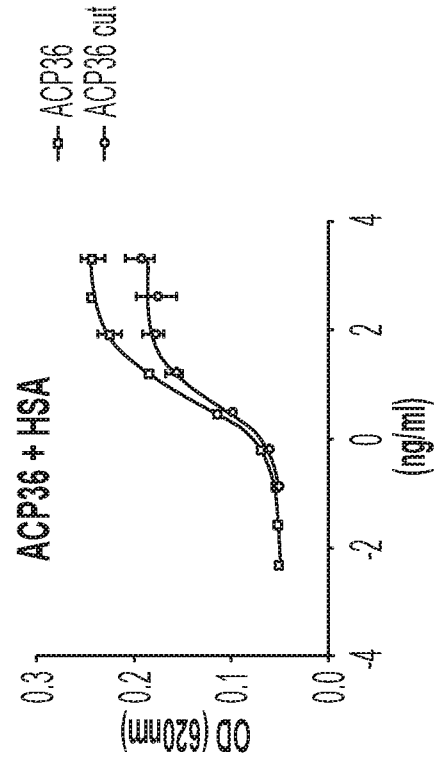
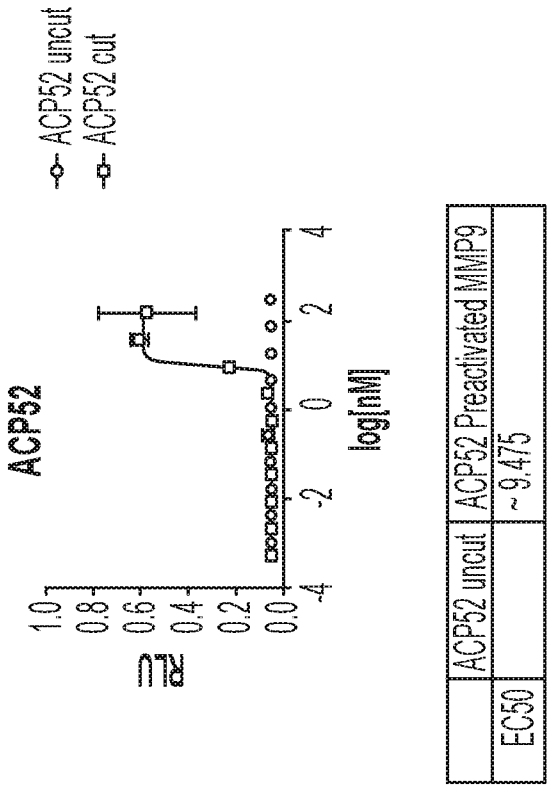
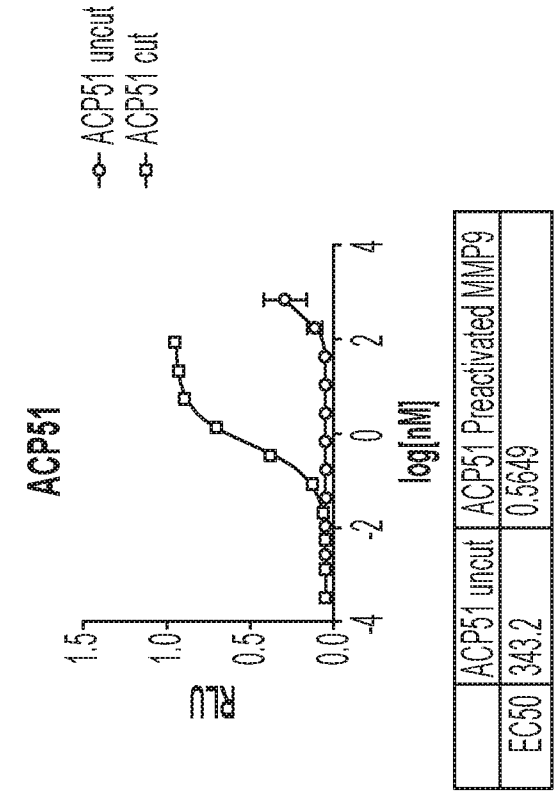


FIG. 11b

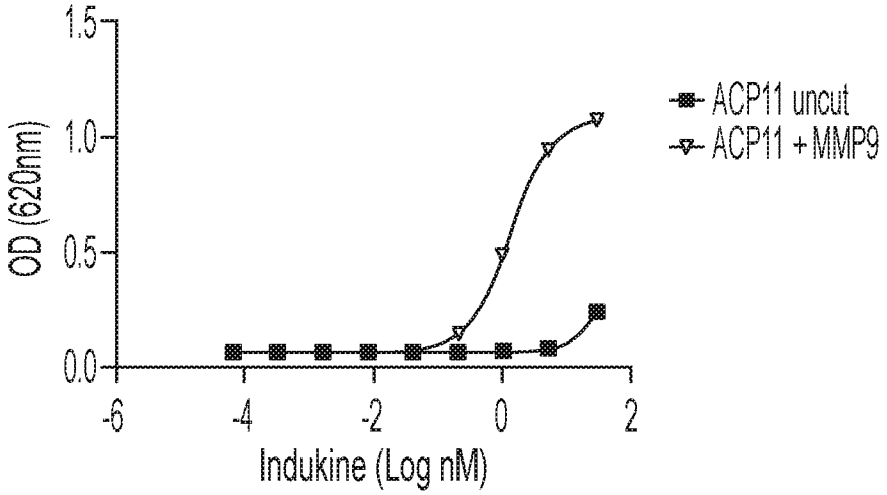


	ACP52 uncut	ACP52 Preactivated MMP9
EC50		~9.475



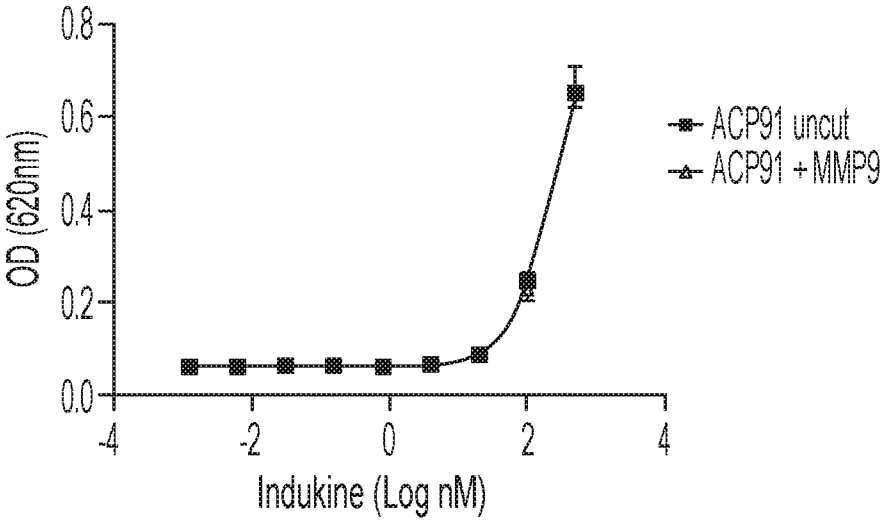
	ACP51 uncut	ACP51 Preactivated MMP9
EC50	343.2	0.5649

FIG. 12



	ACP11 uncut	ACP11 + MMP9
EC50	~ 212.2	1.349

FIG. 13a



	ACP91 uncut	ACP91 + MMP9
EC50	255.9	288.2

FIG. 13b

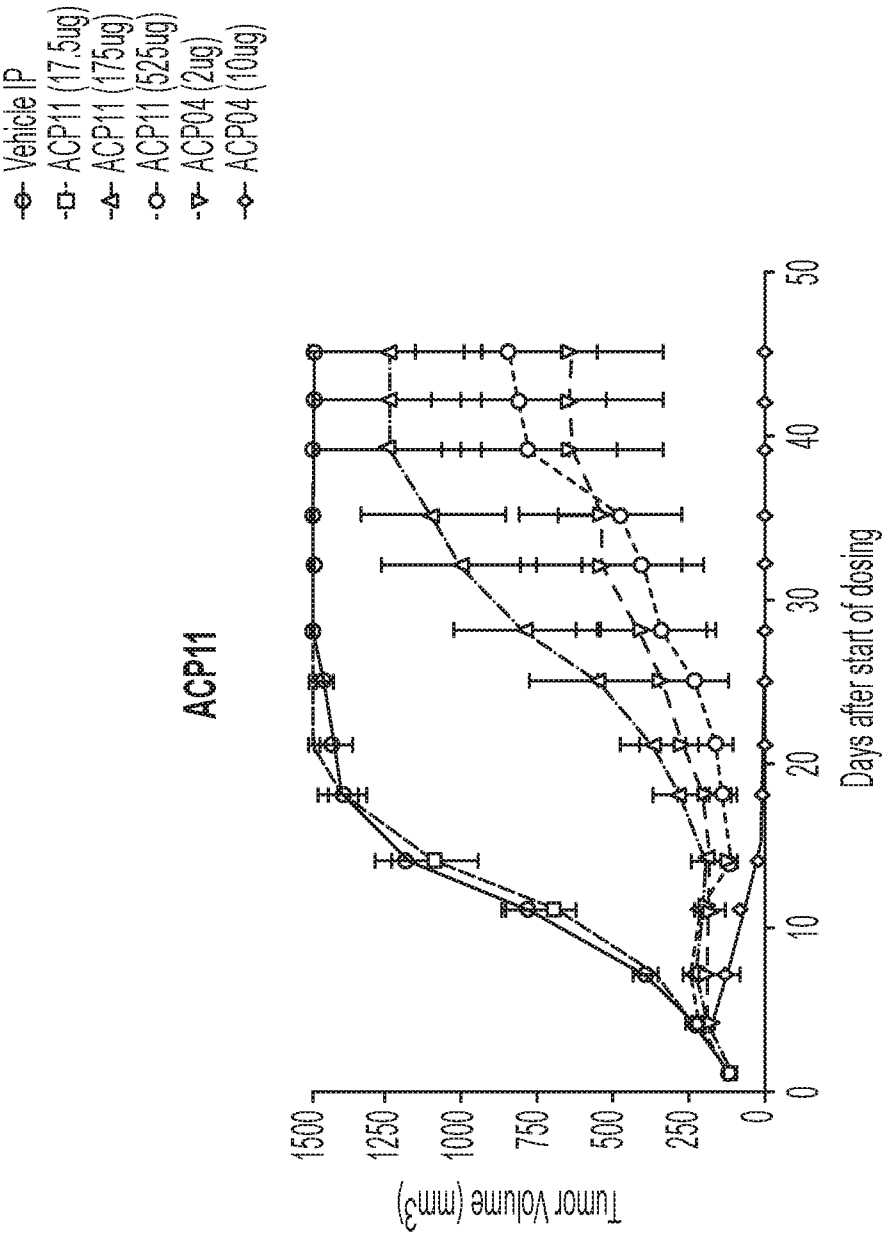

Vehicle IP
ACP11 (17.5ug)
ACP11 (175ug)
ACP11 (525ug)
ACP04 (2ug)
ACP04 (10ug)
ACP11
Tumor Volume (mm3)
1500
1250
1000
750
500
250
0
0
10
20
30
40
50
Days after start of dosing

FIG. 14

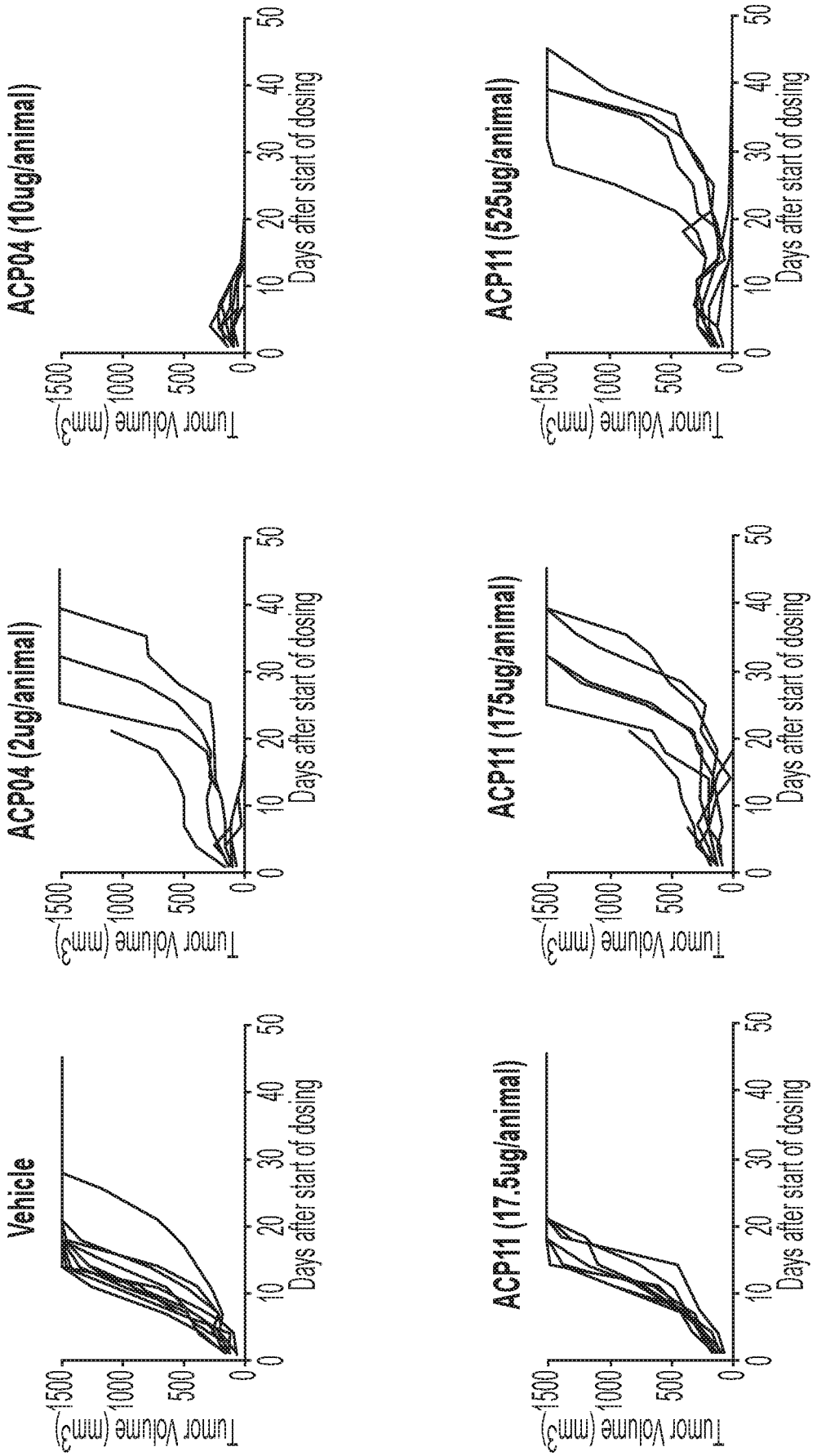

ACP04 (10ug/animal)
ACP11 (525ug/animal)
ACP04 (2ug/animal)
ACP11 (175ug/animal)
Vehicle
ACP11 (17.5ug/animal)
Tumor Volume (mm3)
Days after start of dosing
0
500
1000
1500
0
10
20
30
40
50

FIG. 15

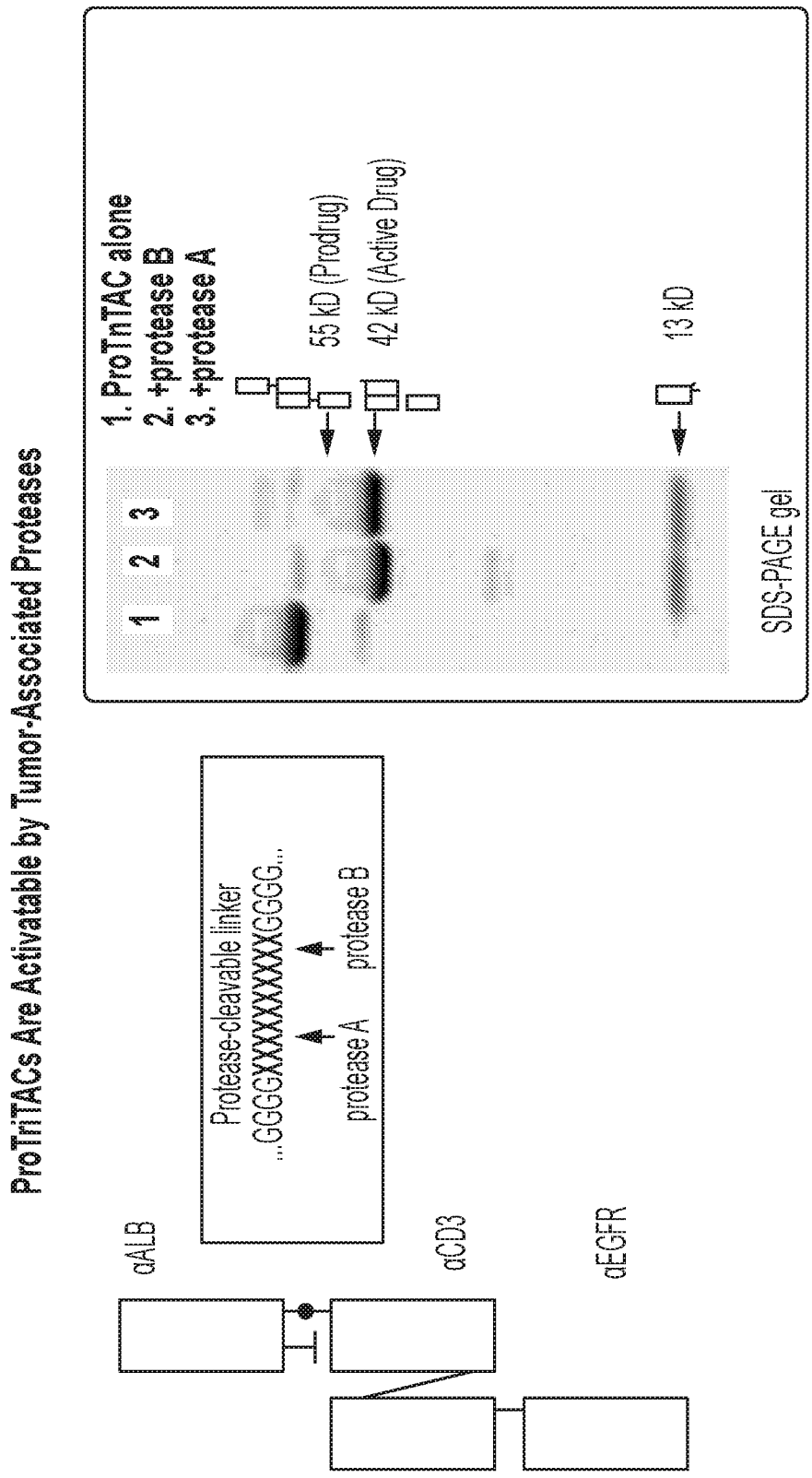
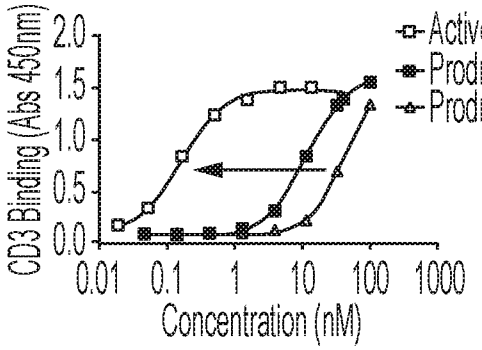

ProTriTACs Are Activatable by Tumor-Associated Proteases
αALB
Protease-cleavable linker
...GGGGXXXXXXXXXGGGG...
protease A
protease B
αCD3
αEGFR
1. ProTriTAC alone
2. +protease B
3. +protease A
55 kD (Prodrug)
42 kD (Active Drug)
13 kD
1 2 3
SDS-PAGE gel
• Proprietary library of substrate linkers with different cleavability engineered

FIG. 16

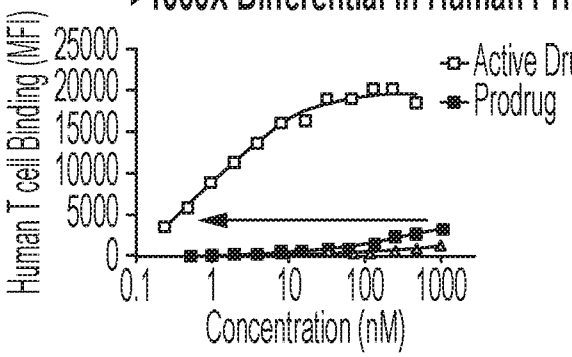
Differential Activity Supported by Various In Vitro Assays

250X Differential in CD3 Binding (ELISA)



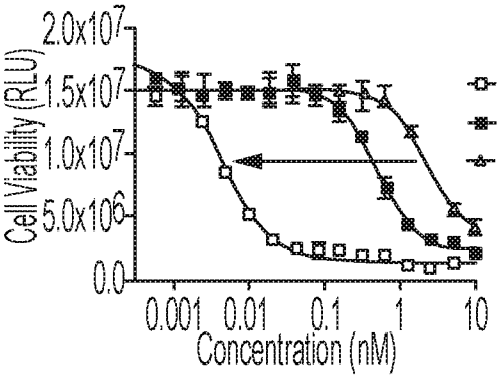
	EC_{50} (nM)	Masking Ratio
Active Drug	0.16	-
Prodrug	11.91	74
Prodrug (non-cleavable)	39.44	247

>1000X Differential in Human Primary T cell Binding (FACS)



	EC50 (nM)	Masking Ratio
Active Drug	1.19	-
Prodrug	>1000	n/a
Prodrug (non-cleavable)	>1000	n/a

550X Differential in T Cell Killing Activity (TDCC)



	EC50 (nM)	Masking Ratio
Active Drug	0.004	-
Prodrug	0.485	121
Prodrug (non-cleavable)	2.197	549

- Plug-and-play: masking demonstrated with >20 binders to 5 different targets

FIG. 17

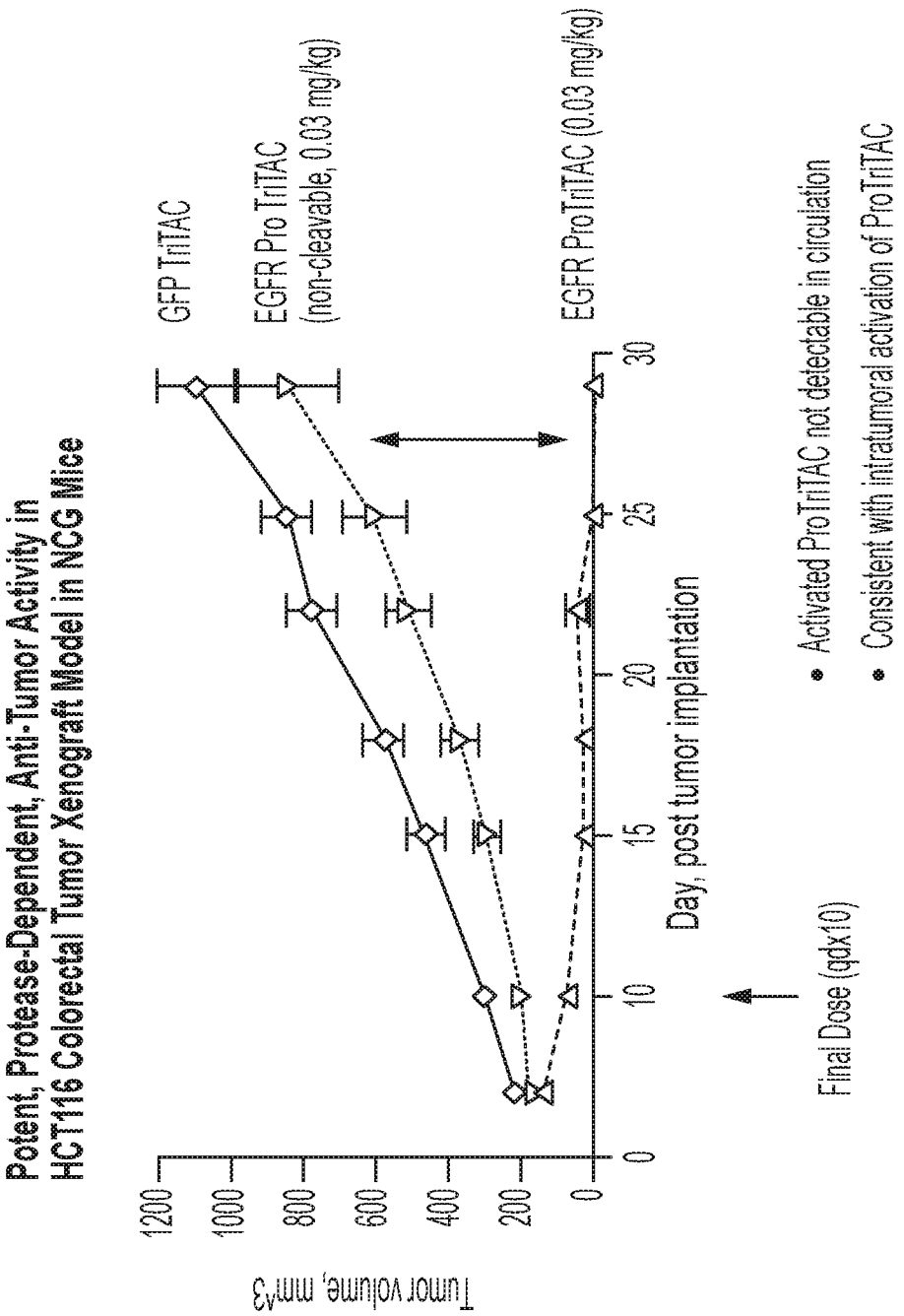

Potent, Protease-Dependent, Anti-Tumor Activity in
HCT116 Colorectal Tumor Xenograft Model in NCG Mice
GFP TriTAC
EGFR Pro TriTAC
(non-cleavable, 0.03 mg/kg)
EGFR Pro TriTAC (0.03 mg/kg)
Tumor volume, mm^3
1200
1000
800
600
400
200
0
0
10
15
20
25
30
Day, post tumor implantation
Final Dose (qdx10)
• Activated Pro TriTAC not detectable in circulation
• Consistent with intratumoral activation of Pro TriTAC

FIG. 18

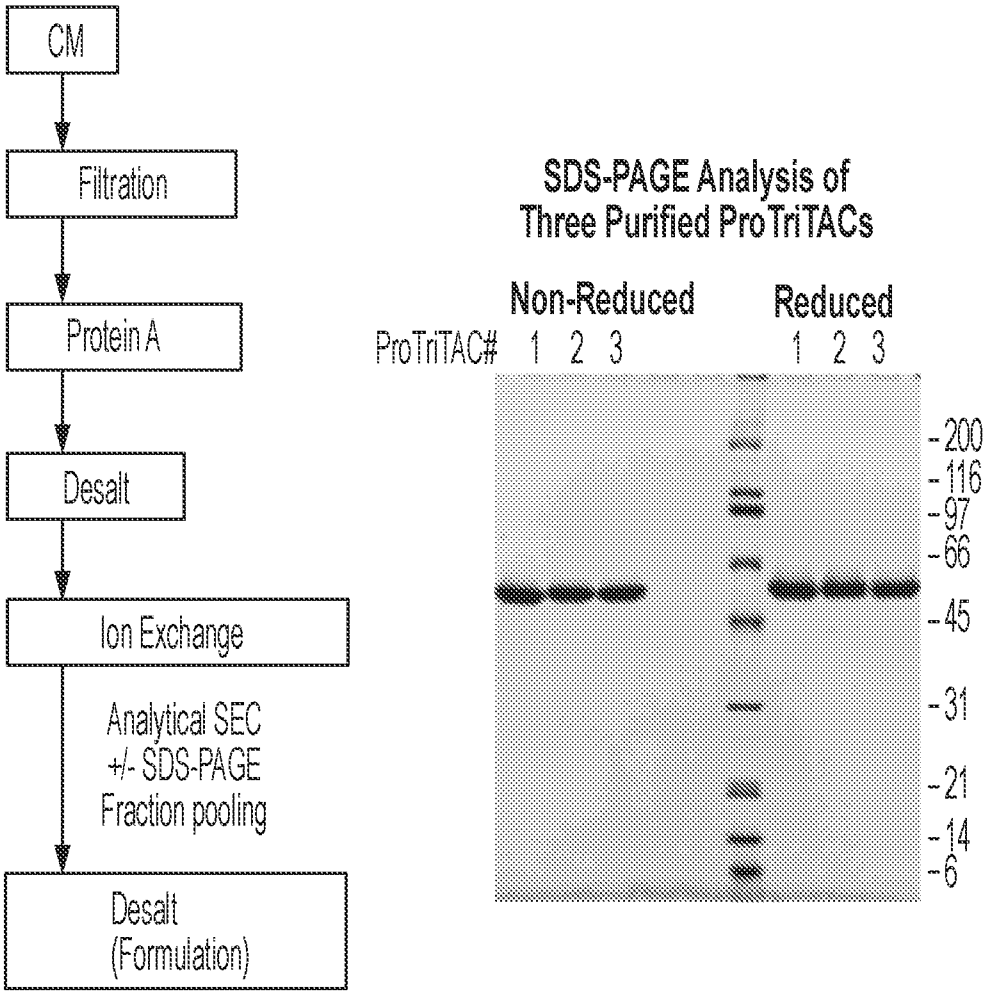
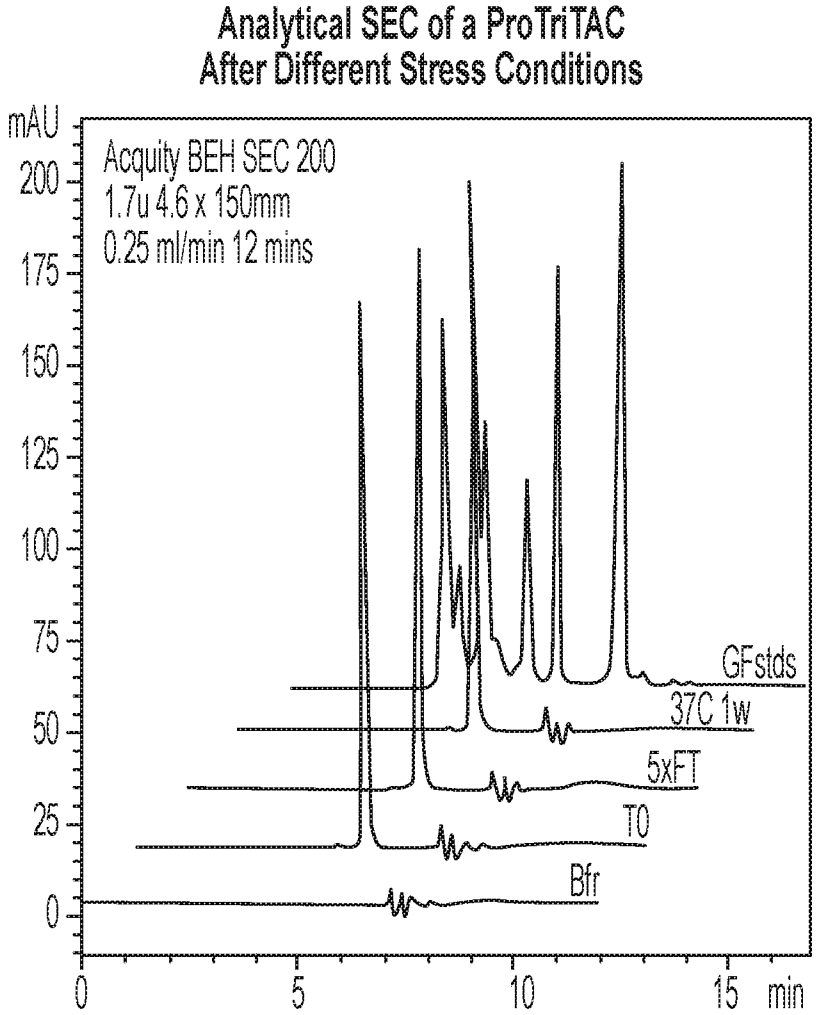

CM
Filtration
Protein A
Desalt
Ion Exchange
Analytical SEC
+/- SDS-PAGE
Fraction pooling
Desalt
(Formulation)
SDS-PAGE Analysis of
Three Purified ProTriTACs
Non-Reduced
Reduced
ProTriTAC# 1 2 3 1 2 3
200
116
97
66
45
31
21
14
6

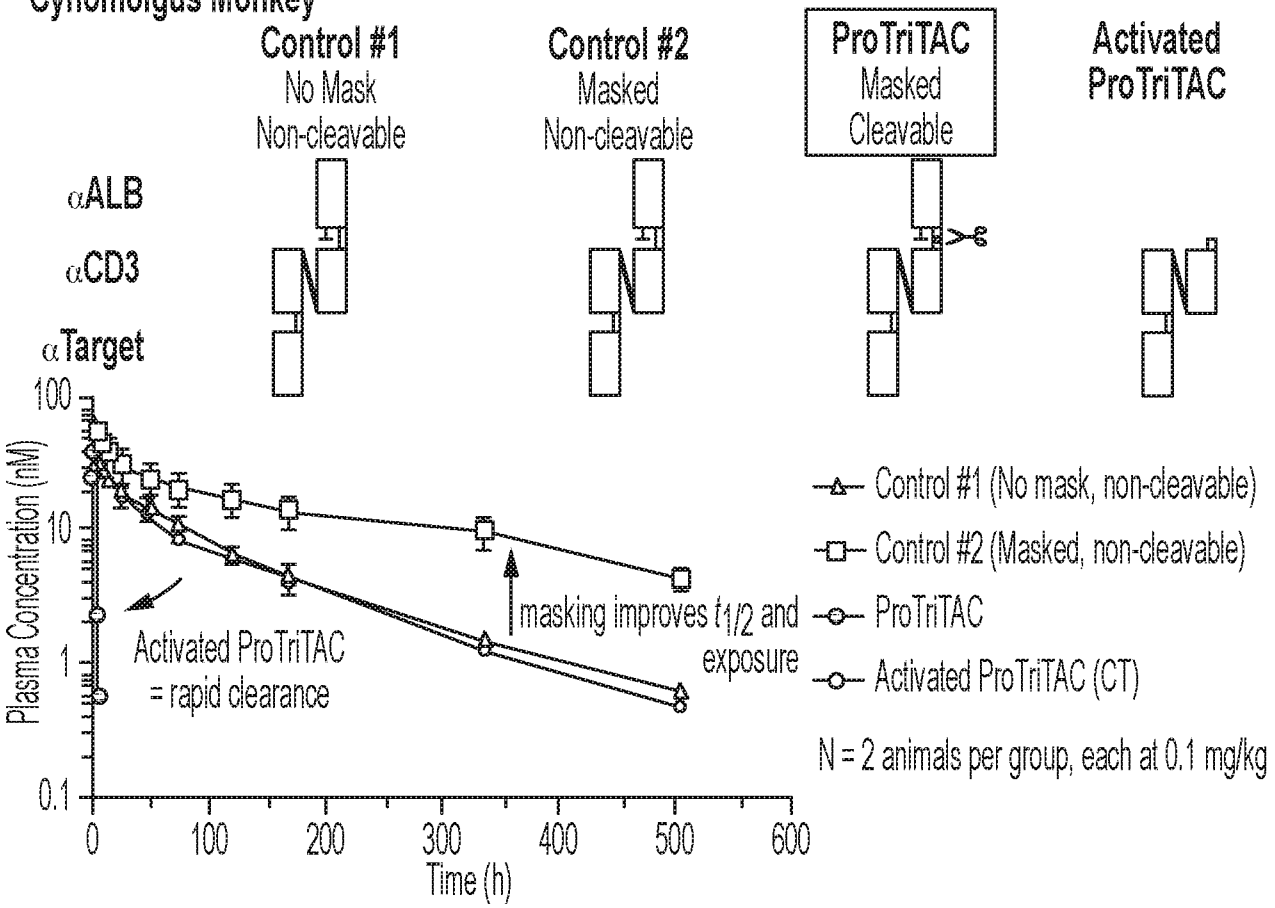
FIG. 19



Condition	%HMW	%Main	%LMW
T0	1.7	96.0	2.3
5x FT	1.6	97.0	1.4
37C 1w	2.6	95.4	2.0

FIG. 20

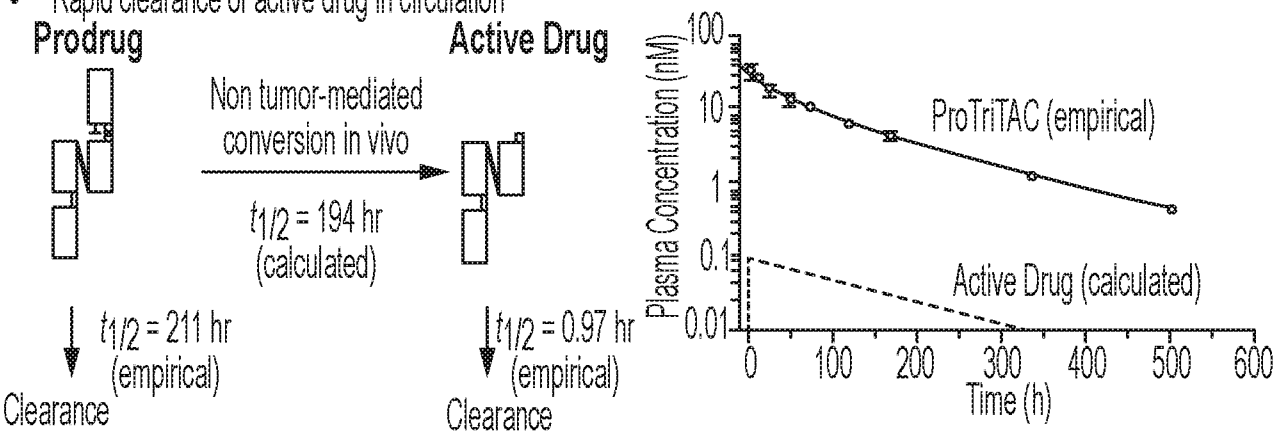
Single Dose PK Study to Investigate Functional Masking and Stability of ProTriTAC in Cynomolgus Monkey



Test Article	Terminal $t_{1/2}$ (hr)	Cmax (nM)	AUC, 0-last (hr*nM)	Clearance (mL/hr/kg)
Control #1 (No mask, non-cleavable)	118	48.2	2490	0.735
Control #2 (Masked, non-cleavable)	211	58.0	7000	0.238
ProTriTAC	101	42.7	2670	0.686
Activated ProTriTAC (CT)	0.969	66.6	41.5	58.5

Dual Protection Against On-Target, Off-Tumor Activity

- Limited peripheral T cell binding (as demonstrated by improved PK from masking)
- Rapid clearance of active drug in circulation



- **Substrate linker is sufficiently stable in circulation:** 50% conversion every 194 hr
- **Active drug does not accumulate in circulation:** below 0.5% of prodrug at all times

FIG. 21