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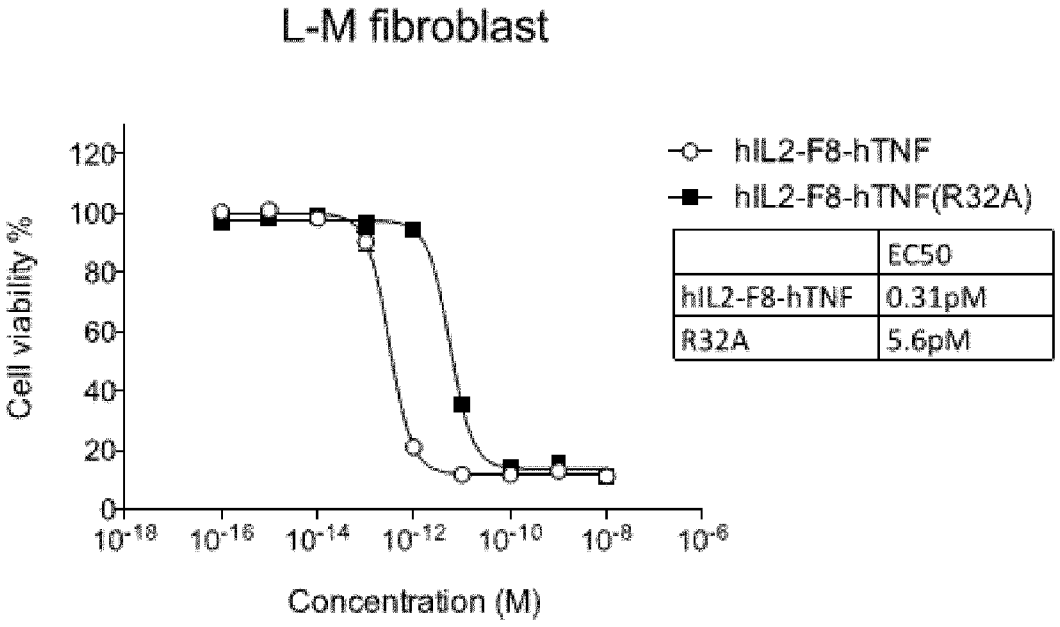

FIGURE 1

(57) Abstract: The present application relates to conjugates comprising interleukin 2 (IL2), and a mutant of tumour necrosis factor, such as tumour necrosis factor alpha (TNFα), and an antibody molecule. The antibody molecule preferably binds to an antigen associated with neoplastic growth and/or angiogenesis, such as the Extra-Domain A (EDA) or Extra-Domain B (EDB) of fibronectin. The conjugate may be used in the treatment of cancer.

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IL2 and TNF Mutant Immunoconjugates

Field

The present invention relates to conjugates comprising interleukin 2 (IL2), a mutant of a tumour necrosis factor, such as tumour necrosis factor alpha (TNFα), and an antibody molecule. The antibody molecule preferably binds to an antigen associated with neoplastic growth and/or angiogenesis, such as the Extra-Domain A (EDA) and the Extra-Domain B (EDB) of fibronectin. The conjugates may be used, for example, in the treatment of cancer.

Background

Many cytokines have shown potent anti-tumour activities in preclinical experiments and represent promising agents for cancer therapy. However, despite encouraging results in animal models, only a few cytokines, such as Proleukin 1 (IL2), Roferon A1 (interferon alpha-2a [IFNα 2a]), Intron A1 (IFNα 2b), Beromun 1 (recombinant TNFα) have been approved as anticancer drugs. Current indications for cytokines include metastatic renal cell cancer, malignant melanoma, hairy cell leukemia, chronic myeloid lymphoma, sarcoma and multiple myeloma. The cytokines may be either administered alone or in combination with chemotherapy.

A further difficulty with pro-inflammatory cytokines in particular is that their use in therapy is often hindered by substantial toxicity even at low doses, which prevents the escalation to therapeutically active doses (Hemmerle et al. (2013) *Br. J. Cancer* 109, 1206-1213).

In an attempt to increase the therapeutic index of certain cytokines, antibody-cytokine fusion proteins (also referred to as “immunocytokines”) have been proposed. In these conjugates, the antibody serves as a “vehicle” for a selective accumulation at the site of disease, while the cytokine payload is responsible for the therapeutic activity (Pasche & Neri, 2012, Drug Discov. Today, 17, 583). Certain immunocytokines based on pro-inflammatory payloads (such as IL2, IL4, IL12, and TNFα) display potent anti-cancer activity in mouse models (Hess *et al.*, 2014, Med. Chem. Comm., 5, 408) and have produced encouraging results in patients with both solid tumours and haematological malignancies (Eigentler *et al.*, 2011, Clin. Cancer Res. 17, 7732-7742; Papadia *et al.*, 2013, J. Surg. Oncol. 107, 173-179; Gutbrodt *et al.*, 2013, Sci. Transl. Med. 5, 201-204; Weide *et al.,* 2014, *Cancer Immunol. Res.* 2, 668-678; Danielli *et al.*, 2015, Cancer Immunol. Immunother. 64, 113-121]. The F8 antibody (specific to the alternatively-spliced EDA domain of fibronectin, a marker of tumour angiogenesis; Rybak et al. (2007) *Cancer Res.* 67, 10948-10957) has been used for tumour targeting, both alone and fused to

either TNF or IL2 (Villa et al. (2008) *Int. J. Cancer* 122, 2405-2413; Hemmerle et al. (2013) *Br. J. Cancer* 109, 1206-1213; Frey et al. (2008) *J. Urol.* 184, 2540-2548).

Constructs that comprise three copies of a single modified cytokine of the TNF superfamily that has reduced activity to its receptor have been reported (WO2015/007903). The constructs are specifically delivered to target cells by a targeting moiety. Modified cytokines used in these constructs include mutant TNF with an activity range between 0.02% and 5 % of wild type TNF, including mutant TNFs with Y87Q, I97S, Y115A, Y87F, Y115G, or I97A substitutions. The effect of R32G is also reported.

In some cases, immunocytokines can mediate tumour eradication in mouse models of cancer when used as single agents (Gutbrodt *et al*., 2013, Sci. Transl. Med. 5, 201-204]. In most cases, however, a single immunocytokine product is not able to induce complete cancer eradication. However, cancer cures have been reported for combinations of immunocytokines with cytotoxic agents (Moschetta *et al.,* 2012, Cancer Res. 72, 1814-1824], intact antibodies (Schliemann *et al.*, 2009, Blood, 113, 2275-2283] and external beam radiation (Zegers *et al.,* 2015, Clin. Cancer Res., 21, 1151-1160).

In addition, several combinations of immunocytokines have been used in therapy. For example, conjugates L19-IL2 and L19-TNFα were able to cure neuroblastoma in a fully syngeneic mouse model of the disease, whereas the individual immunocytokines used as single agents did not result in eradication of the disease (Balza *et al*., 2010, Int. J. Cancer, 127, 101). The combination of IL2 and TNFα payloads has also shown promising results in clinical trials. The fusion proteins L19-IL2 and L19-TNF were shown to potently synergize for the intralesional treatment of certain solid tumours in the mouse (Schwager et al., 2013, J. Invest. Dermatol. 133, 751-758). The corresponding fully human fusion proteins have been administered intralesionally to patients with Stage IIIC melanoma (Danielli *et al*., 2015, Cancer Immunol. Immunother. 64, 113-121), showing better results compared to the intralesional administration of interleukin-2 (Weide *et al*., 2011, Cancer - 116, 4139-4146) or of L19-IL2 (Weide *et al.,* 2014, Cancer Immunol. Immunother. 2, 668-678). However, the genetic fusion of a cytokine to an antibody does not always result in increased efficacy. For example, the fusion of Interleukin-17 to a targeting antibody did not reduce tumour growth (Pasche *et al*., 2012, Angiogenesis 15, 165-169).

There have also been attempts to generate “dual immunocytokines” in which an antibody is genetically fused to two different cytokines. For instance, interleukin-12 (IL12) and TNFα have been incorporated into a single molecular entity. However, these attempts have not been successful and have not led to clinical development programs. Specifically, a triple fusion, consisting of: (i) the L19 antibody in scFv format (specific to the alternatively-spliced EDB domain of fibronectin, a marker of tumour angiogenesis); (ii) murine TNFα; and (iii) murine IL12 in single-chain format has been described (Halin *et al.,* 2003, Cancer Res., 63, 3202–3210). This fusion protein could be expressed and purified to homogeneity. The fusion protein also bound to the cognate antigen with high affinity and specificity, but (unlike L19-TNFα and L19-IL12), it failed to localize to solid tumours *in vivo*, as evidenced by quantitative biodistribution studies in tumour-bearing mice. The behaviour of dual immunocytokines *in vivo* is therefore extremely unpredictable.

Bi-functional cytokine fusion proteins in which the cytokines were linked to an intact whole antibody (or the Fc portion of an antibody) have also been described (Gillies *et al.*, 2002, Cancer Immunol. Immunother. 51, 449). These fusion proteins comprised interleukin-2/interleukin-12 (IL2/IL12), or interleukin-4/granulocyte-macrophage colony-stimulating factor (IL4/GM-CSF). Cytokine activity was retained in constructs where the cytokines were fused in tandem at the carboxyl terminus of the Fc or antibody heavy (H) chain, as well as in constructs where one cytokine was fused at the carboxyl terminus of the H chain while the second cytokine was fused to the amino terminus of either the H or light (L) chain variable region. Antigen binding of the antibody-cytokine fusion proteins was maintained. However, therapeutic activities *in vivo* were reported only for gene therapy applications (i.e. tumour cells transfected with the appropriate IL2/IL12 immunocytokines), but not with therapeutic proteins. Bi-functional cytokine fusion proteins comprising other types of targeting moieties are not reported.

The intrinsic complexity of successfully expressing immunoconjugates containing two different cytokines in a single molecule (also referred to as “dual immunocytokines”) and the unpromising results obtained with such molecules as discussed above (for example in Halin et al (2003)), mean that these molecular formats have not been pursued for clinical applications.

Summary

The present inventors have recognised that the use of a reduced activity tumour necrosis factor (TNF) mutant improves the tolerability of a dual immunocytokine that comprises TNF and IL2, as well as a targeting antibody molecule, without affecting efficacy.

An aspect of the present invention provides a conjugate comprising interleukin-2 (IL2), a TNF mutant having reduced activity, and an antibody molecule which binds an antigen associated with neoplastic growth and/or angiogenesis.

Another aspect of the invention provides a nucleic acid molecule encoding such a conjugate, as well as an expression vector comprising such a nucleic acid. A host cell comprising such a vector is also contemplated.

Another aspect of the invention provides a conjugate described herein for use in a method of treating cancer by targeting IL2 and a TNF mutant, preferably a TNFα mutant, to the neovasculature *in vivo*, as well as a conjugate described herein for use in a method of delivering IL2 and a TNF mutant, preferably a TNFα mutant, to the tumour neovasculature in a patient.

Another aspect of the invention provides a method of treating cancer by targeting IL2 and a TNF mutant, preferably a TNFα mutant, to the neovasculature in a patient, the method comprising administering a therapeutically effective amount of a conjugate described herein to the patient, as well as a method of delivering IL2 and a TNF mutant, preferably a TNFα mutant, to the tumour neovasculature in a patient comprising administering to the patient a conjugate described herein.

In addition, another aspect of the invention provides the use of a conjugate described herein for the preparation of a medicament for the treatment of cancer. The use of a conjugate described herein for the preparation of a medicament for delivery of IL2 and a TNF mutant, preferably a TNFα mutant, to the neovasculature of a tumour is similarly contemplated.

Brief Description of the Figures

Figure 1 shows the cell killing activity of huIL2-F8-huTNFα conjugate and huIL2-F8-huTNFα (R32A) mutant conjugate. The conjugates tested were huIL2-F8-huTNFα and huIL2-F8-huTNFα (R32A) which comprised a mutated TNFα at the position 32, IL2 and the anti-ED-A antibody F8.

The cell killing activity of this mutated conjugate was compared with the cell killing activity observed in the presence of conjugate huIL2-F8-huTNFα. The cell killing activity of the huIL2-F8-huTNFα (R32A) mutant conjugate was lower compared to the huIL2-F8-huTNFα conjugate, as can be seen from the EC50 values. The EC50 value represents the drug concentration required for half-maximal activity.

Figure 2 shows the *in vivo* targeting performance of the huIL2-F8-huTNFα (R32A) mutant conjugate evaluated by biodistribution analysis. The huIL2-F8-huTNFα (R32A) mutant conjugate selectively accumulated in tumour in a mouse model of F9 teratocarcinoma.

Figure 3 shows the IL2 bioactivity assay of the huIL2-L19-huTNFα (R32A) mutant conjugate, based on the proliferation of CTLL-2 cells.

Figure 4 shows the TNF bioactivity assay of the huIL2-L19-huTNFα (R32A) mutant conjugate, based on the killing of HT1080 cells.

Figure 5 shows the quantitative biodistribution analysis of radioiodinated huIL2-L19-huTNFα (R32A) mutant conjugate in immunocompetent mice bearing F9 teratocarcinoma tumours.

Detailed Description

The present invention relates to a conjugate comprising (i) an interleukin-2 (IL2) moiety, (ii) a moiety which is a tumour necrosis factor (TNF) mutant having reduced activity, and (iii) an antibody molecule which binds an antigen associated with neoplastic growth and/or angiogenesis.

The term “antibody molecule” describes an immunoglobulin whether natural or partly or wholly synthetically produced. The term also relates to any polypeptide or protein comprising an antibody antigen-binding site. Antibody molecules may have been isolated or obtained by purification from natural sources, or else obtained by genetic recombination, or by chemical synthesis, and that they may contain unnatural amino acids.

As antibodies can be modified in a number of ways, the term "antibody molecule" should be construed as covering any specific binding member or substance having an antibody antigen-binding site with the required specificity and/or binding to antigen. Thus, this term covers

antibody fragments, in particular antigen-binding fragments, and derivatives, including any polypeptide comprising an antibody antigen-binding site, whether natural or wholly or partially synthetic. Chimeric molecules comprising an antibody antigen-binding site, or equivalent, fused to another polypeptide (e.g. belonging to another antibody class or subclass) are therefore included. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023, and a large body of subsequent literature.

As mentioned above, fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward *et al.* (1989) Nature 341, 544-546; McCafferty *et al.*, (1990) Nature, 348, 552-554; Holt *et al.* (2003) Trends in Biotechnology 21, 484-490), which consists of a VH or a VL domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird *et al.* (1988) Science, 242, 423-426; Huston *et al.* (1988) PNAS USA, 85, 5879-5883); (viii) bispecific single chain Fv dimers (PCT/US92/09965); (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; Holliger *et al.* (1993a), Proc. Natl. Acad. Sci. USA 90 6444-6448) and (x) a single chain diabody format wherein each of the VH and VL domains within a set is connected by a short or 'non-flexible' peptide linker. Fv, scFv or diabody molecules may be stabilized by the incorporation of disulphide bridges linking the VH and VL domains (Reiter *et al.* (1996), Nature Biotech, 14, 1239-1245). A single chain Fv (scFv) may be comprised within a mini-immunoglobulin or small immunoprotein (SIP), e.g. as described in (Li et al., (1997), Protein Engineering, 10: 731-736). A SIP may comprise an scFv molecule fused to the CH4 domain of the human IgE secretory isoform IgE-S2 (ε_{S2}-CH4; Batista et al., (1996), J. Exp. Med., 184: 2197-205) forming a homo-dimeric mini-immunoglobulin antibody molecule. Minibodies comprising a scFv joined to a CH3 domain may also be made (Hu *et al.* (1996), Cancer Res., 56(13):3055-61). Other examples of binding fragments are Fab', which differs from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain, including one or more cysteines from the antibody hinge region, and Fab'-SH, which is a Fab' fragment in which the cysteine residue(s) of the constant domains bear a free thiol group.

The half-life of antibody molecules for use in the conjugates described herein, may be increased by a chemical modification, especially by PEGylation, or by incorporation in a liposome.

Suitable antibody molecules for use in the conjugates described herein include diabodies or, more preferably scFvs. Diabodies and scFvs do not comprise an antibody Fc region, thus potentially reducing the effects of anti-idiotypic reaction. Preferably, the antibody molecule for use in the conjugates described herein is a scFv.

Where the antibody molecule is a scFv, the VH and VL domains of the antibody are preferably linked by a 10 to 20 amino acid linker, by a 14 to 20 amino acid linker, preferably by a 10 to 14 amino acid linker. Suitable linkers are known in the art and available to the skilled person. For example, a linker may have the sequence set forth in SEQ ID NO: 3, SEQ ID NO: 50 or SEQ ID NO: 51

Where the antibody molecule is a diabody, the VH and VL domains may be linked by a 5 to 12 amino acid linker. A diabody comprises two VH-VL molecules which associate to form a dimer. The VH and VL domains of each VH-VL molecule may be linked by a 5 to 12 amino acid linker.

The present inventors have shown that a conjugate comprising IL2; a mutant of TNFα; and an antibody molecule which binds the Extra-Domain A (ED-A) of fibronectin exhibits reduced toxicity compared to a conjugate comprising IL2; TNFα; and an antibody molecule which binds the Extra-Domain A (ED-A) of fibronectin. Furthermore, the present inventors have also shown that a conjugate comprising IL2; a mutant of TNFα; and an antibody molecule which binds the Extra-Domain B (ED-B) isoform of fibronectin exhibits reduced toxicity compared to the recombinant TNFα. Other conjugates comprising IL2 and a mutant of TNF, preferably TNFα, and an antibody molecule which binds an antigen associated with neoplastic growth and/or angiogenesis have similarly reduced toxicity.

The toxicity of a conjugate comprising a TNF mutant as described herein may be reduced compared to the corresponding conjugate comprising wild-type TNF. Reduced toxicity may include improved tolerability in a patient, for example a reduction in one or more adverse symptoms associated with administration of the conjugate(s) to the patient. Adverse symptoms reduced by the toxicity may include weight loss, nausea, vomiting, fever, chills, flushing,

urticaria, rash, pulmonary toxicity, dyspnea, hypotension, anaphylaxis, serum sickness, increased creatinine, headache.

Furthermore, the reduced toxicity of the TNF mutant in the conjugate increases the synergistic effect of the IL2 moiety, which can be administered at a higher dose due to the lower activity of the TNF mutant. The potency matched cytokines in the conjugate may therefore be useful in therapeutic applications.

The present inventors have also shown that a conjugate comprising IL2 and a mutant of TNFα; and an antibody molecule which binds the Extra-Domain A (ED-A) of fibronectin can successfully target tumour neovasculature *in vivo*. Furthermore, the present inventors have also shown that a conjugate comprising IL2 and a mutant of TNFα; and an antibody molecule which binds the Extra-Domain B (ED-B) of fibronectin can successfully target tumour neovasculature *in vivo*. Other conjugates comprising IL2 and a mutant of TNF, preferably TNFα, and an antibody molecule which binds an antigen associated with neoplastic growth and/or angiogenesis will similarly be suitable to target IL2 and mutant of TNF to the tumour neovasculature and thus find application in cancer treatment. A conjugate comprising IL2; TNFα; and an antibody molecule which binds the Extra-Domain A (ED-A) of fibronectin has also been shown to target tumour neovasculature *in vivo* (PCT/EP2016/060128).

Many antigens associated with neoplastic growth and/or angiogenesis are known in the art, as are antibodies capable of binding such antigens. In additions, antibodies against a given antigen can be generated using well-known methods such as those described in the present application. In some embodiments, the antigen may be an extra-cellular matrix component associated with neoplastic growth and/or angiogenesis, such as fibronectins, including the Extra-Domain A (ED-A) isoform of fibronectin (A-FN), the Extra-Domain B (ED-B) isoform of fibronectin (B-FN), tenascin C, the ED-A of fibronectin, the ED-B of fibronectin, or the A1 Domain of Tenascin C. Antibodies which bind the ED-A of fibronectin, and thus also A-FN, are known in the art and include antibody F8. Antibodies which bind the ED-B of fibronectin, or the A1 Domain of Tenascin C (and thus also the B-FN and tenascin C) are also known in the art and include antibodies L19 and F16, respectively. Antibodies which bind the ED-B of fibronectin, or the A1 Domain of Tenascin C, including antibodies L19 and F16, have been shown to be capable of specifically targeting the tumour neovasculature *in vivo.* Thus, a conjugate described herein, comprising IL2, a mutant of TNF, preferably TNFα, and an antibody molecule which binds an

antigen associated with neoplastic growth and/or angiogenesis, preferably exhibits reduced toxicity when administered to a patient, compared with administration of a conjugate comprising IL2, TNF and the antibody molecule, to the patient.

Other antigens which are associated with neoplastic growth and/or angiogenesis include carbonic anhydrase IX (a marker of renal cell carcinoma), A33 and CEA (good markers of colorectal cancer), HER2 (a marker of breast cancer), PSMA (a marker of prostate cancer) and fibroblast activation protein (a protease, present both as membrane bound protein and as shed protein, on activated fibroblasts and on certain types of tumour cells). Conjugates comprising IL2 and a mutant of TNF, preferably TNFα, and an antibody molecule which binds antigens such as carbonic anhydrase IX, A33, CEA, HER2, PSMA, or fibroblast activation protein are similarly suitable to target IL2 and TNF to the tumour neovasculature and thus find application in cancer treatment and will exhibit reduced toxicity.

In some preferred embodiments, an antibody molecule for use as described herein may have the CDRs and/or the VH and/or VL domains of antibodies F8, L19 or F16 described herein. An antibody molecule for use as described herein preferably has the CDRs of antibody F8 set forth in SEQ ID NOs 6-11. More preferably, an antibody for use as described herein may comprise the VH and/or VL domains of antibody F8 set forth in SEQ ID NOs 2 and 4. Yet more preferably, an antibody for use as described herein comprises the VH and VL domains of antibody F8 set forth in SEQ ID NOs 2 and 4. The F8 antibody is preferably in scFv or diabody format, most preferably in scFv format. Where the F8 antibody is in scFv format, the antibody molecule for use as described herein preferably has the amino acid sequence set forth in SEQ ID NO: 5.

Another antibody molecule for use as described herein preferably has the CDRs of antibody L19 set forth in SEQ ID NOs 18-23. More preferably, an antibody for use as described herein may comprise the VH and/or VL domains of antibody L19 set forth in SEQ ID NOs 24 and 25. Yet more preferably, an antibody for use as described herein comprises the VH and VL domains of antibody L19 set forth in SEQ ID NOs 24 and 25. The L19 antibody is preferably in scFv or diabody format, most preferably in scFv format. Where the L19 antibody is in scFv format, the antibody molecule for use as described herein preferably has the amino acid sequence set forth in SEQ ID NO: 26.

An antibody molecule for use as described herein may bind the A-FN and/or the ED-A of fibronectin, with the same affinity as anti-ED-A antibody F8 e.g. in scFv format, or with an affinity that is better. An antibody molecule for use as described herein may bind the B-FN and/or the ED-B of fibronectin, with the same affinity as anti-ED-B antibody L19 e.g. in scFv format, or with an affinity that is better. An antibody molecule for use as described herein may bind Tenascin C and/or the A1 domain of tenascin C, with the same affinity as anti-Tenascin C antibody F16 e.g. in scFv format, or with an affinity that is better.

An antibody molecule for use as described herein may bind to the same epitope on A-FN and/or the ED-A of fibronectin as anti-ED-A antibody F8. An antibody molecule of the present invention may bind to the same epitope on B-FN and/or the ED-B of fibronectin as anti-ED-B antibody L19. An antibody molecule of the present invention may bind to the same epitope on tenascin C and/or the A1 domain of tenascin C as antibody F16.

Variants of antibody molecules disclosed herein may be produced and used in the present invention. The techniques required to make substitutions within amino acid sequences of CDRs, antibody VH or VL domains, in particular the framework regions of the VH and VL domains, and antibody molecules generally are available in the art. Variant sequences may be made, with substitutions that may or may not be predicted to have a minimal or beneficial effect on activity, and tested for ability to bind A-FN and/or the ED-A of fibronectin, B-FN and/or the ED-B of fibronectin, tenascin C and/or the A1 domain of tenascin C, and/or for any other desired property.

It is contemplated that from 1 to 5, e.g. from 1 to 4, including 1 to 3, or 1 or 2, or 3 or 4, amino acid alterations (addition, deletion, substitution and/or insertion of an amino acid residue) may be made in one or more of the CDRs and/or the VH and/or the VL domain of an antibody molecule as described herein. Thus, an antibody molecule which binds the FN-A, FN-B, or tenascin C, may comprise the CDRs and/or the VH and/or the VL domain of antibody F8, L19, or F16 described herein with 5 or fewer, for example, 5, 4, 3, 2 or 1 amino acid alterations within the CDRs and/or the VH and/or the VL domain. For example, an antibody molecule which binds the FN-A, FN-B, or tenascin C, may comprise the VH and/or the VL domain of antibody F8, L19, or F16 described herein with 5 or fewer, for example, 5, 4, 3, 2 or 1 amino acid alterations within the framework region of the VH and/or VL domain. An antibody molecule that binds the FN-A or ED-A of fibronectin, as referred to herein, thus may comprise the VH domain shown in SEQ ID

NO: 2 and/or the VL domain shown in SEQ ID NO: 4 with 5 or fewer, for example, 5, 4, 3, 2 or 1 amino acid alterations within the framework region of the VH and/or VL domain. Such an antibody molecule may bind the ED-A isoform or ED-A of fibronectin with the same or substantially the same, affinity as an antibody molecule comprising the VH domain shown in SEQ ID NO: 2 and the VL domain shown in SEQ ID NO: 4 or may bind the ED-A isoform or ED-A of fibronectin with a higher affinity than an antibody molecule comprising the VH domain shown in SEQ ID NO: 2 and the VL domain shown in SEQ ID NO: 4. An antibody molecule that binds the FN-B or ED-B of fibronectin, as referred to herein, thus may comprise the VH domain shown in SEQ ID NO: 24 and/or the VL domain shown in SEQ ID NO: 25 with 5 or fewer, for example, 5, 4, 3, 2 or 1 amino acid alterations within the framework region of the VH and/or VL domain. Such an antibody molecule may bind the ED-B isoform or ED-B of fibronectin with the same or substantially the same, affinity as an antibody molecule comprising the VH domain shown in SEQ ID NO: 24 and the VL domain shown in SEQ ID NO: 25 or may bind the ED-B isoform or ED-B of fibronectin with a higher affinity than an antibody molecule comprising the VH domain shown in SEQ ID NO: 24 and the VL domain shown in SEQ ID NO: 25.

An antibody molecule for use as described herein may comprise a VH and/or VL domain that has at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, sequence identity to the VH and/or VL domain, as applicable, of antibody F8, L19, or F16 set forth in SEQ ID NOs 2, 4, 24, 25, 33, and 34. An antibody molecule for use as described herein may have at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, sequence identity to the amino acid sequence of the F8, L19, or F16 antibodies set forth in SEQ ID NOs 5, 26, 35, and 46, respectively.

An antigen binding site is the part of a molecule that recognises and binds to all or part of a target antigen. In an antibody molecule, it is referred to as the antibody antigen-binding site or paratope, and comprises the part of the antibody that recognises and binds to all or part of the target antigen. Where an antigen is large, an antibody may only bind to a particular part of the antigen, which part is termed an epitope. An antibody antigen-binding site may be provided by one or more antibody variable domains. An antibody antigen-binding site preferably comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

An antigen binding site may be provided by means of arrangement of complementarity determining regions (CDRs). The structure for carrying a CDR or a set of CDRs will generally be

an antibody heavy or light chain sequence or substantial portion thereof in which the CDR or set of CDRs is located at a location corresponding to the CDR or set of CDRs of naturally occurring VH and VL antibody variable domains encoded by rearranged immunoglobulin genes. The structures and locations of immunoglobulin variable domains may be determined by reference to Kabat *et al*. (1987) (Sequences of Proteins of Immunological Interest. 4th Edition. US Department of Health and Human Services.), and updates thereof, now available on the Internet (at immuno.bme.nwu.edu or find "Kabat" using any search engine).

By CDR region or CDR, it is intended to indicate the hypervariable regions of the heavy and light chains of the immunoglobulin as defined by Kabat *et al*. (1987) Sequences of Proteins of Immunological Interest, 4th Edition, US Department of Health and Human Services (Kabat *et al*., (1991a), Sequences of Proteins of Immunological Interest, 5th Edition, US Department of Health and Human Services, Public Service, NIH, Washington, and later editions). An antibody typically contains 3 heavy chain CDRs and 3 light chain CDRs. The term "CDR" or "CDRs" may indicate, according to the case, one of these regions or several, or even the whole, of these regions which contain the majority of the amino acid residues responsible for the binding by affinity of the antibody for the antigen or the epitope which it recognizes.

Among the six short CDR sequences, the third CDR of the heavy chain (HCDR3) has a greater size variability (greater diversity essentially due to the mechanisms of arrangement of the genes which give rise to it). It can be as short as 2 amino acids although the longest size known is 26. Functionally, HCDR3 plays a role in part in the determination of the specificity of the antibody (Segal *et al*., (1974), PNAS, 71:4298-4302; Amit *et al*., (1986), Science, 233:747-753; Chothia *et al*., (1987), J. Mol. Biol., 196:901-917; Chothia *et al*., (1989), Nature, 342:877-883; Caton *et al*., (1990), J. Immunol., 144:1965-1968; Sharon *et al*., (1990a), PNAS, 87:4814-4817; Sharon *et al*., (1990b), J. Immunol., 144:4863-4869; Kabat *et al*., (1991b), J. Immunol., 147:1709-1719).

The antigen-binding site of an antibody molecule for use as described herein preferably has the CDRs of antibody F8 set forth in SEQ ID NOs 6-11, the CDRs of antibody L19 set forth in SEQ ID Nos 18-23, or the CDRs of antibody F16 set forth in SEQ ID NOs 27-32. Most preferably, the antigen binding site of an antibody molecule for use as described herein has the CDRs of antibody F8 set forth in SEQ ID NOs 6-11 or the CDRs of antibody L19 set forth in SEQ ID Nos 18-23.

Various methods are available in the art for obtaining antibodies molecules against a target antigen. The antibody molecules for use in the conjugates described herein are preferably monoclonal antibodies, especially of human, murine, chimeric or humanized origin, which can be obtained according to the standard methods well known to the person skilled in the art. An antibody molecule for use in the conjugates described herein is most preferably a human antibody molecule.

It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or chimeric molecules that bind the target antigen. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the CDRs, of an antibody molecule to the constant regions, or constant regions plus framework regions, of a different immunoglobulin (see, for instance, EP-A-184187, GB 2188638A or EP-A-239400, and a large body of subsequent literature). A hybridoma or other cell producing an antibody may also be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

Techniques available in the art of antibody engineering have made it possible to isolate human and humanised antibodies. For example, human hybridomas can be made as described by Kontermann & Dubel (2001), S, *Antibody Engineering,* Springer-Verlag New York, LLC; ISBN: 3540413545. Phage display, another established technique for generating specific binding members has been described in detail in many publications such as WO92/01047 (discussed further below) and US patents US5969108, US5565332, US5733743, US5858657, US5871907, US5872215, US5885793, US5962255, US6140471, US6172197, US6225447, US6291650, US6492160, US6521404 and Kontermann & Dubel (2001), S, *Antibody Engineering,* Springer-Verlag New York, LLC; ISBN: 3540413545. Transgenic mice in which the mouse antibody genes are inactivated and functionally replaced with human antibody genes while leaving intact other components of the mouse immune system, can be used for isolating human antibodies (Mendez *et al*., (1997), Nature Genet, 15(2): 146–156).

In general, for the preparation of monoclonal antibodies or their functional fragments, especially of murine origin, it is possible to refer to techniques which are described in particular in the manual "Antibodies" (Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor N.Y., pp. 726, 1988) or to the technique of preparation from hybridomas described by Kohler and Milstein, 1975, Nature, 256:495-497.

Monoclonal antibodies can be obtained, for example, from an animal cell immunized against the an antigen associated with neoplastic growth and/or angiogenesis, such as A-FN, B-FN, tenascin C, the ED-A of fibronectin, the ED-B of fibronectin, or the A1 Domain of Tenascin C, according to the usual working methods, by genetic recombination starting with a nucleic acid sequence contained in the cDNA sequence coding for A-FN, B-FN, or tenascin C, or fragment thereof, or by peptide synthesis starting from a sequence of amino acids comprised in the peptide sequence of the A-FN, B-FN, or tenascin C, and/or a fragment thereof.

Synthetic antibody molecules may be created by expression from genes generated by means of oligonucleotides synthesized and assembled within suitable expression vectors, for example as described by Knappik *et al*. (2000) J. Mol. Biol. 296, 57-86 or Krebs *et al*. (2001) Journal of Immunological Methods, 254 67–84.

Alternatively, one or more antibody molecules for an antigen associated with neoplastic growth and/or angiogenesis, such as the A-FN, the ED-A, B-FN, the ED-B, tenascin C, or the A1 domain of tenascin C may be obtained by bringing into contact a library of antibody molecules and the antigen or a fragment thereof, e.g. a fragment comprising or consisting of ED-A, ED-B, or the A1 domain of tenascin C, or a peptide fragment thereof, and selecting one or more antibody molecules of the library able to bind the antigen.

An antibody library may be screened using Iterative Colony Filter Screening (ICFS). In ICFS, bacteria containing the DNA encoding several binding specificities are grown in a liquid medium and, once the stage of exponential growth has been reached, some billions of them are distributed onto a growth support consisting of a suitably pre-treated membrane filter which is incubated until completely confluent bacterial colonies appear. A second trap substrate consists of another membrane filter, pre-humidified and covered with the desired antigen.

The trap membrane filter is then placed onto a plate containing a suitable culture medium and covered with the growth filter with the surface covered with bacterial colonies pointing upwards. The sandwich thus obtained is incubated at room temperature for about 16 h. It is thus possible to obtain the expression of the genes encoding antibody fragments, such as scFvs, having a spreading action, so that those fragments binding specifically with the antigen which is present on the trap membrane are trapped. The trap membrane may then be treated to identify bound

antibody fragments, such as scFvs, for example using colorimetric techniques commonly used to this purpose.

The position of the identified fragments, for example as coloured spots, on the trap filter allows one to go back to the corresponding bacterial colonies which are present on the growth membrane and produce the antibody fragments trapped. Colonies are gathered and grown and the bacteria are distributed onto a new culture membrane, repeating the procedures described above. Analogous cycles are then carried out until the positive signals on the trap membrane correspond to single positive colonies, each of which represents a potential source of monoclonal antibody fragments directed against the antigen used in the selection. ICFS is described in e.g. WO02/46455.

A library may also be displayed on particles or molecular complexes, e.g. replicable genetic packages such bacteriophage (e.g. T7) particles, or other *in vitro* display systems, each particle or molecular complex containing nucleic acid encoding the antibody VH variable domain displayed on it, and optionally also a displayed VL domain if present. Phage display is described in WO92/01047 and e.g. US patents US5969108, US5565332, US5733743, US5858657, US5871907, US5872215, US5885793, US5962255, US6140471, US6172197, US6225447, US6291650, US6492160 and US6521404.

Following selection of antibody molecules able to bind the antigen and displayed on bacteriophage or other library particles or molecular complexes, nucleic acid may be taken from a bacteriophage or other particle or molecular complex displaying a said selected antibody molecule. Such nucleic acid may be used in subsequent production of an antibody molecule or an antibody VH or VL variable domain by expression from nucleic acid with the sequence of nucleic acid taken from a bacteriophage or other particle or molecular complex displaying a said selected antibody molecule.

Ability to bind an antigen associated with neoplastic growth and/or angiogenesis, such as the A-FN, B-FN, the ED-A, or the ED-B of fibronectin, tenascin C or the A1 domain of tenascin C or other target antigen or isoform may be further tested, e.g. ability to compete with an antibody specific for the A-FN, B-FN, the ED-A, or the ED-B of fibronectin, tenascin C or the A1 domain of tenascin C, such as antibody F8, L19, or F16.

Novel VH or VL regions carrying CDR-derived sequences for use as described herein may be also generated using random mutagenesis of one or more selected VH and/or VL genes to generate mutations within the entire variable domain. In some embodiments one or two amino acid substitutions are made within an entire variable domain or set of CDRs. Another method that may be used is to direct mutagenesis to CDR regions of VH or VL genes.

Variable domains employed as described herein may be obtained or derived from any germ-line or rearranged human variable domain, or may be a synthetic variable domain based on consensus or actual sequences of known human variable domains. A variable domain can be derived from a non-human antibody. A CDR sequence for use as described herein (e.g. CDR3) may be introduced into a repertoire of variable domains lacking a CDR (e.g. CDR3), using recombinant DNA technology. For example, Marks *et al*. (1992) describe methods of producing repertoires of antibody variable domains in which consensus primers directed at or adjacent to the 5' end of the variable domain area are used in conjunction with consensus primers to the third framework region of human VH genes to provide a repertoire of VH variable domains lacking a CDR3. Marks *et al*. further describe how this repertoire may be combined with a CDR3 of a particular antibody. Using analogous techniques, the CDR3-derived sequences of the present invention may be shuffled with repertoires of VH or VL domains lacking a CDR3, and the shuffled complete VH or VL domains combined with a cognate VL or VH domain to provide antibody molecules for use as described herein. The repertoire may then be displayed in a suitable host system such as the phage display system of WO92/01047, or any of a subsequent large body of literature, including Kay, Winter & McCafferty (1996), so that suitable antibody molecules may be selected. A repertoire may consist of from anything from 10^4 individual members upwards, for example at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9 or at least 10^{10} members.

An antigen associated with neoplastic growth and/or angiogenesis, such as the A-FN, B-FN, the ED-A, or the ED-B of fibronectin, tenascin C or the A1 domain of tenascin C may be used in a screen for antibody molecules, e.g. antibody molecules, for use as described herein. The screen may a screen of a repertoire as disclosed elsewhere herein.

Similarly, one or more, or all three CDRs may be grafted into a repertoire of VH or VL domains that are then screened for an antibody molecule or antibody molecules for an antigen associated with neoplastic growth and/or angiogenesis, such as A-FN, B-FN, the ED-A, or the

ED-B of fibronectin, tenascin C or the A1 domain of tenascin C. One or more of the HCDR1, HCDR2 and HCDR3 of antibody F8, L19, or F16, or the set of HCDRs of antibody F8, L19, or F16 may be employed, and/or one or more of the LCDR1, LCDR2 and LCDR3 of antibody F8, L19, or F16 the set of LCDRs of antibody F8, L19, or F16 may be employed.

A substantial portion of an immunoglobulin variable domain may comprise at least the three CDR regions, together with their intervening framework regions. The portion may also include at least about 50% of either or both of the first and fourth framework regions, the 50% being the C-terminal 50% of the first framework region and the N-terminal 50% of the fourth framework region. Additional residues at the N-terminal or C-terminal end of the substantial part of the variable domain may be those not normally associated with naturally occurring variable domain regions. For example, construction of antibody molecules of the present invention made by recombinant DNA techniques may result in the introduction of N- or C-terminal residues encoded by linkers introduced to facilitate cloning or other manipulation steps. Other manipulation steps include the introduction of linkers to join variable domains disclosed elsewhere herein to further protein sequences including antibody constant regions, other variable domains (for example in the production of diabodies) or detectable/functional labels as discussed in more detail elsewhere herein.

Although antibody molecules may comprise a pair of VH and VL domains, single binding domains based on either VH or VL domain sequences may also be used as described herein. It is known that single immunoglobulin domains, especially VH domains, are capable of binding target antigens in a specific manner. For example, see the discussion of dAbs above.

In the case of either of the single binding domains, these domains may be used to screen for complementary domains capable of forming a two-domain antibody molecule able to bind an antigen associated with neoplastic growth and/or angiogenesis, such as A-FN, B-FN, the ED-A, or the ED-B of fibronectin, tenascin C or the A1 domain of tenascin C. This may be achieved by phage display screening methods using the so-called hierarchical dual combinatorial approach as disclosed in WO92/01047, in which an individual colony containing either an H or L chain clone is used to infect a complete library of clones encoding the other chain (L or H) and the resulting two-chain antibody molecule is selected in accordance with phage display techniques such as those described in that reference. This technique is also disclosed in Marks 1992.

Fragments of whole antibodies for use as described herein can be obtained starting from any of the antibody molecules described herein, e.g. antibody molecules comprising VH and/or VL domains or CDRs of any of antibodies described herein, by methods such as digestion by enzymes, such as pepsin or papain and/or by cleavage of the disulfide bridges by chemical reduction. In another manner, antibody fragments may be obtained by techniques of genetic recombination likewise well known to the person skilled in the art or else by peptide synthesis by means of, for example, automatic peptide synthesizers such as those supplied by the company Applied Biosystems, etc., or by nucleic acid synthesis and expression.

A conjugate as described herein comprises IL2 and a mutant of TNF, preferably TNFα, and an antibody molecule which binds an antigen associated with neoplastic growth and/or angiogenesis, as described herein. The antibody molecule is preferably a scFv or a diabody, most preferably a scFv, as described herein.

IL2 is preferably human IL2.

The IL2 preferably comprises or consist of the sequence set forth in SEQ ID NO: 12. Typically, IL2 has at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 12. IL2 in conjugates of the invention retains a biological activity of human IL2, e.g. the ability to inhibit cell proliferation.

TNF is preferably human TNF. Where the tumour necrosis factor is TNFα, the TNFα is preferably human TNFα.

The TNF mutant in conjugates described herein is a mutant of TNF which retains biological function of human TNF, e.g. the ability to inhibit cell proliferation but has a reduced activity.

The TNF mutant may comprise one or more mutations which reduce activity relative to the wild-type TNF which lacks the one or more mutations i.e. the TNF mutant is less potent than wild-type TNF. For example, the TNF mutant may comprise a mutation at the position corresponding to position 32 in SEQ ID NO: 15 or position 52 of SEQ ID NO: 17. In some embodiments, the R at said position may be substituted for a different amino acid, preferably an amino acid other than G, for example a non-polar amino acid, preferably A, F, or V, most preferably A. The

sequences of examples of suitable TNF mutants are set forth in SEQ ID NO: 37, 39, 54-55, 56-57, respectively.

The identity of the residue at the position in a TNF mutant corresponding to position 32 in SEQ ID NO: 15 or position 52 of SEQ ID NO: 17 is shown herein to affect protein yield on expression in a recombinant system. For example, the presence of W at this position leads to substantially no expression in a recombinant system and the presence of A at this position leads to unexpectedly high yields in a recombinant system.

Human TNFα consists of a 35 amino acid cytoplasmic domain, a 20 amino acid transmembrane domain and a 177 amino acid extracellular domain. The 177 amino acid extracellular domain is cleaved to produce a 157 amino acid soluble form, which is biologically active, and which forms a non-covalently linked trimer in solution. In the context of the present invention, the human TNFα is a mutant of TNFα which is preferably the soluble form of the extracellular domain of human TNFα, or the extracellular domain of human TNFα. The sequence of the soluble form of the extracellular domain of human TNFα is shown in SEQ ID NO: 15 Typically, the mutant TNFα has at least 70%, more preferably one of at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 15 with one or more mutations which reduce activity, for example a mutation at the position corresponding to position 32 in SEQ ID NO: 15. The sequence of the extracellular domain of human TNFα is shown in SEQ ID NO: 17. In this case, the mutant TNFα may have at least 70%, more preferably one of at least at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to the amino acid sequence set forth in SEQ ID NO: 17 with one or more mutations which reduce activity, for example a mutation at the position corresponding to position 52 in SEQ ID NO: 17.

The inventors have shown that a conjugate of the present invention, and in particular the TNFα present in a conjugate of the present invention, wherein the arginine residue of TNFα at position 32 of SEQ ID NO: 15 or at position 52 of SEQ ID NO: 17 is substituted with alanine, exhibits reduced activity. Thus, the mutant of TNFα may comprise or consist of the sequence shown in SEQ ID NO: 15 or 17, except that the residue at position 32 of SEQ ID NO: 15 or at position 52 of SEQ ID NO: 17 is an alanine residue rather than an arginine residue. This sequence is shown in SEQ ID NO: 37 or 39. The mutant of TNFα thus preferably comprises or consist of the

sequence set forth in SEQ ID NO: 37. Typically, the mutant of TNFα has at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 37 with an A at the position corresponding to position 32 in SEQ ID NO: 37. Thus, alternatively the TNFα may comprise or consist of the sequence set forth in SEQ ID NO: 39. In this case, the TNFα may have at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100%, sequence identity to the amino acid sequence set forth in SEQ ID NO: 39 with an A at the position corresponding to position 52 in SEQ ID NO: 39.

Most preferably, the IL2 comprise the sequence set forth in SEQ ID NO: 12 and/or the TNFα comprise the sequence set forth in SEQ ID NO: 37.

Mutants of TNFα proteins may be tested *in vivo* and *in vitro* assays. Suitable assays include but are not limited to activity assays and binding assays. The substitution or deletion of arginine residue at position 32 (Arg 32) has been described in the prior art. For example, arginine residue has been proposed to be substituted by serine, glutamine, asparagine, aspartic acid, glutamic acid, histidine, tryptophan, threonine or tyrosine (US 7101974; US 5422104; WO1988/006625; Yamagishi *et al.*, Protein Eng. (1990) 3:713-9)). Furthermore, Arg32 has also been proposed to be deleted in EP158286. Mutants wherein Arg 32 has been substituted by tryptophan have shown a loss of cytotoxic activity (Van Ostade et al.The Embo Journal (1991) 10:827-836). Mutants wherein arginine at position 29 and/or 31 and/or 32 is substituted by tryptophan or tyrosine, show a significant difference between binding affinity to the human p75 TNF Receptor and to the human p55-TNF Receptor (US 5,422,104). US 7,101,974 described TNFα variants which interact with the wild-type TNFα to form mixed trimers incapable of activating receptor signalling. In this last example, Arg32 is substituted by aspartic acid, glutamic acid or histidine.

Preferably, the antibody molecule is connected to the IL2 and the TNF mutant, preferably TNFα mutant, through linkers, for example peptide linkers. Alternatively, the antibody molecule and IL2 and/or a mutant of tumour necrosis factor, may be connected directly, e.g. through a chemical bond. Where the antibody molecule is linked to IL2 and a mutant of tumour necrosis factor by means of one or more peptide linkers, the conjugate may be a fusion protein. By “fusion protein” is meant a polypeptide that is a translation product resulting from the fusion of two or more genes or nucleic acid coding sequences into one open reading frame (ORF).

The chemical bond may be, for example, a covalent or ionic bond. Examples of covalent bonds include peptide bonds (amide bonds) and disulphide bonds. The antibody molecule and IL2 and/or TNF mutant, preferably TNFα mutant, may be covalently linked, for example by peptide bonds (amide bonds). Thus, the antibody molecule, in particular a scFv portion of an antibody molecule, and IL2 and/or the TNF mutant, preferably TNFα mutant, may be produced as a fusion protein.

Where the antibody molecule is a two-chain or multi-chain molecule (e.g. a diabody), IL2 and/or the TNF mutant may be conjugated as a fusion protein with one or more polypeptide chains in the antibody molecule.

The peptide linker connecting the antibody molecule and IL2 and/or the TNF mutant, may be a flexible peptide linker. Suitable examples of peptide linker sequences are known in the art. The linker may be 10-20 amino acids, preferably 10-15 amino acids in length. Most preferably, the linker is 11-15 amino acids in length. The linker may have the sequence set forth in SEQ ID NO: 13, SEQ ID NO: 14 or SEQ ID NO: 49. In some preferred embodiments, the IL2 and the TNF mutant may be linked to the antibody molecule by the linkers set forth in SEQ ID NO: 13 and SEQ ID NO: 14, respectively. In other preferred embodiments, the IL2 and the TNF mutant may be linked to the antibody molecule by the linkers set forth in SEQ ID NO: 49 and SEQ ID NO: 14, respectively.

For example, in the conjugates exemplified in Example 2, IL2 was conjugated to the VH domain of the F8 scFv and the TNFα or the TNFα mutant was conjugated to the VL domain of the F8 scFv, each via a peptide linker as shown in SEQ ID NO: 1 and SEQ ID NO: 36 respectively. In the conjugate exemplified in Example 4, IL2 was conjugated to the VH domain of the L19 scFv and the TNFα or the TNFα mutant was conjugated to the VL domain of the L19 scFv, each via a peptide linker as shown in SEQ ID NO: 70 and SEQ ID NO: 44, respectively.

However, it is expected that the conjugate comprising IL2 and a TNF mutant, preferably a TNFα mutant, and an antibody molecule which binds an antigen associated with neoplastic growth and/or angiogenesis would show the same or similar tumour targeting properties, and/or therapeutic efficacy as the tumour necrosis factor and IL2 were conjugated to the antibody molecule. Thus, where the antibody molecule is, or comprises, an scFv, the IL2 may be linked

to the N-terminus of the VH domain of the scFv via a peptide linker and the mutant of TNF may be linked to the C-terminus of the VL domain of the scFv via a peptide linker. Alternatively, where the antibody molecule is, or comprises, an scFv, the mutant of TNF may be linked to the N-terminus of the VH domain of the scFv via a peptide linker and the IL2 may be linked to the C-terminus of the VL domain of the scFv via a peptide linker. It is expected that a conjugate would have the same or similar tumour targeting properties, and/or therapeutic efficacy, and/or cell killing activity if both IL2 and a mutant of TNF, preferably TNFα, were conjugated to the VH domain of the antibody. As a further alternative, the IL2 and TNF mutant, preferably TNFα mutant, may therefore be linked to the C-terminus of the VL domain of the antibody, e.g. in scFv format, via a peptide linker. As a yet further alternative the IL2 and TNF mutant, preferably TNFα mutant, may be linked to the N-terminus of the VH domain of the antibody, e.g. in scFv format, via a peptide linker. In the latter two conjugates, the IL2 and TNF may be in any order and/or may optionally be linked to one another via a peptide linker. Suitable peptide linkers are described herein.

Conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 36 or may be a variant thereof. A variant may have at least 70%, more preferably at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the reference sequence e.g. the amino acid sequence shown in SEQ ID NO: 36. Preferably, the residue at the position in the variant corresponding to position 432 of SEQ ID NO: 36 is A. For example, a conjugate that is a variant of SEQ ID NO: 36 may comprise an A residue at position 432.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 1 with an R to A mutation at position 432 or SEQ ID NO: 16 with an R to A mutation at position 452 or may be a variant of one of these sequences. A variant may have at least 70%, more preferably at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the reference sequence e.g. the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 16. Preferably, the residue at the position corresponding to position 432 in a variant of SEQ ID NO: 1 is A and the residue at the position corresponding to position 452 in a variant of SEQ ID NO: 16 is A. For example, a conjugate that is a variant of SEQ ID NO: 1 or SEQ ID NO: 16 may comprise an A residue at position 432 or 452 respectively.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 38 or may be a variant thereof. A variant may have at least 70%, more preferably at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the reference sequence e.g. the amino acid sequence shown in SEQ ID NO: 38. Preferably, the residue at the position in the variant corresponding to position 452 of SEQ ID NO: 38 is A. For example, a conjugate that is a variant of SEQ ID NO: 38 may comprise an A residue at position 452.

Alternatively, conjugates described herein may comprise or consist of one of the sequences shown in SEQ ID NOs: 58 to 63 or may be a variant thereof. A variant may have at least 70%, more preferably at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the reference sequence e.g. one of the amino acid sequences shown in SEQ ID NOs: 58 to 63. Preferably, the residue at the position corresponding to position 432 in a variant of SEQ ID NO: 58, 60, or 62 is W, F, or V, respectively. Preferably, the residue at the position corresponding to position 452 in a variant of SEQ ID NO: 59, 61 or 63 is W, F, or V, respectively.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 40 or may be a variant thereof. A variant may have at least 70%, more preferably at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO: 40. Preferably, the residue at the position in the variant corresponding to position 427 of SEQ ID NO: 40 is A. For example, a conjugate that is a variant of SEQ ID NO: 40 may comprise an A residue at position 427.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 41 or may be a variant thereof. A variant may have at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the reference sequence e.g. the amino acid sequence shown in SEQ ID NO: 41. Preferably, the residue at the position in the variant corresponding to position 447 of SEQ ID NO: 41 is A. For example, a conjugate that is a variant of SEQ ID NO: 41 may comprise an A residue at position 447.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 42 or may be a variant thereof. A variant may have at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence shown in SEQ ID NO: 42. Preferably, the residue at the position in the variant corresponding to position 428 of SEQ ID NO: 42 is A. For example, a conjugate that is a variant of SEQ ID NO: 42 may comprise an A residue at position 428.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 43 or may be a variant thereof. A variant may have at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence shown in SEQ ID NO: 43. Preferably, the residue at the position in the variant corresponding to position 448 of SEQ ID NO: 43 is A. For example, a conjugate that is a variant of SEQ ID NO: 43 may comprise an A residue at position 448.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 44 or may be a variant thereof. A variant may have at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence shown in SEQ ID NO: 44. Preferably, the residue at the position in the variant corresponding to position 430 of SEQ ID NO: 44 is A. For example, a conjugate that is a variant of SEQ ID NO: 44 may comprise an A residue at position 430.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 45 or may be a variant thereof. A variant may have at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence shown in SEQ ID NO: 45. Preferably, the residue at the position in the variant corresponding to position 450 of SEQ ID NO: 45 is A. For example, a conjugate that is a variant of SEQ ID NO: 45 may comprise an A residue at position 450.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 70 with an R to A mutation at position 430 or SEQ ID NO: 71 with an R to A mutation at position 450 or may be a variant of one of these sequences. A variant may have at least 70%, more preferably at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the reference sequence e.g. the amino acid sequence shown in SEQ ID NO: 70 or SEQ ID NO: 71.

Preferably, the residue at the position corresponding to position 430 in a variant of SEQ ID NO: 70 is A and the residue at the position corresponding to position 450 in a variant of SEQ ID NO: 71 is A. For example, a conjugate that is a variant of SEQ ID NO: 70 or SEQ ID NO: 71 may comprise an A residue at position 430 or 450 respectively.

Alternatively, conjugates described herein may comprise or consist of the sequences shown in SEQ ID NOs: 64 to 69 or may be a variant thereof. A variant may have at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequences shown in SEQ ID NOs: 64 to 69. Preferably, the residue at the position corresponding to position 430 in a variant of SEQ ID NO: 64, 66 or 68 is W, F or V, respectively. Preferably, the residue at the position corresponding to position 450 in a variant of SEQ ID NO: 65, 67 or 69 is W, F or V, respectively.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 47 or may be a variant thereof. A variant may have at least 70%, more preferably at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the amino acid sequence shown in SEQ ID NO: 47. Preferably, the residue at the position in the variant corresponding to position 431 of SEQ ID NO: 47 is A. For example, a conjugate that is a variant of SEQ ID NO: 47 may comprise an A residue at position 431.

Alternatively, conjugates described herein may comprise or consist of the sequence shown in SEQ ID NO: 48 or may be a variant thereof. A variant may have at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the reference sequence e.g. the amino acid sequence shown in SEQ ID NO: 48. Preferably, the residue at the position in the variant corresponding to position 451 of SEQ ID NO: 48 is A. For example, a conjugate that is a variant of SEQ ID NO: 48 may comprise an A residue at position 451.

Alternatively, conjugates described herein may comprise or consist of the sequences shown in SEQ ID NOs: 72 to 77 or may be a variant thereof. A variant may have at least 70%, more preferably one of at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the reference sequence e.g. the amino acid sequences shown in SEQ ID NOs: 72 to 77. Preferably, the residue at the position corresponding to position 431 in a variant of SEQ ID

NO: 72, 74 or 76 is W, F, or V, respectively. Preferably, the residue at the position corresponding to position 451 in a variant of SEQ ID NO: 73, 75 or 77 is W, F, or V, respectively.

Without being limited by any theoretical explanation, a conjugate described herein comprising a TNF mutant may form a homotrimer in solution. Such a trimeric conjugate would comprise three molecules of active IL2 to one molecule of active TNF with reduced activity (in trimeric structure). This may be advantageous as IL2-based immunocytokines are typically used in the clinic at higher doses compared to TNFα-based immunocytokines. For example, the recommended dose of L19-IL2 was found to be 4 mg in patients with cancer [Johannsen et al. (2010) *Eur. J. Cancer*], while the recommended dose of L19-TNFα is in the 1-1.5 mg dose range [Spitaleri et al. (2012) *J. Clin. Oncol. Cancer Res.*]. Furthermore, higher doses of the conjugates described herein may be used as the mutant of TNF has a reduced activity, compared to a conjugate comprising a wild type TNF and IL2. Thus, the conjugates described herein may have advantageous properties with respect to administration regimens.

Also provided is an isolated nucleic acid molecule encoding a conjugate as described herein. Nucleic acid molecules may comprise DNA and/or RNA and may be partially or wholly synthetic. Reference to a nucleotide sequence as set out herein encompasses a DNA molecule with the specified sequence, and encompasses a RNA molecule with the specified sequence in which U is substituted for T, unless context requires otherwise.

Further provided are constructs in the form of plasmids, vectors (e.g. expression vectors), transcription or expression cassettes which comprise such nucleic acids. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids e.g. phagemid, or viral e.g. 'phage, as appropriate. For further details see, for example, Sambrook & Russell (2001) Molecular Cloning: a Laboratory Manual: 3rd edition, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in the preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Ausubel et al. (1999) 4th eds., Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, John Wiley & Sons.

A recombinant host cell that comprises one or more constructs as described above is also provided. Suitable host cells include bacteria, mammalian cells, plant cells, filamentous fungi, yeast and baculovirus systems and transgenic plants and animals.

Conjugates described herein may be produced using such a recombinant host cell. The production method may comprise expressing a nucleic acid or construct as described above. Expression may conveniently be achieved by culturing the recombinant host cell under appropriate conditions for production of the conjugate. Following production the conjugate may be isolated and/or purified using any suitable technique, and then used as appropriate. The conjugate may be formulated into a composition including at least one additional component, such as a pharmaceutically acceptable excipient.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. The expression of antibodies, including conjugates thereof, in prokaryotic cells is well established in the art. For a review, see for example Plückthun (1991), Bio/Technology 9: 545-551. A common bacterial host is *E.coli*.

Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for production of conjugates for example Chadd et al. (2001), Current Opinion in Biotechnology 12: 188-194); Andersen et al. (2002) Current Opinion in Biotechnology 13: 117; Larrick & Thomas (2001) Current Opinion in Biotechnology 12:411-418. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney cells, NS0 mouse melanoma cells, YB2/0 rat myeloma cells, human embryonic kidney cells, human embryonic retina cells and many others.

A method comprising introducing a nucleic acid or construct disclosed herein into a host cell is also described. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. Introducing nucleic acid in the host cell, in particular a eukaryotic cell may use a viral or a plasmid based system. The plasmid system may be maintained episomally or may be incorporated into the host cell or into an artificial chromosome. Incorporation may be either by random or targeted integration of one or more

copies at single or multiple loci. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage.

The nucleic acid may or construct be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences that promote recombination with the genome, in accordance with standard techniques.

The term “isolated” refers to the state in which conjugates described herein, antibodies for use as described herein, or nucleic acid encoding such conjugates, will generally be in accordance with the present invention. Thus, conjugates described herein, antibodies for use as described herein, or nucleic acid encoding such conjugates may be provided in isolated and/or purified, e.g. from the environment in which they are prepared (such as cell culture), in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially free of nucleic acid other than the sequence encoding a polypeptide with the required function. Isolated members and isolated nucleic acids will be free or substantially free of material with which they are found in the environment in which they are prepared (e.g. cell culture) when such preparation is by recombinant DNA technology practised *in vitro* or *in vivo*. Specific conjugates and nucleic acids may be formulated with diluents or adjuvants and still for practical purposes be isolated - for example the members may be mixed with pharmaceutically acceptable carriers or diluents when used in therapy. Specific conjugates may be glycosylated, either naturally or by systems of heterologous eukaryotic cells (e.g. CHO or NS0 (ECACC 85110503) cells, or they may be (for example if produced by expression in a prokaryotic cell) unglycosylated.

Heterogeneous preparations of conjugates may also be used as described herein. For example, such preparations may be mixtures of conjugates comprising antibody molecules with full-length heavy chains and heavy chains lacking the C-terminal lysine, with various degrees of glycosylation and/or with derivatized amino acids, such as cyclization of an N-terminal glutamic acid to form a pyroglutamic acid residue.

Fibronectin is an antigen that is subject to alternative splicing, and a number of alternative isoforms of fibronectin are known, including alternatively spliced isoforms A-FN and B-FN, comprising domains ED-A or ED-B respectively, which are known markers of angiogenesis. An antibody molecule, as referred to herein, may selectively bind to isoforms of fibronectin selectively expressed in the neovasculature. An antibody molecule may bind fibronectin isoform

A-FN, e.g. it may bind domain ED-A (extra domain A). An antibody molecule may bind ED-B (extra domain B).

Fibronectin Extra Domain-A (EDA or ED-A) is also known as ED, extra type III repeat A (EIIIA) or EDI. The sequence of human ED-A has been published by Kornblihtt *et al*. (1984), Nucleic Acids Res. 12, 5853-5868 and Paolella *et al* . (1988), Nucleic Acids Res. 16, 3545-3557. The sequence of human ED-A is also available on the SwissProt database as amino acids 1631-1720 (Fibronectin type-III 12; extra domain 2) of the amino acid sequence deposited under accession number P02751. The sequence of mouse ED-A is available on the SwissProt database as amino acids 1721-1810 (Fibronectin type-III 13; extra domain 2) of the amino acid sequence deposited under accession number P11276.

The ED-A isoform of fibronectin (A-FN) contains the Extra Domain-A (ED-A). The sequence of the human A-FN can be deduced from the corresponding human fibronectin precursor sequence which is available on the SwissProt database under accession number P02751. The sequence of the mouse A-FN can be deduced from the corresponding mouse fibronectin precursor sequence which is available on the SwissProt database under accession number P11276. The A-FN may be the human ED-A isoform of fibronectin. The ED-A may be the Extra Domain-A of human fibronectin.

ED-A is a 90 amino acid sequence which is inserted into fibronectin (FN) by alternative splicing and is located between domain 11 and 12 of FN (Borsi *et al*. (1987), *J. Cell. Biol.*, 104, 595-600). ED-A is mainly absent in the plasma form of FN but is abundant during embryogenesis, tissue remodelling, fibrosis, cardiac transplantation and solid tumour growth.

Fibronectin isoform B-FN is one of the best known markers angiogenesis (US 10/382,107, WO01/62298). An extra domain “ED-B” of 91 amino acids is found in the B-FN isoform and is identical in mouse, rat, rabbit, dog and man. B-FN accumulates around neovascular structures in aggressive tumours and other tissues undergoing angiogenesis, such as the endometrium in the proliferative phase and some ocular structures in pathological conditions, but is otherwise undetectable in normal adult tissues.

Tenascin-C is a large hexameric glycoprotein of the extracellular matrix which modulates cellular adhesion. It is involved in processes such as cell proliferation and cell migration and is

associated with changes in tissue architecture as occurring during morphogenesis and embryogenesis as well as under tumourigenesis or angiogenesis. Several isoforms of tenascin-C can be generated as a result of alternative splicing which may lead to the inclusion of (multiple) domains in the central part of this protein, ranging from domain A1 to domain D (Borsi L *et al* Int J Cancer 1992; 52:688-692, Carnemolla B *et al*. Eur J Biochem 1992; 205:561-567, WO2006/050834). An antibody molecule, as referred to herein, may bind tenascin-C. An antibody molecule may bind tenascin-C domain A1.

Cancer, as referred to herein, may be a cancer which expresses, or has been shown to express, an antigen associated with neoplastic growth and/or angiogenesis, such as an extracellular matrix component associated with neoplastic growth and/or angiogenesis. Preferably, the cancer is a cancer which expresses, or has been shown to express, the ED-A isoform of fibronectin, the ED-B isoform of fibronectin and/or alternatively spliced tenascin C. More preferably the cancer expresses the ED-A isoform of fibronectin. For example, the cancer may be any type of solid or non-solid cancer or malignant lymphoma. The cancer may be selected from the group consisting of skin cancer (in particular melanoma), head and neck cancer, kidney cancer, sarcoma, germ cell cancer (such as teratocarcinoma), liver cancer, lymphoma (such as Hodgkin's or non-Hodgkin's lymphoma), leukaemia (e.g. acute myeloid leukaemia), skin cancer, bladder cancer, breast cancer, uterine cancer, ovarian cancer, prostate cancer, lung cancer, colorectal cancer, cervical cancer, oesophageal cancer, pancreatic cancer, stomach cancer, and cerebral cancer. Cancers may be familial or sporadic. Cancers may be metastatic or non-metastatic. Preferably, the cancer is a cancer selected from the group consisting of a melanoma, head and neck cancer, kidney cancer, and a sarcoma. The reference to a cancer as mentioned above normally refers to a malignant transformation of the cells in question. Thus, kidney cancer, for example, refers to a malignant transformation of cells in the kidney. The cancer may be located at its primary location, such as the kidney in the case of kidney cancer, or at a distant location in the case of metastases. A tumour as referred to herein may be the result of any of the cancers mentioned above. Preferably, a tumour is the result of a melanoma, head and neck cancer, kidney cancer, or a sarcoma. A tumour which is the result of a particular cancer includes both a primary tumour and tumour metastases of said cancer. Thus, a tumour which is the result of head and neck cancer, for example, includes both a primary tumour of head and neck and cancer and metastases of head and neck cancer found in other parts of a patient's body.

Conjugates described herein may have anti-tumour activity and thus find application in cancer treatment. Without being limited by any theoretical explanation, it is expected that the conjugates will show potent anti-tumour activity as a result of excellent tumour targeting properties, as demonstrated in Examples 3 and 4 below. The conjugates described herein are thus designed to be used in methods of treatment of patients, preferably human patients. Conjugates of the present invention may in particular be used in the treatment of cancer.

Accordingly, the invention provides methods of treatment comprising administration of a conjugate described above, pharmaceutical compositions comprising such conjugates, and use of such conjugates in the manufacture of a medicament for administration, for example in a method of making a medicament or pharmaceutical composition comprising formulating the conjugate with a pharmaceutically acceptable excipient. Pharmaceutically acceptable vehicles are well known and will be adapted by the person skilled in the art as a function of the nature and of the mode of administration of the active compound(s) chosen.

Conjugates described herein will usually be administered in the form of a pharmaceutical composition, which may comprise at least one component in addition to the antibody molecule. Thus, pharmaceutical compositions described herein, and for use in accordance with the present invention, may comprise, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be by injection, e.g. intravenous, intratumoral or subcutaneous. Preferably, the conjugate of the present invention is administered intratumorally.

Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's

Injection, Lactated Ringer's Injection. Preservatives, stabilizers, buffers, antioxidants and/or other additives may be employed, as required. Many methods for the preparation of pharmaceutical formulations are known to those skilled in the art. See e.g. Robinson ed., Sustained and Controlled Release Drug Delivery Systems, Marcel Dekker, Inc., New York, 1978.

A composition comprising a conjugate described herein may be administered alone or in combination with other cancer treatments, concurrently or sequentially or as a combined preparation with another therapeutic agent or agents, for the treatment of cancer. For example, a conjugate of the invention may be used in combination with an existing therapeutic agent for cancer.

A conjugate described herein may be used in the manufacture of a medicament. The medicament may be for separate or combined administration to an individual, and accordingly may comprise the conjugate and the additional component as a combined preparation or as separate preparations. Separate preparations may be used to facilitate separate and sequential or simultaneous administration, and allow administration of the components by different routes.

Compositions provided may be administered to mammals, preferably humans. Administration may be in a "therapeutically effective amount", this being sufficient to show benefit to a patient. Such benefit may be at least amelioration of at least one symptom. Thus “treatment” of a specified disease refers to amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated, the particular patient being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the composition, the type of conjugate, the method of administration, the scheduling of administration and other factors known to medical practitioners. Prescription of treatment, e.g. decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors, and may depend on the severity of the symptoms and/or progression of a disease being treated. Appropriate doses of antibody are well known in the art (Ledermann et al. (1991) Int. J. Cancer 47: 659-664; and Bagshawe et al. (1991) Antibody, Immunoconjugates and Radiopharmaceuticals 4: 915-922). Specific dosages indicated herein, or in the Physician's Desk Reference (2003) as appropriate for the type of medicament being administered, may be used. A therapeutically effective amount or suitable dose of a conjugate for use as described herein can be determined by

comparing its *in vitro* activity and *in vivo* activity in an animal model. Methods for extrapolation of effective dosages in mice and other test animals to humans are known. The precise dose will depend upon a number of factors, including whether the antibody is for diagnosis, prevention or for treatment, the size and location of the area to be treated, the precise nature of the conjugate. A typical conjugate dose will be in the range 10 µg to 500 µg /kg for systemic applications. An initial higher loading dose, followed by one or more lower doses, may be administered. This is a dose for a single treatment of an adult patient, which may be proportionally adjusted for children and infants, and also adjusted according to conjugate format in proportion to molecular weight. Treatments may be repeated at daily, twice-weekly, weekly or monthly intervals, at the discretion of the physician. Treatments may be every two to four weeks for subcutaneous administration and every four to eight weeks for intravenous administration. In some embodiments of the present invention, treatment is periodic, and the period between administrations is about two weeks or more, e.g. about three weeks or more, about four weeks or more, or about once a month. In other embodiments of the invention, treatment may be given before, and/or after surgery, and may be administered or applied directly at the anatomical site of surgical treatment.

Further aspects and embodiments of the invention will be apparent to those skilled in the art given the present disclosure including the following experimental exemplification.

All documents mentioned in this specification are incorporated herein by reference in their entirety for all purposes.

"and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example, "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein.

Unless context dictates otherwise, the descriptions and definitions of the features set out above are not limited to any particular aspect or embodiment of the invention and apply equally to all aspects and embodiments which are described.

Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures described above.

Examples

Example 1 – Production and analysis of huIL2-F8-huTNFα conjugate, huIL2-F8-huTNFα mutant conjugates, huIL2-L19-huTNFα conjugate and huIL2-L19-huTNFα mutant conjugate.

Various conjugates with human TNF α mutants were prepared and characterised by FPLC, SDS-PAGE and MS. The results are summarized in Table 1. Little or no expression of the R32W mutant was observed in either the IL2-L19-TNFα or the IL2-F8-TNFα immunocytokines. Yields of the R32A mutant were unexpectedly high for both immunocytokines.

	Protein	Mutation	Dialysis Buffer	Yield (mg/L)	FPLC Profile	SDS-PAGE	MS	SEQ ID NO
1	IL2-L19-TNF α	-	PBS	1.6	✓	✓	✓	70
2	IL2-L19-TNF α	R32W	PBS	0.4	✓	✓	✓	64
3	IL2-L19-TNF α	R32A	PBS	2.2	✓	✓	✓	44
4	IL2-F8-TNF α	-	PBS	1.4	✓	✓	✓	1
5	IL2-F8-TNF α	R32W	PBS	-	×	×	×	58
6	IL2-F8-TNF α	R32A	PBS	3.4	✓	✓	✓	36
7	IL2-F8-TNF α	R32F	MES	1.9	✓	✓	✓	60
8	IL2-F8-TNF α	R32V	MES	3.2	✓	✓	✓	62

Table 1

Example 2 – Effect of conjugate format on cell killing activity

The fusion proteins could be expressed and purified to homogeneity. The purified huIL2-F8-huTNFα conjugate (SEQ ID NO: 1) and huIL2-F8-huTNFα (R32A) mutant conjugate (SEQ ID NO: 36) were analysed by routine experiment on an ÄKTA-FPLC system with a Superdex 200 HR 10/30 column and characterized by SDS–PAGE analysis under non-reducing and reducing conditions.

To test the significance of the TNFα mutation in the conjugate on cell killing activity, the activity of the two fusion proteins was tested in a cell killing assay employing the L M fibroblast cell line. The assay was performed in the presence of 2 µg/mL actinomycin D (Sigma-Aldrich). Cells were seeded in 96-well plates in the culture medium supplemented with increasing concentrations of huIL2-F8-huTNFα (SEQ ID NO: 1), or huIL2-F8-huTNFα (R32A) (SEQ ID NO: 36) as indicated in Figure 1. The F8 antibody was in scFv format in all of the conjugates tested. The results are shown in Figure 1. Results are expressed as the percentage of cell viability

compared to cells treated with actinomycin D only (used as the negative control). The results demonstrate that the cell killing activity of the huIL2-F8-huTNFα (R32A) mutant conjugate was lower compared to the huIL2-F8-huTNFα conjugate, as can be seen from the EC50 values reported in Figure 1. The EC50 value represents the drug concentration required for half-maximal activity.

Example 3 – Biodistribution analysis of huIL2-F8-huTNF (R32A) mutant conjugate

The *in vivo* targeting performance of huIL2-F8-huTNF (R32A) mutant conjugate was evaluated by biodistribution analysis. The fusion protein was purified over size exclusion chromatography and then radioiodinated with Iodine 125. A total of 12µg (~9.6µCi) of the fusion protein preparation were injected into the tail vein of immunocompetent 129Sv mice bearing subcutaneously implanted F9 murine teratocarcinomas. Mice were sacrificed 24 h after injection. Organs were weighed and radioactivity was counted with a Packard Cobra gamma counter. The radioactive content of representative organs was recorded and expressed as percentage injected dose over gram of tissue (%ID/g). The results show a preferential and selective accumulation of huIL2-F8-huTNFα (R32A) mutant conjugate in the tumour (Figure 2).

Example 4 – Production and analysis of huIL2-L19-huTNFα (R32A) mutant conjugates

Protein Characterization

The fusion protein huIL2-L19-huTNFα (R32A) (SEQ ID NO: 44) was purified from the cell culture medium to homogeneity by protein A chromatography and analysed by SDS-PAGE, ESI-MS and size exclusion chromatography (Superdex200 10/300GL, GE Healthcare).

The biological activity of TNF and IL2 was determined on HT1080 and CTLL2 cells, respectively.

The huIL2-L19-huTNFα (R32A) mutant conjugate was well-behaved in biochemical assays, selectively localized to solid tumours *in vivo* and displayed a matched *in vitro* activity of the IL2 and TNF moieties, using cellular assays based on the proliferation of murine CTLL-2 lymphocytes (Figure 3) and on the killing of human HT-1080 tumour cell line (Figure 4).

Biodistribution studies

The *in vivo* EDB targeting performance of huIL2-L19-huTNF (R32A) mutant conjugate was evaluated by biodistribution analysis. 10 µg of radioiodinated fusion protein was injected into the lateral tail vein of F9 tumour-bearing mice. Mice were sacrificed 24h after injection, organs were excised, weighed and the radioactivity of organs and tumours was measured using a Cobra γ

counter and expressed as percentage of injected dose per gram of tissue (%ID/g ± SEM), (n = 3 mice per group). The results show a preferential and selective accumulation of huIL2-L19-huTNFα (R32A) mutant conjugate in the tumour (Figure 5).

Sequence listing

1. Amino acid sequence of the hulL2-F8-huTNFα [soluble form] conjugate (SEQ ID NO: 1)

The amino acid sequence of the hulL2-F8-huTNFα [soluble form] conjugate (human IL2 – linker - F8 VH – linker - F8 VL – linker – human TNFα [soluble form]) is shown below. The linker sequences are underlined. The human TNFα in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTL
VTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLN
RRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYE
PIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

2. Amino acid sequence of the F8 VH domain (SEQ ID NO: 2)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSL
RAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSS

3. Amino acid sequence of the linker linking the VH domain to the -VL domain of the antibody (SEQ ID NO: 3)

GGGGSGGGGSGGGG

4. Amino acid sequence of the F8 VL domain (SEQ ID NO: 4)

EIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAV
YYCQQMRGRPPTFGQGTKVEIK

5. Amino acid sequence of the F8 scFv (SEQ ID NO: 5)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSL
RAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQK
PGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIK

6. Amino acid sequences of the F8 CDR's

F8 CDR1 VH – LFT (SEQ ID NO: 6)

F8 CDR2 VH – SGSGGS (SEQ ID NO: 7)

F8 CDR3 VH – STHLYL (SEQ ID NO: 8)

F8 CDR1 VL – MPF (SEQ ID NO: 9)

F8 CDR2 VL – GASSRAT (SEQ ID NO: 10)

F8 CDR3 VL – MRGRPP (SEQ ID NO: 11)

7. Amino acid sequence of human IL2 (hulL2) in the conjugates (SEQ ID NO: 12)

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT

8. Amino acid sequence of the linker linking the antibody molecule and IL2 and/or the TNF mutant (SEQ ID NO: 13)

GDGSSGGSGGAS

9. Amino acid sequence of the linker linking the antibody molecule and IL2 and/or the TNF mutant (SEQ ID NO: 14)

SSSSGSSSSGSSSSG

10. Amino acid sequence of the soluble form of the extracellular domain of human TNFα (huTNFα) (SEQ ID NO: 15)

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVS
YQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

11. Amino acid sequence of the hulL2-F8-huTNFα [extracellular domain] conjugate (SEQ ID NO: 16)

The amino acid sequence of the hulL2-F8-huTNFα [extracellular domain] conjugate (human IL2 – linker - F8 VH – linker - F8 VL – linker – human TNFα [extracellular domain]) is shown below. The linker sequences are underlined. The human TNFα in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTL
VTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSD
KPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSA
IKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

12. Amino acid sequence of the extracellular domain of human TNFα (huTNFα) (SEQ ID NO:17)

GPQREEFPRDLSLISPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKG
QGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYF
GIIAL

13. Amino acid sequence of L19 CDR's

L19 CDR1 VH - Ser Phe Ser Met Ser (SEQ ID NO: 18)

L19 CDR2 VH - Ser Ile Ser Gly Ser Ser Gly Thr Thr Tyr Tyr Ala Asp Ser Val Lys (SEQ ID NO: 19)

L19 CDR3 VH - Pro Phe Pro Tyr Phe Asp Tyr (SEQ ID NO: 20)

L19 CDR1 VL - Arg Ala Ser Gln Ser Val Ser Ser Ser Phe Leu Ala (SEQ ID NO: 21)

L19 CDR2 VL - Tyr Ala Ser Ser Arg Ala Thr (SEQ ID NO: 22)

L19 CDR3 VL - Gln Gln Thr Gly Arg Ile Pro Pro Thr (SEQ ID NO: 23)

14. Amino acid sequence of L19 VH domain (SEQ ID NO: 24)

Glu Val Gln Leu Leu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Phe
Ser Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
Ser Ser Ile Ser Gly Ser Ser Gly Thr Thr Tyr Tyr Ala Asp Ser Val
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
Ala Lys Pro Phe Pro Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val
Thr Val Ser Ser

15. Amino acid sequence of L19 VL domain (SEQ ID NO: 25)

Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly
Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Ser
Phe Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu
Ile Tyr Tyr Ala Ser Ser Arg Ala Thr Gly Ile Pro Asp Arg Phe Ser
Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg Leu Glu
Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Thr Gly Arg Ile Pro
Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys

16. Amino acid sequence of scFv(L19) (SEQ ID NO: 26)

Glu Val Gln Leu Leu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Phe
Ser Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
Ser Ser Ile Ser Gly Ser Ser Gly Thr Thr Tyr Tyr Ala Asp Ser Val
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
Ala Lys Pro Phe Pro Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val
Thr Val Ser Ser Gly Asp Gly Ser Ser Gly Gly Ser Gly Gly Ala Ser
Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly
Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Ser
Phe Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu
Ile Tyr Tyr Ala Ser Ser Arg Ala Thr Gly Ile Pro Asp Arg Phe Ser
Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg Leu Glu
Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Thr Gly Arg Ile Pro
Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys

17. Amino acid sequence of F16 CDR's

F16 CDR1 VH – RYGMS (SEQ ID NO: 27)

F16 CDR2 VH – AISGSGGSTYYADSVKG (SEQ ID NO: 28)

F16 CDR3 VH – AHNAFDY (SEQ ID NO: 29)

F16 CDR1 VL – QGDSLRSYYAS (SEQ ID NO: 30)

F16 CDR2 VL – GKNNRPS (SEQ ID NO: 31)

F16 CDR3 VL – NSSVYTMPPVV (SEQ ID NO: 32)

18. Amino acid sequence F16 VH domain (SEQ ID NO: 33)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSL
RAEDTAVYYCAKAHNAFDYWGQGTLVTVSR

19. Amino acid sequence F16 VL domain (SEQ ID NO: 34)

SSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAEDEADYY
CNSSVYTMPPVVFGGGTKLTVLG

20. Amino acid sequence of the scFv(F16) (SEQ ID NO: 35)

The VH and VL domain linker sequence is shown underlined

EVQLLESGGGLVQPGGSLRLSCAASGFTFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSL
RAEDTAVYYCAKAHNAFDYWGQGTLVTVSRGGGSGGGSGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLV
IYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLG

21. Amino acid sequence of the hulL2-F8-huTNFα (R32A) mutant [soluble form] conjugate (SEQ ID NO: 36)

The amino acid sequence of the hulL2-F8-huTNFα (R32A) mutant [soluble form] conjugate (human IL2 – linker - F8 VH – linker - F8 VL – linker – human TNFα (R32A) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32A is underlined in bold. The mutant of human TNFα (R32A) in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTL
VTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLN
RAANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYE
PIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

22. Amino acid sequence of the soluble form of the extracellular domain of human TNFα (R32A) mutant (huTNFα R32A) (SEQ ID NO: 37). The R32A is underlined in bold.

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRAANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVS
YQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

23. Amino acid sequence of the hulL2-F8-huTNFα (R52A) mutant (huTNFα R52A) [extracellular domain] conjugate (SEQ ID NO: 38)

The amino acid sequence of the hulL2-F8-huTNFα (R52A) mutant [extracellular domain] conjugate (human IL2 – linker - F8 VH – linker - F8 VL – linker – human TNFα (R52A) mutant [extracellular domain]) is shown below. The linker sequences are underlined and the R52A is in underlined in bold. The human TNFα (R52A) mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTL
VTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSD

KPVAHVVANPQAEGQLQWLNR**A**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSA
IKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

24. Amino acid sequence of the extracellular domain of human TNFα (R52A) mutant (huTNFα R52A) [extracellular domain] (SEQ ID NO: 39). R52A is underlined in bold.

GPQREEFPRDLSLISPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNR**A**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKG
QGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYF
GIIAL

25. Amino acid sequence of the hulL2-F16-huTNFα (R32A) mutant [soluble form] conjugate (SEQ ID NO: 40)

The amino acid sequence of the hulL2-F16-huTNFα (R32A) mutant [soluble form] conjugate (human IL2 – linker – F16 VH – linker – F16 VL – linker – human TNFα (R32A) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32A is underlined in bold. The human TNFα mutant in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHNAFDYWGQGTLVT
VSRGGGSGGGSGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLT
ITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLGSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNR**A**ANA
LLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLG
GVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

26. Amino acid sequence of the hulL2-F16-huTNFα (R52A) mutant [extracellular domain] conjugate (SEQ ID NO: 41)

The amino acid sequence of the hulL2-F16-huTNFα (R52A) mutant [extracellular domain] conjugate (human IL2 – linker – F16 VH – linker – F16 VL – linker – human TNFα (R52A) mutant) is shown below. The linker sequences are underlined and the R52A is underlined in bold. The human TNFα mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHNAFDYWGQGTLVT
VSRGGGSGGGSGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLT
ITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLGSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDKPVAH
VVANPQAEGQLQWLNR**A**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC
QRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

27. Amino acid sequence of the hulL2-L19-huTNFα (R32A) mutant [soluble form] conjugate (SEQ ID NO: 42)

The amino acid sequence of the hulL2-L19-huTNFα (R32A) mutant [soluble form] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα (R32A) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32A is underlined in bold. The human TNFα mutant in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTLVT

VSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSGTD
FTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRAAN
ALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYL
GGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

28. Amino acid sequence of the hulL2-L19-huTNFα (R52A) mutant [extracellular domain] conjugate (SEQ ID NO: 43)

The amino acid sequence of the hulL2-L19-huTNFα (R52A) mutant [extracellular domain] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα (R52A) mutant) is shown below. The linker sequences are underlined and the R52A is underlined in bold. The human TNFα mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTLVT
VSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSGTD
FTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDKPVA
HVVANPQAEGQLQWLNRAANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSP
CQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

29. Amino acid sequence of the hulL2-L19-huTNFα (R32A) mutant [soluble form] conjugate (SEQ ID NO: 44)

The amino acid sequence of the hulL2-L19-huTNFα (R32A) mutant [soluble form] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα (R32A) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32A is underlined in bold. The human TNFα mutant in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAAS
GFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTL
VTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSG
TDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRA
ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPI
YLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

30. Amino acid sequence of the hulL2-L19-huTNFα (R52A) mutant [extracellular domain] conjugate (SEQ ID NO: 45)

The amino acid sequence of the hulL2-L19-huTNFα (R52A) mutant [extracellular domain] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα (R52A) mutant) is shown below. The linker sequences are underlined and the R52A is underlined in bold. The human TNFα mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAAS
GFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTL
VTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSG
TDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDKP
VAHVVANPQAEGQLQWLNRAANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIK
SPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

31. Amino acid sequence of the scFv(F16) (SEQ ID NO: 46)

The VH and VL domain linker sequence is shown underlined

EVQLLESGGGLVQPGGSLRLSCAASGFTFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSL
RAEDTAVYYCAKAHNAFDYWGQGTLVTVSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQA
PVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLG

32. Amino acid sequence of the hulL2-F16-huTNFα (R32A) mutant [soluble form] conjugate (SEQ ID NO: 47)

The amino acid sequence of the hulL2-F16-huTNFα (R32A) mutant [soluble form] conjugate (human IL2 – linker – F16 VH – linker – F16 VL – linker – human TNFα (R32A) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32A is underlined in bold. The human TNFα mutant in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHNAFDYWGQGTLVT
VSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNT
ASLTITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLGSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNR
AANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEP
IYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

33. Amino acid sequence of the hulL2-F16-huTNFα (R52A) mutant [extracellular domain] conjugate (SEQ ID NO: 48)

The amino acid sequence of the hulL2-F16-huTNFα (R52A) mutant [extracellular domain] conjugate (human IL2 – linker – F16 VH – linker – F16 VL – linker – human TNFα (R52A) mutant) is shown below. The linker sequences are underlined and the R52A is underlined in bold. The human TNFα mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHNAFDYWGQGTLVT
VSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNT
ASLTITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLGSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDK
PVAHVVANPQAEGQLQWLNR**A**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAI
KSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

34. Amino acid sequence of the linker linking the antibody molecule and IL2 and/or the TNF mutant (SEQ ID NO: 49)

GGGGSGGGGSGGGG

35. Amino acid sequence of the linker linking the VH domain to the VL domain of the antibody (SEQ ID NO: 50)

GDGSSGGSGGAS

36. Amino acid sequence of the linker linking the VH domain to the VL domain of the antibody (SEQ ID NO: 51)

GGGSGGGSGG

37. Amino acid sequence of the soluble form of the extracellular domain of human TNFα (R32W) mutant (huTNFα R32W) (SEQ ID NO: 52). The R32W is underlined in bold.

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNR**W**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVS
YQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

38. Amino acid sequence of the extracellular domain of human TNFα (R52W) mutant (huTNFα R52W) (SEQ ID NO: 53). R52W is underlined in bold.

GPQREEFPRDLSLISPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNR**W**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKG
QGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYF
GIIAL

39. Amino acid sequence of the soluble form of the extracellular domain of human TNFα (R32F) mutant (huTNFα R32F) (SEQ ID NO: 54). The R32F is underlined in bold.

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNR**F**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVS
YQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

40. Amino acid sequence of the extracellular domain of human TNFα (R52F) mutant (huTNFα R52F) (SEQ ID NO: 55). R52F is underlined in bold.

GPQREEFPRDLSLISPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNR**F**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKG
QGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYF
GIIAL

41. Amino acid sequence of the soluble form of the extracellular domain of human TNFα (R32V) mutant (huTNFα R32V) (SEQ ID NO: 56). The R32V is underlined in bold.

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNR**V**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVS
YQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

42. Amino acid sequence of the extracellular domain of human TNFα (R52V) mutant (huTNFα R52V) (SEQ ID NO: 57). R52V is in underlined in bold.

GPQREEFPRDLSLISPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNR**V**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKG
QGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYF
GIIAL

43. Amino acid sequence of the hulL2-F8-huTNFα (R32W) mutant [soluble form] conjugate (SEQ ID NO: 58)

The amino acid sequence of the hulL2-F8-huTNFα (R32W) mutant [soluble form] conjugate (human IL2 – linker - F8 VH – linker - F8 VL – linker – human TNFα (R32W) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32W is underlined in bold. The mutant of human TNFα (R32W) in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTL
VTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLN
R**W**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYE
PIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

44. Amino acid sequence of the hulL2-F8-huTNFα (R52W) mutant (huTNFα R52W) [extracellular domain] conjugate (SEQ ID NO: 59)

The amino acid sequence of the hulL2-F8-huTNFα (R52W) mutant [extracellular domain] conjugate (human IL2 – linker - F8 VH – linker - F8 VL – linker – human TNFα (R52W) mutant [extracellular domain]) is shown below. The linker sequences are underlined and the R52W is underlined in bold. The human TNFα (R52W) mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTL
VTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSD
KPVAHVVANPQAEGQLQWLNRWANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSA
IKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

45. Amino acid sequence of the hulL2-F8-huTNFα (R32F) mutant [soluble form] conjugate (SEQ ID NO: 60)

The amino acid sequence of the hulL2-F8-huTNFα (R32F) mutant [soluble form] conjugate (human IL2 – linker - F8 VH – linker - F8 VL – linker – human TNFα (R32F) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32F is underlined in bold. The mutant of human TNFα (R32F) in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTL
VTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLN
RFANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYE
PIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

46. Amino acid sequence of the hulL2-F8-huTNFα (R52F) mutant (huTNFα R52F) [extracellular domain] conjugate (SEQ ID NO: 61)

The amino acid sequence of the hulL2-F8-huTNFα (R52F) mutant [extracellular domain] conjugate (human IL2 – linker - F8 VH – linker - F8 VL – linker – human TNFα (R52F) mutant [extracellular domain]) is shown below. The linker sequences are underlined and the R52F is underlined in bold. The human TNFα (R52F) mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTL
VTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSD
KPVAHVVANPQAEGQLQWLNRFANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSA
IKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

47. Amino acid sequence of the hulL2-F8-huTNFα (R32V) mutant [soluble form] conjugate (SEQ ID NO: 62)

The amino acid sequence of the hulL2-F8-huTNFα (R32V) mutant [soluble form] conjugate (human IL2 – linker - F8 VH – linker - F8 VL – linker – human TNFα (R32V) mutant [soluble form]) is shown below. The

linker sequences are underlined and the R32V is underlined in bold. The mutant of human TNFα (R32V) in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTL
VTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLN
R**V**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYE
PIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

48. Amino acid sequence of the hulL2-F8-huTNFα (R52V) mutant (huTNFα R52V) [extracellular domain] conjugate (SEQ ID NO: 63)

The amino acid sequence of the hulL2-F8-huTNFα (R52V) mutant [extracellular domain] conjugate (human IL2 – linker - F8 VH – linker - F8 VL – linker – human TNFα (R52V) mutant [extracellular domain]) is shown below. The linker sequences are underlined and the R52V is underlined in bold. The human TNFα (R52V) mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTL
VTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQMRGRPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSD
KPVAHVVANPQAEGQLQWLNR**V**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSA
IKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

49. Amino acid sequence of the hulL2-L19-huTNFα (R32W) mutant [soluble form] conjugate (SEQ ID NO: 64)

The amino acid sequence of the hulL2-L19-huTNFα (R32W) mutant [soluble form] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα (R32W) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32W is underlined in bold. The human TNFα mutant in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAAS
GFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTL
VTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSG
TDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNR**W**
ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPI
YLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

50. Amino acid sequence of the hulL2-L19-huTNFα (R52W) mutant [extracellular domain] conjugate (SEQ ID NO: 65)

The amino acid sequence of the hulL2-L19-huTNFα (R52W) mutant [extracellular domain] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα (R52W) mutant) is shown below. The linker sequences are underlined and the R52W is underlined in bold. The human TNFα mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAAS
GFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTL

VTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSG
TDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDKP
VAHVVANPQAEGQLQWLNRWANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIK
SPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

51. Amino acid sequence of the hulL2-L19-huTNFα (R32F) mutant [soluble form] conjugate (SEQ ID NO: 66)

The amino acid sequence of the hulL2-L19-huTNFα (R32F) mutant [soluble form] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα (R32F) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32F is underlined in bold. The human TNFα mutant in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAAS
GFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTL
VTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSG
TDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRF
ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPI
YLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

52. Amino acid sequence of the hulL2-L19-huTNFα (R52F) mutant [extracellular domain] conjugate (SEQ ID NO: 67)

The amino acid sequence of the hulL2-L19-huTNFα (R52F) mutant [extracellular domain] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα (R52F) mutant) is shown below. The linker sequences are underlined and the R52F is underlined in bold. The human TNFα mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAAS
GFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTL
VTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSG
TDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDKP
VAHVVANPQAEGQLQWLNRFANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIK
SPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

53. Amino acid sequence of the hulL2-L19-huTNFα (R32V) mutant [soluble form] conjugate (SEQ ID NO: 68)

The amino acid sequence of the hulL2-L19-huTNFα (R32V) mutant [soluble form] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα (R32V) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32V is underlined in bold. The human TNFα mutant in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAAS
GFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTL
VTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSG
TDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRV
ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPI
YLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

54. Amino acid sequence of the hulL2-L19-huTNFα (R52V) mutant [extracellular domain] conjugate (SEQ ID NO: 69)

The amino acid sequence of the hulL2-L19-huTNFα (R52V) mutant [extracellular domain] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα (R52V) mutant) is shown below. The linker sequences are underlined and the R52V is underlined in bold. The human TNFα mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAAS
GFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTL
VTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSG
TDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDKP
VAHVVANPQAEGQLQWLNRVANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIK
SPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

55. Amino acid sequence of the hulL2-L19-huTNFα [soluble form] conjugate (SEQ ID NO: 70)

The amino acid sequence of the hulL2-L19-huTNFα [soluble form] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα [soluble form]) is shown below. The linker sequences are underlined. The human TNFα in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAAS
GFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTL
VTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSG
TDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRR
ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPI
YLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

56. Amino acid sequence of the hulL2-L19-huTNFα [extracellular domain] conjugate (SEQ ID NO: 71)

The amino acid sequence of the hulL2-L19-huTNFα [extracellular domain] conjugate (human IL2 – linker – L19 VH – linker – L19 VL – linker – human TNFα) is shown below. The linker sequences are underlined. The human TNFα in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGGGGSGGGGSGGGGEVQLLESGGGLVQPGGSLRLSCAAS
GFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTL
VTVSSGDGSSGGSGGASEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSG
TDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDKP
VAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIK
SPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

57. Amino acid sequence of the hulL2-F16-huTNFα (R32W) mutant [soluble form] conjugate (SEQ ID NO: 72)

The amino acid sequence of the hulL2-F16-huTNFα (R32W) mutant [soluble form] conjugate (human IL2 – linker – F16 VH – linker – F16 VL – linker – human TNFα (R32W) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32W is underlined in bold. The human TNFα mutant in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF

TFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHNAFDYWGQGTLVT
VSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNT
ASLTITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLGSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNR
WANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEP
IYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

58. Amino acid sequence of the hulL2-F16-huTNFα (R52W) mutant [extracellular domain] conjugate (SEQ ID NO: 73)

The amino acid sequence of the hulL2-F16-huTNFα (R52W) mutant [extracellular domain] conjugate (human IL2 – linker – F16 VH – linker – F16 VL – linker – human TNFα (R52W) mutant) is shown below. The linker sequences are underlined and the R52W is underlined in bold. The human TNFα mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHNAFDYWGQGTLVT
VSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNT
ASLTITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLGSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDK
PVAHVVANPQAEGQLQWLNR**W**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAI
KSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

59. Amino acid sequence of the hulL2-F16-huTNFα (R32F) mutant [soluble form] conjugate (SEQ ID NO: 74)

The amino acid sequence of the hulL2-F16-huTNFα (R32F) mutant [soluble form] conjugate (human IL2 – linker – F16 VH – linker – F16 VL – linker – human TNFα (R32F) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32F is underlined in bold. The human TNFα mutant in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHNAFDYWGQGTLVT
VSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNT
ASLTITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLGSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNR
FANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEP
IYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

60. Amino acid sequence of the hulL2-F16-huTNFα (R52F) mutant [extracellular domain] conjugate (SEQ ID NO: 75)

The amino acid sequence of the hulL2-F16-huTNFα (R52F) mutant [extracellular domain] conjugate (human IL2 – linker – F16 VH – linker – F16 VL – linker – human TNFα (R52F) mutant) is shown below. The linker sequences are underlined and the R52F is underlined in bold. The human TNFα mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHNAFDYWGQGTLVT
VSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNT
ASLTITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLGSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDK
PVAHVVANPQAEGQLQWLNR**F**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAI
KSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

61. Amino acid sequence of the hulL2-F16-huTNFα (R32V) mutant [soluble form] conjugate (SEQ ID NO: 76)

The amino acid sequence of the hulL2-F16-huTNFα (R32V) mutant [soluble form] conjugate (human IL2 – linker – F16 VH – linker – F16 VL – linker – human TNFα (R32V) mutant [soluble form]) is shown below. The linker sequences are underlined and the R32V is underlined in bold. The human TNFα mutant in this conjugate is the soluble form of the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHNAFDYWGQGTLVT
VSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNT
ASLTITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLGSSSSGSSSSGSSSSGVRSSSRTPSDKPVAHVVANPQAEGQLQWLNR
VANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEP
IYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

62. Amino acid sequence of the hulL2-F16-huTNFα (R52V) mutant [extracellular domain] conjugate (SEQ ID NO: 77)

The amino acid sequence of the hulL2-F16-huTNFα (R52V) mutant [extracellular domain] conjugate (human IL2 – linker – F16 VH – linker – F16 VL – linker – human TNFα (R52V) mutant) is shown below. The linker sequences are underlined and the R52V is underlined in bold. The human TNFα mutant in this conjugate is the extracellular domain of TNFα.

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLI
SNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIISTLTGDGSSGGSGGASEVQLLESGGGLVQPGGSLRLSCAASGF
TFSRYGMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKAHNAFDYWGQGTLVT
VSRGGGGSGGGGSGGGGSSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNT
ASLTITGAQAEDEADYYCNSSVYTMPPVVFGGGTKLTVLGSSSSGSSSSGSSSSGGPQREEFPRDLSLISPLAQAVRSSSRTPSDK
PVAHVVANPQAEGQLQWLNR**V**ANALLANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAI
KSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

Claims

1. A conjugate comprising interleukin-2 (IL2), a tumour necrosis factor (TNF) mutant, and an antibody molecule which binds an antigen associated with neoplastic growth and/or angiogenesis, wherein the TNF mutant has reduced activity relative to the wild type TNF.

2. The conjugate according to claim 1 wherein the TNF mutant comprises the amino acid sequence of wild-type TNF with one or more amino acid substitutions, deletions or insertions which reduce the activity of the TNF mutant relative to the wild type TNF.

3. The conjugate according to claim 1 or claim 2, wherein the TNF is TNFα.

4. The conjugate according to claim 3 wherein the TNFα is human TNFα.

5. The conjugate according to any one of the preceding claims wherein the TNF mutant has a mutation at position corresponding to R32 of SEQ ID NO: 15 or R52 of SEQ ID NO: 17.

6. The conjugate according to claim 5 wherein the R at said position is substituted for a non-polar amino acid.

7. The conjugate according to claim 6 wherein the TNF mutant has an R to A mutation at said position.

8. The conjugate according to any one of the preceding claims wherein the TNF mutant comprises the amino acid sequence of SEQ ID NO: 37 or SEQ ID NO: 39 or a variant thereof.

9. The conjugate according to any one of claims 1 to 8, wherein the TNFα is linked to the antibody molecule via a peptide linker.

10. The conjugate according to claim 9, wherein the peptide linker has the amino acid sequence of SEQ ID NO: 14.

11. The conjugate according to any one of the preceding claims, wherein the IL2 is human IL2.

12. The conjugate according to claim 11, wherein the IL2 comprises the sequence of SEQ ID NO: 12.

13. The conjugate according to any one of claims 1 to 12, wherein the IL2 is linked to the antibody molecule by a peptide linker.

14. The conjugate according to claim 13 wherein the peptide linker has the amino acid sequence of SEQ ID NO: 13 or SEQ ID NO: 49.

15. The conjugate according to any one of claims 1 to 14, wherein the antibody molecule comprises a single chain Fv (scFv).

16. The conjugate according to any one of claims 1 to 14, wherein the antibody molecule comprises a diabody.

17. The conjugate according to any one of the preceding claims, wherein the antibody molecule binds fibronectin.

18. The conjugate according to claim 17, wherein the antibody molecule binds the Extra Domain-A (ED-A) of fibronectin.

19. The conjugate according to claim 18, wherein the antibody molecule comprises an antigen binding site having the complementarity determining regions (CDRs) of antibody F8 set forth in SEQ ID NOs 6-11.

20. The conjugate according to claim 19, wherein the antibody molecule comprises the VH and VL domains of antibody F8 set forth in SEQ ID NOs 2 and 4 or variants thereof.

21. The conjugate according to claim 20, wherein the antibody molecule is an scFv, and wherein the VH domain and the VL domain of the scFv are linked by a 10 to 20 amino acid linker.

22. The conjugate according to claim 17 to 21, wherein the antibody molecule comprises the amino acid sequence of scFv F8 set forth in SEQ ID NO: 5 or a variant thereof.

23. The conjugate according to claim 17, wherein the antibody molecule binds the Extra Domain-B (ED-B) of fibronectin.

24. The conjugate according to claim 23, wherein the antibody molecule comprises an antigen binding site having the complementarity determining regions (CDRs) of antibody L19 set forth in SEQ ID NOs 18-23.

25. The conjugate according to claim 24, wherein the antibody molecule comprises the VH and VL domains of antibody L19 set forth in SEQ ID NOs 24 and 25 or variants thereof.

26. The conjugate according to claim 25, wherein the antibody molecule is an scFv, and wherein the VH domain and the VL domain of the scFv are linked by a 10 to 20 amino acid linker.

27. The conjugate according to any one of claims 23 to 26, wherein the antibody molecule comprises the amino acid sequence of scFv L19 set forth in SEQ ID NO: 26 or a variant thereof.

28. The conjugate according to any one of claims 1 to 16, wherein the antibody molecule binds the A1 Domain of Tenascin C.

29. The conjugate according to claim 28, wherein the antibody molecule comprises an antigen binding site having the complementarity determining regions (CDRs) of antibody F16 set forth in SEQ ID NOs 27-32.

30. The conjugate according to claim 29, wherein the antibody molecule comprises the VH and VL domains of antibody F16 set forth in SEQ ID NOs 33 and 34 or variants thereof.

31. The conjugate according to claim 30, wherein the antibody molecule is an scFv, and wherein the VH domain and the VL domain of the scFv are linked by a 10 to 20 amino acid linker.

32. The conjugate according to any one of claims 28 to 31, wherein the antibody molecule comprises the amino acid sequence of scFv F16 set forth in SEQ ID NO: 35 or SEQ ID NO: 46 or a variant thereof.

33. The conjugate according to any one of claims 1 to 32, wherein the antibody molecule is, or comprises, a single chain Fv (scFv) and, wherein the IL2 is linked to the N-terminus of the VH domain of the scFv via a peptide linker and the TNF mutant is linked to the C-terminus of the VL domain of the scFv via a peptide linker.

34. The conjugate according to any one of claims 1 to 32, wherein the antibody molecule is, or comprises, a single chain Fv (scFv) and, wherein the TNF mutant is linked to the N-terminus of the VH domain of the scFv via a peptide linker and the IL2 is linked to the C-terminus of the VL domain of the scFv via a peptide linker.

35. The conjugate according to any one of claims 1 to 32, wherein the antibody molecule is, or comprises, a single chain Fv (scFv) and, wherein the IL2 and the TNF mutant are linked to C-terminus of the VL domain of the scFv via a peptide linker or the IL2 and the TNFα are linked to the N-terminus of the scFv via a peptide linker.

36. The conjugate according to any one of claims 33, 34 or 35, wherein the peptide linker is 10 to 20 amino acids long.

37. The conjugate according to any one of claims 1 to 22, wherein the conjugate comprises the amino acid sequence of SEQ ID NO: 1 with an R to A mutation at position 432 or the amino acid sequence of SEQ ID NO: 16 with an R to A mutation at position 452.

38. The conjugate according to any one of claims 1 to 22, wherein the conjugate comprises the amino acid sequence of SEQ ID NO: 36, SEQ ID NO: 38 or a variant thereof.

39. The conjugate according to any one of claims 1 to 17 and claims 23 to 27, wherein the conjugate comprises the amino acid sequence of SEQ ID NO: 70 with an R to A mutation at position 430 or the amino acid sequence of SEQ ID NO: 71 with an R to A mutation at position 450.

40. The conjugate according to any one of claims 1 to 17 and claims 23 to 27, wherein the conjugate comprises the amino acid sequence of SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45 or a variant thereof.

41. The conjugate according to any one of claims 1 to 16 and claims 28 to 32, wherein the conjugate comprises the amino acid sequence of SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 47, SEQ ID NO: 48 or a variant thereof.

42. A nucleic acid molecule encoding a conjugate according to any one of claims 1 to 41.

43. An expression vector comprising the nucleic acid of claim 42.

44. A host cell comprising the vector of claim 43.

45. A method of producing a conjugate according to any one of claims 1 to 41, the method comprising culturing the host cell of claim 44 under conditions for expression of the conjugate.

46. The method of claim 45 further comprising isolating and/or purifying the conjugate.

47. The conjugate according to any one of claims 1 to 41 for use in a method of treating cancer by targeting IL2 and TNF to the neovasculature *in vivo*.

48. The conjugate according to any one of claims 1 to 41 for use in a method of delivering IL2 and TNF to the tumour neovasculature in a patient.

49. A method of treating cancer by targeting IL2 and TNF to the neovasculature in a patient, the method comprising administering a therapeutically effective amount of a conjugate according to any of claims 1 to 41 to the patient.

50. A method of delivering IL2 and TNF to the tumour neovasculature in a patient comprising administering to the patient a conjugate according to any one of claims 1 to 41.

51. The conjugate for use according to claim 47, or the method according to claim 49, wherein the cancer is a melanoma, head and neck cancer, kidney cancer, or a sarcoma.

52. The conjugate for use according to claim 48, or the method according to claim 50, wherein the tumour is the result of a melanoma, head and neck cancer, kidney cancer, or a sarcoma.

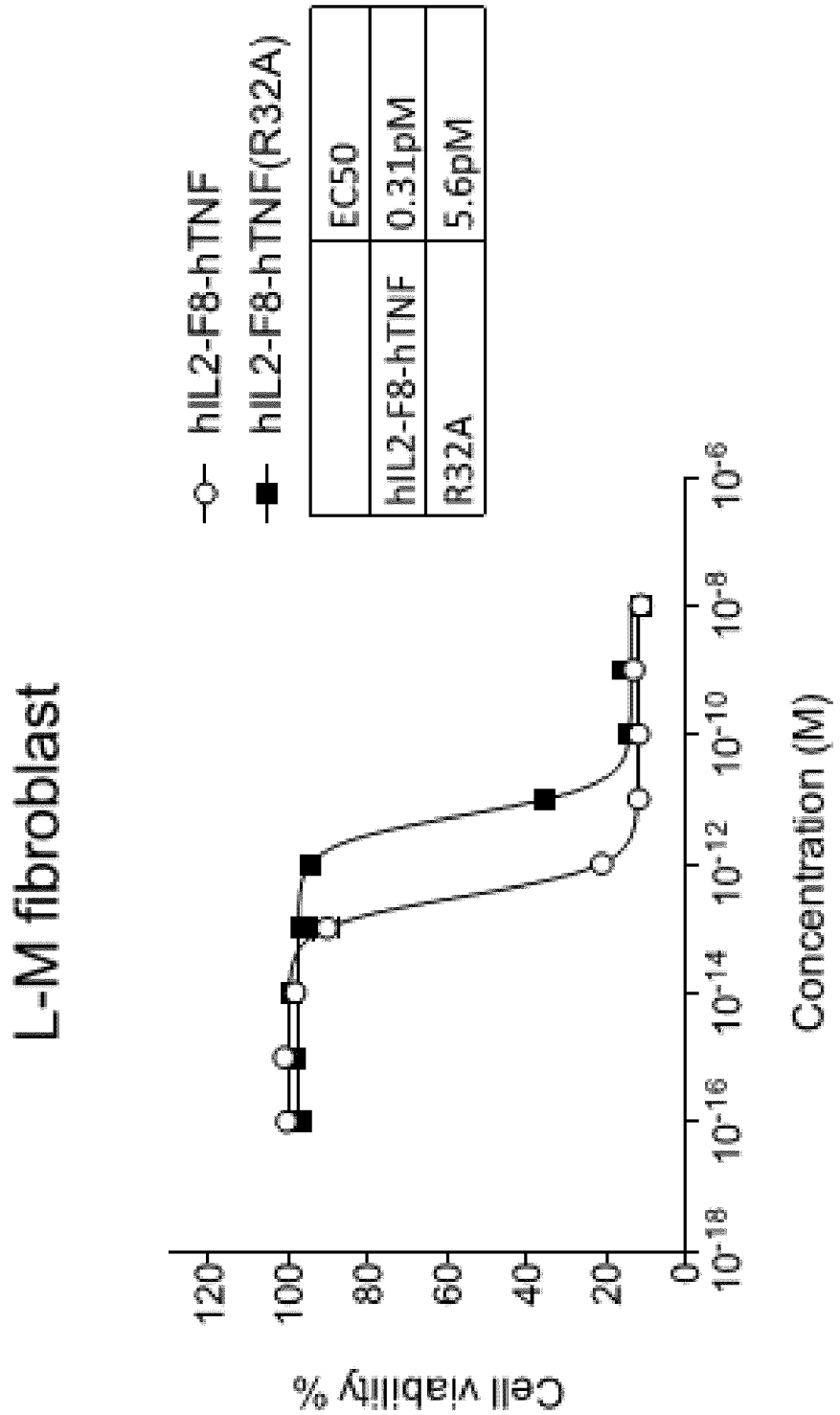
L-M fibroblast
hIL2-F8-hTNF
hIL2-F8-hTNF(R32A)
EC50
hIL2-F8-hTNF 0.31pM
R32A 5.6pM
Cell viability %
120
100
80
60
40
20
0
10^{-18}
10^{-16}
10^{-14}
10^{-12}
10^{-10}
10^{-8}
10^{-6}
Concentration (M)

FIGURE 1

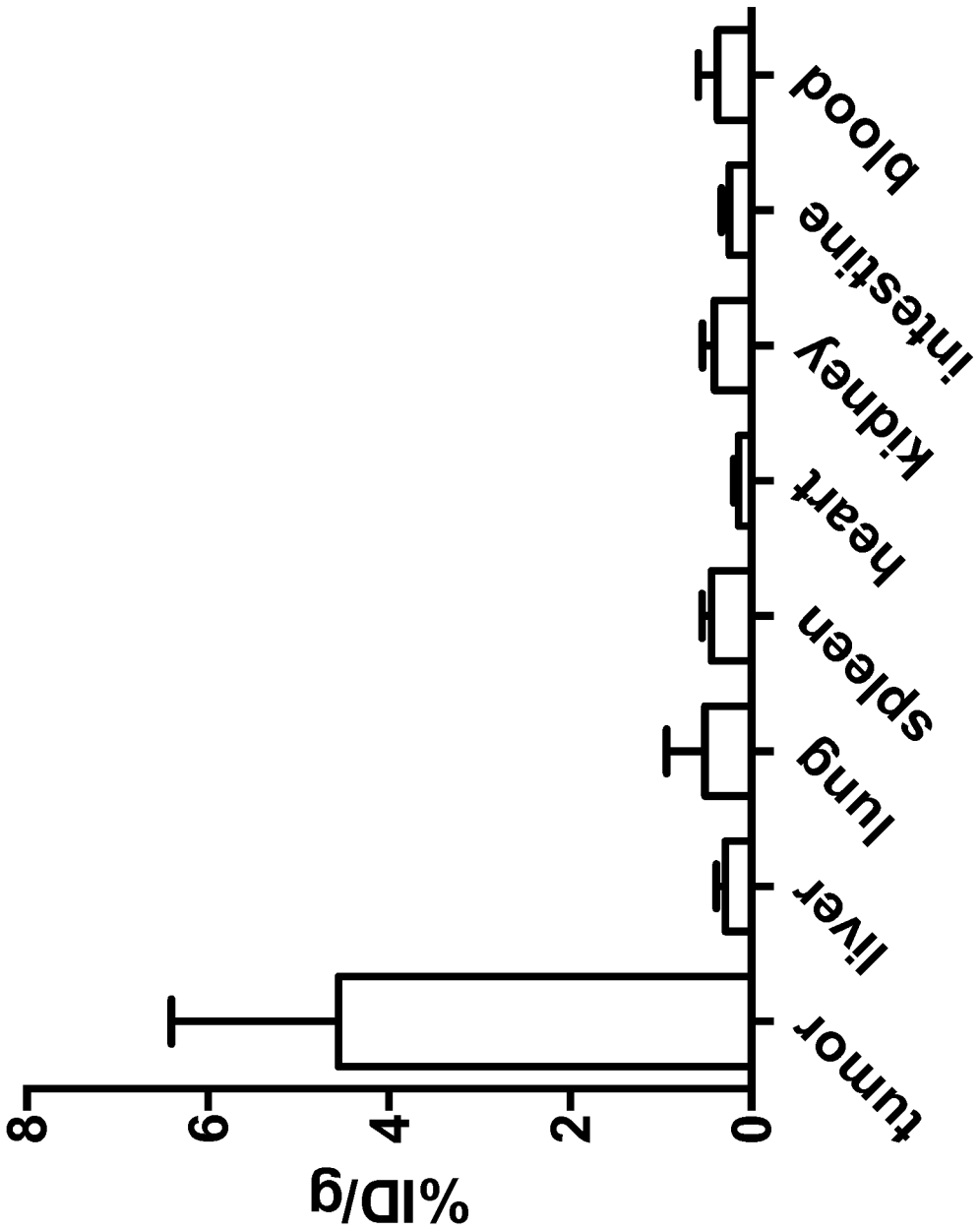
%ID/g
0
2
4
6
8
tumor
liver
lung
spleen
heart
kidney
intestine
blood

FIGURE 2

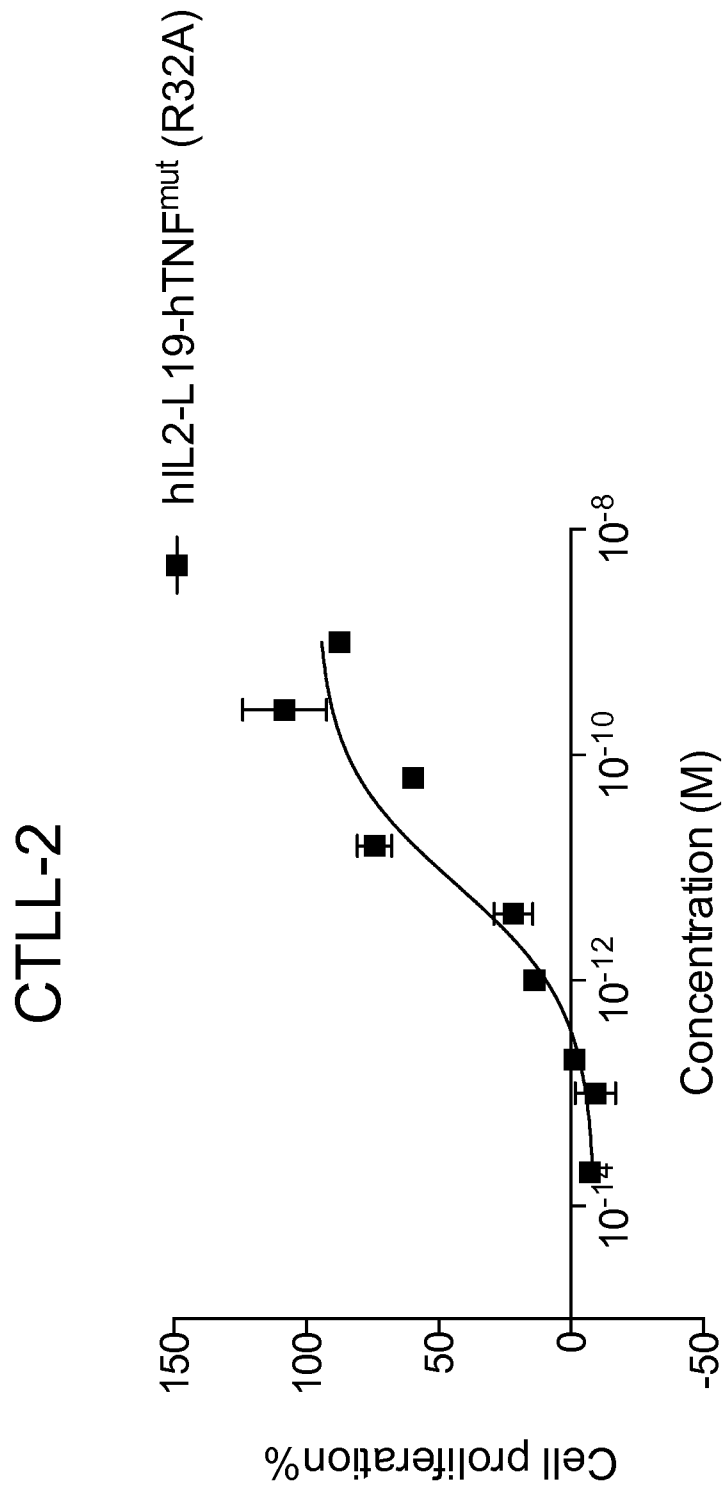
CTLL-2
hIL2-L19-hTNFmut (R32A)
Cell proliferation%
150
100
50
0
-50
10-14
10-12
10-10
10-8
Concentration (M)

Figure 3

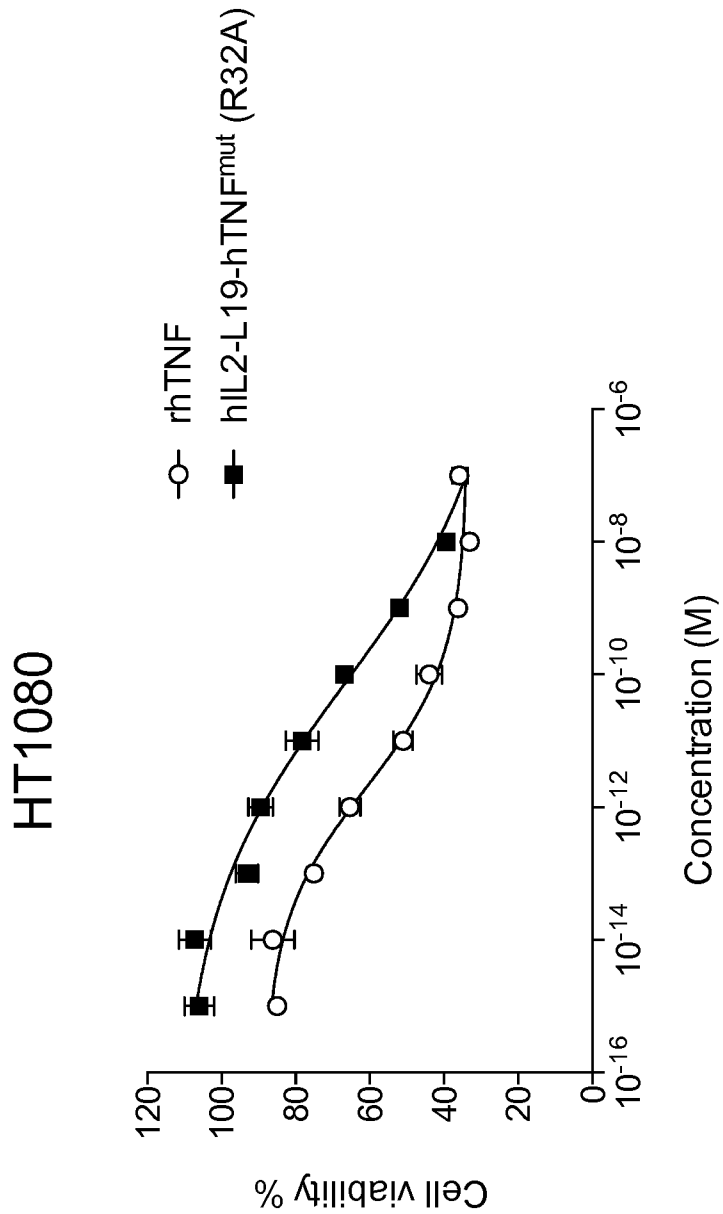
HT1080
rhTNF
hIL2-L19-hTNFmut (R32A)
Cell viability %
120
100
80
60
40
20
0
10-16
10-14
10-12
10-10
10-8
10-6
Concentration (M)

Figure 4

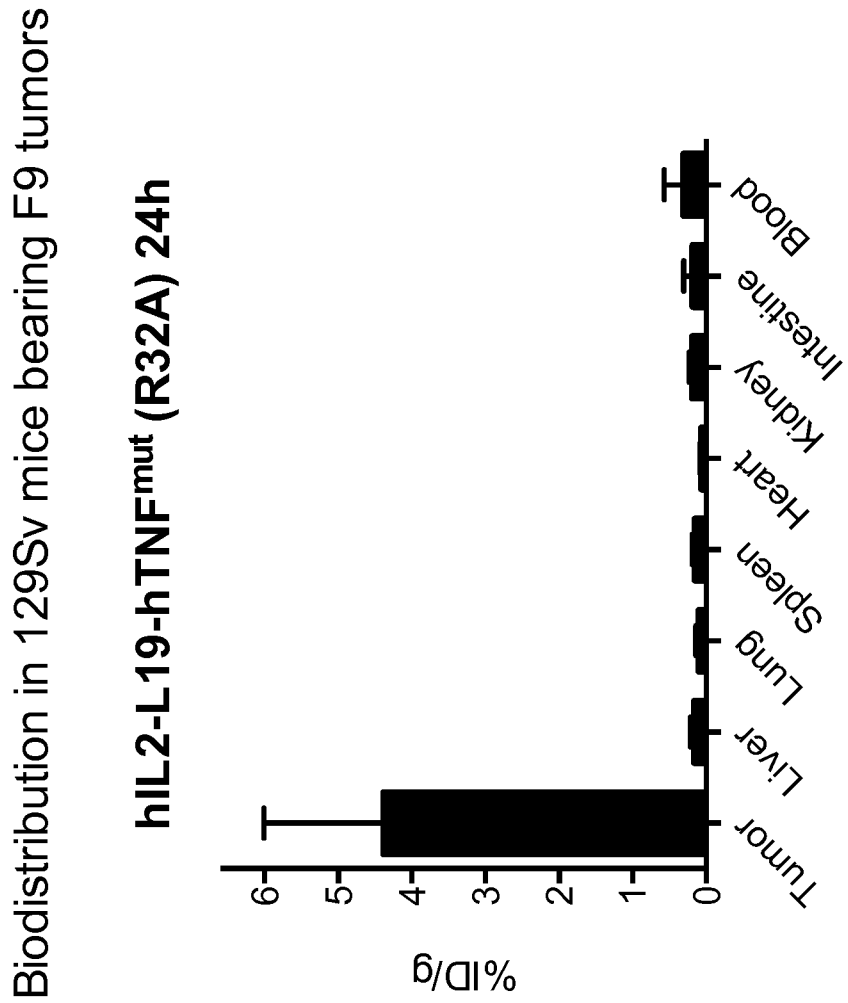
Biodistribution in 129Sv mice bearing F9 tumors
hIL2-L19-hTNFmut (R32A) 24h
%ID/g
0
1
2
3
4
5
6
Tumor
Liver
Lung
Spleen
Heart
Kidney
Intestine
Blood
n = 3
incorporation rate: 18.7%
injected dose: 10 µg/mice

Figure 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/078652

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/525 C07K14/55 C07K16/18 A61K38/20 A61K38/19
A61K47/66 C07K19/00 A61P35/00
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PRETTO F ET AL: "Preclinical evaluation of IL2-based immunocytokines supports their use in combination with dacarbazine, paclitaxel and TNF-based immunotherapy", CANCER IMMUNOLOGY, IMMUNOTHERAPY, vol. 63, no. 9, 1 September 2014 (2014-09-01), pages 901-910, XP002760021, SPRINGER, BERLIN/HEIDELBERG ISSN: 0340-7004, DOI: 10.1007/S00262-014-1562-7 [retrieved on 2014-06-04] figure 3 -----	1-36,38, 40-52
X	WO 2013/045125 A1 (PHILOGEN SPA [IT]; SCHWAGER KATHRIN [CH]) 4 April 2013 (2013-04-04) example 1 ----- -/--	1-36,38, 40-52

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 2017	24/01/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Brouns, Gaby

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; April 1996 (1996-04), SHINGAROVA L N ET AL: "[Human tumor necrosis factor mutants: preparation and some properties].", XP002776913, Database accession no. NLM8768260 abstract & SHINGAROVA L N ET AL: "[Human tumor necrosis factor mutants: preparation and some properties].", BIOORGANICHESKAIA KHIMIIA APR 1996, vol. 22, no. 4, April 1996 (1996-04), pages 243-251, ISSN: 0132-3423 -----	1-52
A	OSTADE X VAN ET AL: "Localization of the active site of human tumour necrosis factor (hTNF) by mutational analysis", EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 10, no. 4, 1 April 1991 (1991-04-01), pages 827-836, XP000293038, WILEY, DE ISSN: 0261-4189 cited in the application table I -----	1-52
A	T. LIST ET AL: "A Chemically Defined Trifunctional Antibody-Cytokine-Drug Conjugate with Potent Antitumor Activity", MOLECULAR CANCER THERAPEUTICS, vol. 13, no. 11, 9 September 2014 (2014-09-09), pages 2641-2652, XP055289329, US ISSN: 1535-7163, DOI: 10.1158/1535-7163.MCT-14-0599 ----- -/--	1-52

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NERI DARIO ET AL: "Immunocytokines for cancer treatment: past, present and future", CURRENT OPINION IN IMMUNOLOGY, vol. 40, 6 April 2016 (2016-04-06), pages 96-102, XP029551352, ELSEVIER, OXFORD, GB ISSN: 0952-7915, DOI: 10.1016/J.COI.2016.03.006 the whole document	1-52
A	NATALIA NUÑEZ-PRADO ET AL: "The coming of age of engineered multivalent antibodies", DRUG DISCOVERY TODAY, vol. 20, no. 5, 1 May 2015 (2015-05-01), pages 588-594, XP055225083, AMSTERDAM, NL ISSN: 1359-6446, DOI: 10.1016/j.drudis.2015.02.013 the whole document	1-52
A	WO 2015/007903 A1 (VIB VZW [BE]; UNIV GENT [BE]; CENTRE NAT RECH SCIENT [FR]; UNIVERSITÉ) 22 January 2015 (2015-01-22)	1-52
X,P	WO 2016/180715 A1 (PHILOGEN SPA [IT]) 17 November 2016 (2016-11-17) the whole document	1-36,38, 40-52
T	ROBERTO DE LUCA ET AL: "Potency-matched Dual Cytokine-Antibody Fusion Proteins for Cancer Therapy", MOLECULAR CANCER THERAPEUTICS, vol. 16, no. 11, 1 November 2017 (2017-11-01), pages 2442-2451, XP055436538, US ISSN: 1535-7163, DOI: 10.1158/1535-7163.MCT-17-0211 the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/078652

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		SG 11201600168U A	26-02-2016
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