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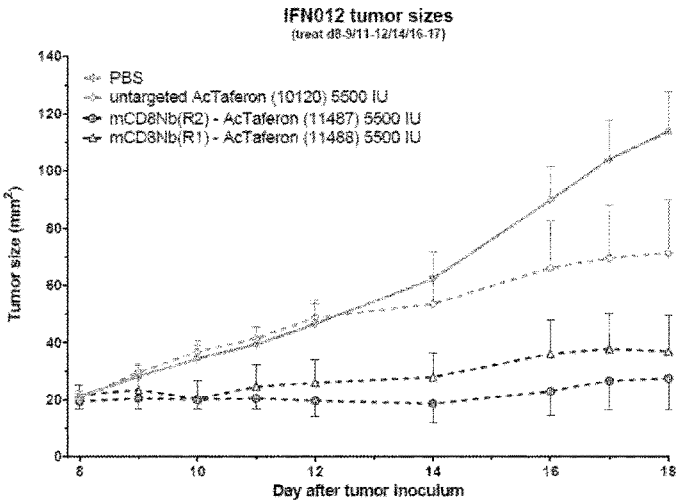
[Continued on next page]

(54) **Title**: CD8 BINDING AGENTS

FIGURE 3



(57) **Abstract**: The present invention relates, in part, to agents that bind CD8 and their use as therapeutic and diagnostic agents. The present invention further relates to pharmaceutical compositions comprising the CD8 binding agents and their use in the treatment of various diseases, including, for example, cancers.

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CD8 BINDING AGENTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Nos. 62/291,769, filed February 5, 2016; 62/335,880, filed May 13, 2016; 62/411,805, filed October 24, 2016; 62/291,772, filed February 5, 2016; 62/291,774, filed February 5, 2016; 62/335,965, filed May 13, 2016; 62/291,776, filed February 5, 2016; 62/335,968, filed May 13, 2016; 62/335,979, filed May 13, 2016; 62/336,030, filed May 13, 2016, 62/353,607, filed June 23, 2016; and 62/291,779, filed February 5, 2016, the entire contents of all of which are herein incorporated by reference.

FIELD

The present invention relates, in part, to binding agents (*e.g.*, antibodies, such as, without limitation, VHHs) which bind CD8 and their use as therapeutic and diagnostic agents.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: ORN-011PC_Sequence_listing date recorded: February 1, 2017; file size: 265 KB).

BACKGROUND

Despite major advances in medicine, cancer remains one of the leading causes of death globally, with an estimated 12.7 million cases each year. One of the major stumbling blocks in designing effective anticancer therapy is cancer immune evasion, in which cancer cells evade immune surveillance and destruction thereby resulting in clinically overt cancer. Mechanisms of immune evasion include the selection of tumor variants resistant to immune effectors and the progressive formation of an immune suppressive environment within the tumor.

$CD8^+$ T lymphocytes (also known as cytotoxic T cells or CTLs) play an important role in host defense against a wide gamut of viral, protozoan and intracellular bacterial pathogens and are important effectors in antitumor immunity. Generally, CD8+ CTLs curb cancer development by mechanisms including production of interferon (IFN)-γ and cytotoxins, exocytosis of lytic proteins (*e.g.*, perforin, granzymes), and receptor-ligand binding of FAS molecules. However, tumors can evade immune surveillance by crippling CTL functionality via, for instance, production of immune suppressive cytokines and engagement of immune checkpoint inhibition, either by the cancer cells themselves or by non-cancerous cells present in the tumor microenvironment. Further still, cancer cells have been shown to delete CTLs through apoptosis.

Current treatments for cancer include chemotherapy, radiation therapy, immunotherapy, targeted therapy, and surgery which all have limitations and detrimental side effects.

Furthermore, there are a number of non-oncology indications that are effected by the immune system, such as autoimmune diseases, have limited treatment options that do not provide desirable therapeutic effects.

Accordingly, there remains a need for improved immunotherapeutic agents, including, for example, those that can effectively derail tumor evasion and enhance anti-tumor immunity as mediated, for example, by CTLs.

SUMMARY

In various aspects, the present invention relates to CD8 binding agents having at least one targeting moiety that specifically binds to CD8. In various embodiments, these CD8 binding agents bind to, but do not functionally modulate (including, without limitation, partially or fully neutralizing) CD8. Therefore, in various embodiments, the present CD8 binding agents have use in, for instance, recruiting a CD8-expressing cell to a site of interest while still allowing the CD8-expressing cell to signal via CD8 (*i.e.* the binding of the CD8 binding agent does not reduce or eliminate CD8 signaling at the site of interest). In an embodiment, the targeting moiety is a single domain antibody (NANOBODY or VHH). In various embodiments, the CD8 binding agent further comprises a signaling agent, *e.g.*, without limitation, an interferon, an interleukin, and a tumor necrosis factor, that may be modified to attenuate activity. In various embodiments, the CD8 binding agent comprises additional targeting moieties that bind to other antigens of interest. In an embodiment, the other antigens of interest are present on tumor cells. In another embodiment, the other antigens of interest are present on immune cells. In these embodiments, the present CD8 binding agent may directly or indirectly recruit an immune cell, *e.g.* an immune cell that can kill and/or suppress a tumor cell (*e.g.*, cytotoxic T cells), to a site of action (such as, by way of non-limiting example, the tumor microenvironment).

In various embodiments, the present CD8 binding agents find use in the treatment of various diseases or disorders such as cancer, infections, immune disorders, autoimmune diseases, and other diseases and disorders, and the present invention encompasses various methods of treatment.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1, panel A, provides histograms showing the binding of six VHHs specific for mouse CD8 to CHO cells transfected with CD8α. Panel B provides histograms showing mouse splenocytes stained with six VHHs specific for mouse CD8. The relative binding affinities of the six VHHs are as follows: I-11269 > I-11265 and I-11268> I-11278 and I-11287.

Figure 2 provides histograms depicting the effects of various VHHs specific for mouse CD8 on OVA-induced T cell proliferation and activation.

Figure 3 shows that the administration of a fusion of a VHH specific for mouse CD8 with a modified human interferon (Q124R mutant) led to reductions in tumor size. mCD8Nb(R1) corresponds to clone R1CDE28 and mCD8Nb(R2) corresponds to clone R2CDE71.

Figure 4 shows that the administration of a fusion of a VHH specific for mouse CD8 with a modified human interferon (Q124R mutant) did not result in weight loss or hematological toxicity. In all panels, the histograms

from left to right are: naive mice, PBS, untargeted chimera (*i.e.* a modified IFN, Q124R, targeting moiety for an irrelevant target), and two CD8 VHHs fused to a modified human IFN (Q124R). mCD8Nb(R1) corresponds to clone R1CDE28 and mCD8Nb(R2) corresponds to clone R2CDE71.

Figure 5 depicts the binding characteristics of anti-human CD8 VHHs on HekT cells.

Figure 6, panels A-C, depict the binding characteristics of anti-human CD8 VHHs. Panel A shows cellular florescence as detected by flow cytometry. Panel B shows median fluorescence intensity (MFI), which was calculated for five VHH dilutions and compared to binding obtained with a control VHH. Panel C shows the percentage of human peripheral blood mononuclear cells (PMBCs) that bind the CD8 VHH (His+) among CD3- antigen presenting cells. Calculation was performed for five VHH dilutions and compared to binding obtained with the control VHH.

Figure 7 shows a mouse tumor growth study in which C57BL/6 mice were inoculated subcutaneously with B16 melanoma tumor cells. Perilesional treatment with the indicated treatment agents was started when tumors reached certain size as measured by caliper. Graph shows the evolution of tumor size over the indicated time.

Figure 8 shows a mouse tumor growth study in which mice were inoculated with 4T1 mammary tumor cells. The mice were treated with the indicated agents. Graphs show the evolution of tumor size over the indicated time.

Figure 9 shows a mouse tumor growth study in which mice were inoculated with 4T1 mammary tumor cells. The mice were then treated with the indicated agents with or without doxorubicin. Graphs show the evolution of tumor size over the indicated time.

Figure 10 shows, in panels A, B, and C, human CD8 targeting of mono-specific chimeras. Zebra-plot of CD8 versus pSTAT1 staining of stimulated PBMCs is shown in panel A. Panels B and C: mean fluorescent intensities (MFI) of pSTAT1 staining of CD8-positive (panel B) or CD8-negative (panel C) are plotted (in both panels B and C, the order of the curves at X-axis point 100 is: anti-human CD8 VHH/human IFN R149A, anti-BclI10 VHH/human IFN R149A, and anti-human CD8 VHH/human IFN R33A/E120A).

DETAILED DESCRIPTION

The present invention is based, in part, on the discovery of agents (*e.g.* antibodies such as, by way of non-limiting example, VHHs) that recognize and bind to CD8. In various embodiments, these CD8 binding agents bind to, but do not functionally modulate CD8. In various embodiments, these CD8 binding agents may bind and directly or indirectly recruit immune cells to sites in need of therapeutic action (*e.g.* a tumor). The present invention provides pharmaceutical compositions comprising the CD8 binding agents and their use in the treatment of various diseases.

CD8 Binding Agents

In various embodiments, the present CD8 binding agent is a protein-based agent capable of specific binding to CD8. In various embodiments, the present CD8 binding agent is a protein-based agent capable of specific binding to CD8 without functionally modulation (*e.g.* partial or complete neutralization) of CD8. CD8 is a

heterodimeric type I transmembrane glycoprotein, whose α and β chains are both composed of an immunoglobulin (Ig)-like extracellular domain connected by an extended O-glycosylated stalk to a single-pass transmembrane domain and a short cytoplasmic tail (Li *et al.*, 2013). The cytoplasmic region of the α-chain contains two cysteine motifs that serve as a docking site for src tyrosine kinase p56lck (Lck). In contrast, this Lck binding domain appears to be absent from the β chain, suggesting that the CD8 β chain is not involved in downstream signaling (Artyomov *et al.*, 2010). CD8 functions as a co-receptor for the T-cell receptor with its principle role being the recruitment of Lck to the TCR-pMHC complex following co-receptor binding to MHC (Turner *et al.*, 1990, Veillette *et al.*, 1988). The increase in the local concentration of this kinase activates a signaling cascade that recruits and activates ζ-chain-associated protein kinase 70 (ZAP-70), subsequently leading to the amplification or enhancement of T-cell activation signals (Purbhoo *et al.*, 2001, Laugel *et al.*, 2007a).

In various embodiments, the CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that recognizes an epitope present on the CD8 α and/or β chains. In an embodiment, the antigen-recognition domain recognizes one or more linear epitopes on the CD8 α and/or β chains. As used herein, a linear epitope refers to any continuous sequence of amino acids present on the CD8 α and/or β chains. In another embodiment, the antigen-recognition domain recognizes one or more conformational epitopes present on the CD8 α and/or β chains. As used herein, a conformation epitope refers to one or more sections of amino acids (which may be discontinuous) which form a three-dimensional surface with features and/or shapes and/or tertiary structures capable of being recognized by an antigen recognition domain.

In various embodiments, the CD8 binding agent of the present invention may bind to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants of human CD8 α and/or β chains. In various embodiments, the CD8 binding agent of the invention may bind to any forms of the human CD8 α and/or β chains, including monomeric, dimeric, heterodimeric, multimeric and associated forms. In an embodiment, the CD8 binding agent binds to the monomeric form of CD8 α chain or CD8 β chain. In another embodiment, the CD8 binding agent binds to a homodimeric form comprised of two CD8 α chains or two CD8 β chains. In a further embodiment, the CD8 binding agent binds to a heterodimeric form comprised of one CD8 α chain and one CD8 β chain.

In an embodiment, the present CD8 binding agent comprises a targeting moiety with an antigen recognition domain that recognizes one or more epitopes present on the human CD8 α chain. In an embodiment, the human CD8 α chain comprises the amino acid sequence of:

Isoform 1 (SEQ ID NO:1)

MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPT
SGCSWLFQPRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLT
LSDFRRENEGYYFCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIA

SQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLY
CNHRNRRRVCKCPRPVVKSGDKPSLSARYV.

In an embodiment, the human CD8 α chain comprises the amino acid sequence of:

Isoform 2 (SEQ ID NO:2)

MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPT
SGCSWLFQPRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLT
LSDFRRENEGYYFCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIA
SQPLSLRPEACRPAAGGAGNRRRVCKCPRPVVKSGDKPSLSARYV.

In an embodiment, the human CD8 α chain comprises the amino acid sequence of:

Isoform 3 (SEQ ID NO:3)

MRNQAPGRPKGATFPPRRPTGSRAPPLAPELRAKQRPGERVMALPVTALL
LPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQ
PRGAAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENE
GYYFCSALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPE
ACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRRR
VCKCPRPVVKSGDKPSLSARYV.

In an embodiment, the present CD8 binding agent comprises a targeting moiety with an antigen recognition domain that recognizes one or more epitopes present on the human CD8 β chain. In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 1 (SEQ ID NO:4)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGPLCSPITLGLLVAGVLVLLVSLGVAIHLCCRRRRARLRFMKQ
FYK.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 2 (SEQ ID NO:5)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGPLCSPITLGLLVAGVLVLLVSLGVAIHLCCRRRRARLRFMKQ
LRLHPLEKCSRMDY.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 3 (SEQ ID NO:6)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGRRRRARLRFMKQPQGEGISGTFVPQCLHGYYSNTTTSQKL
LNPWILKT.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 4 (SEQ ID NO:7)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGPLCSPITLGLLVAGVLVLLVSLGVAIHLCCRRRRARLRFMKQ
KFNIVCLKISGFTTCCCFQILQISREYGFGVLLQKDIGQ.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 5 (SEQ ID NO:8)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGPLCSPITLGLLVAGVLVLLVSLGVAIHLCCRRRRARLRFMKQ
PQGEGISGTFVPQCLHGYYSNTTTSQKLLNPWILKT.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 6 (SEQ ID NO:9)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGRRRRARLRFMKQFYK.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 7 (SEQ ID NO:10)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC

RLPRPETQKDFTNKQRIGFWCPATKRHRSVMSTMWKNERRDTFNPGEFN
GC.

In an embodiment, the human CD8 β chain comprises the amino acid sequence of:

Isoform 8 (SEQ ID NO:11)

MRPRLWLLLAAQLTVLHGNSVLQQTPAYIKVQTNKMVMLSCEAKISLSNMR
IYWLRQRQAPSSDSHHEFLALWDSAKGTIHGEEVEQEKIAVFRDASRFILNL
TSVKPEDSGIYFCMIVGSPELTFGKGTQLSVVDFLPTTAQPTKKSTLKKRVC
RLPRPETQKGLKGKVYQEPLSPNACMDTTAILQPHRSCLTHGS.

In various embodiments, the present CD8 binding agent comprises a targeting moiety capable of specific binding. In various embodiments, the CD8 binding agent comprises a targeting moiety having an antigen recognition domain such as an antibody or derivatives thereof. In an embodiment, the CD8 binding agent comprises a targeting moiety which is an antibody. In various embodiments, the antibody is a full-length multimeric protein that includes two heavy chains and two light chains. Each heavy chain includes one variable region (*e.g.*, V_H) and at least three constant regions (*e.g.*, CH_1, CH_2 and CH_3), and each light chain includes one variable region (V_L) and one constant region (C_L). The variable regions determine the specificity of the antibody. Each variable region comprises three hypervariable regions also known as complementarity determining regions (CDRs) flanked by four relatively conserved framework regions (FRs). The three CDRs, referred to as CDR1, CDR2, and CDR3, contribute to the antibody binding specificity. In some embodiments, the antibody is a chimeric antibody. In some embodiments, the antibody is a humanized antibody.

In some embodiments, the CD8 binding agent comprises a targeting moiety which is an antibody derivative or format. In some embodiments, the present CD8 binding agent comprises a targeting moiety which is a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein (cysteine knot protein, knottin), a DARPin; a Tetranectin; an Affibody; a Transbody; an Anticalin; an AdNectin; an Affilin; an Affimer, a Microbody; a peptide aptamer; an alterase; a plastic antibody; a phylomer; a stradobody; a maxibody; an evibody; a fynomer, an armadillo repeat protein, a Kunitz domain, an avimer, an atrimer, a probody, an immunobody, a triomab, a troybody; a pepbody; a vaccibody, a UniBody; a DuoBody, a Fv, a Fab, a Fab', a $F(ab')_2$, a peptide mimetic molecule, or a synthetic molecule, as described in US Patent Nos. or Patent Publication Nos. US 7,417,130, US 2004/132094, US 5,831,012, US 2004/023334, US 7,250,297, US 6,818,418, US 2004/209243, US 7,838,629, US 7,186,524, US 6,004,746, US 5,475,096, US 2004/146938, US 2004/157209, US 6,994,982, US 6,794,144, US 2010/239633, US 7,803,907, US 2010/119446, and/or US 7,166,697, the contents of which are hereby incorporated by reference in their entireties. *See* also, Storz MAbs. 2011 May-Jun; 3(3): 310–317.

In some embodiments, the CD8 binding agent comprises a targeting moiety which is a single-domain antibody, such as a VHH. The VHH may be derived from, for example, an organism that produces VHH antibody such as a camelid, a shark, or the VHH may be a designed VHH. VHHs are antibody-derived therapeutic proteins that

contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. VHH technology is based on fully functional antibodies from camelids that lack light chains. These heavy-chain antibodies contain a single variable domain (V_HH) and two constant domains (CH2 and CH3). VHHs are commercially available under the trademark of NANOBODY or NANOBODIES. In an embodiment, the CD8 binding agent comprises a VHH.

In some embodiments, the CD8 binding agent comprises a targeting moiety which is a VHH comprising a single amino acid chain having four "framework regions" or FRs and three "complementary determining regions" or CDRs. As used herein, "framework region" or "FR" refers to a region in the variable domain which is located between the CDRs. As used herein, "complementary determining region" or "CDR" refers to variable regions in VHHs that contains the amino acid sequences capable of specifically binding to antigenic targets.

In various embodiments, the CD8 binding agent comprises a VHH having a variable domain comprising at least one CDR1, CDR2, and/or CDR3 sequences.

In some embodiments, the CDR1 sequence is selected from:

GFTFDDYAMS (SEQ ID NO:12) or

GFTFDDYAIG (SEQ ID NO:13).

In some embodiments, the CDR2 sequence is selected from:

TINWNGGSAEYAEPVKG (SEQ ID NO:14) or

CIRVSDGSTYYADPVKG (SEQ ID NO:15).

In some embodiments, the CDR3 sequence is selected from:

KDADLVWYNLS (SEQ ID NO:16) or

KDADLVWYNLR (SEQ ID NO:17) or

AGSLYTCVQSIVVVPARPYYDMDY (SEQ ID NO:18).

In various embodiments, the CD8 binding agent comprises SEQ ID NO:12, SEQ ID NO:14, and SEQ ID NO:16.
In various embodiments, the CD8 binding agent comprises SEQ ID NO:12, SEQ ID NO:14, and SEQ ID NO:17.
In various embodiments, the CD8 binding agent comprises SEQ ID NO:12, SEQ ID NO:14, and SEQ ID NO:18.

In various embodiments, the CD8 binding agent comprises SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO:16.
In various embodiments, the CD8 binding agent comprises SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO:17.
In various embodiments, the CD8 binding agent comprises SEQ ID NO:12, SEQ ID NO:15, and SEQ ID NO:18.

In various embodiments, the CD8 binding agent comprises SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:16.
In various embodiments, the CD8 binding agent comprises SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:17.
In various embodiments, the CD8 binding agent comprises SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:18.

In various embodiments, the CD8 binding agent comprises SEQ ID NO:13, SEQ ID NO:15, and SEQ ID NO:16. In various embodiments, the CD8 binding agent comprises SEQ ID NO:13, SEQ ID NO:15, and SEQ ID NO:17. In various embodiments, the CD8 binding agent comprises SEQ ID NO:13, SEQ ID NO:15, and SEQ ID NO:18.

In various embodiments, the CD8 binding agent comprises an amino acid sequence selected from the following sequences:

R3HCD27 (SEQ ID NO:19)

QVQLQESGGGSVQPGGSLRLSCAASGFTFDDYAMSWVRQVPGKGLEWV
STINWNGGSAEYAEPVKGRFTISRDNAKNTVYLQMNSLKLEDTAVYYCAK
DADLVWYNLSTGQGTQVTVSSAAAYPYDVPDYGS

or

R3HCD129 (SEQ ID NO:20)

QVQLQESGGGLVQPGGSLRLSCAASGFTFDDYAMSWVRQVPGKGLEWV
STINWNGGSAEYAEPVKGRFTISRDNAKNTVYLQMNSLKLEDTAVYYCAK
DADLVWYNLRTGQGTQVTVSSAAAYPYDVPDYGS

or

R2HCD26 (SEQ ID NO:21)

QVQLQESGGGLVQAGGSLRLSCAASGFTFDDYAIGWFRQAPGKEREGVS
CIRVSDGSTYYADPVKGRFTISSDNAKNTVYLQMNSLKPEDAAVYYCAAGS
LYTCVQSIVVVPARPYYDMDYWGKGTQVTVSSAAAYPYDVPDYGS.

In various embodiments, the CD8 binding agent comprises an amino acid sequence described in US Patent Publication No. 2014/0271462, the entire contents of which are incorporated by reference. In various embodiments, the CD8 binding agent comprises an amino acid sequence described in Table 0.1, Table 0.2, Table 0.3, and/or Figures 1A-12I of US Patent Publication No. 2014/0271462, the entire contents of which are incorporated by reference. In various embodiments, the CD8 binding agent comprises a HCDR1 of a HCDR1 of SEQ ID NO: 22 or 23 and/or a HCDR2 of HCDR1 of SEQ ID NO: 22 or 23 and/or a HCDR3 of HCDR1 of SEQ ID NO: 22 or 23 and/or a LCDR1 of LCDR1 of SEQ ID NO: 24 and/or a LCDR2 of LCDR1 of SEQ ID NO: 24 and/or a LCDR3 of LCDR1 of SEQ ID NO: 24.

SEQ ID NO: 22:

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

Ala Val Tyr Tyr Cys Gly Arg Gly Tyr Gly Tyr Tyr Val Phe Asp His Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser.

SEQ ID NO: 23:

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

SEQ ID NO: 24:

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

In various embodiments, the present invention contemplates the use of any natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as "analogs") of the CD8 binding agent of the invention as described herein. In various embodiments, the amino acid sequence of the CD8 binding agent further includes an amino acid analog, an amino acid derivative, or other non-classical amino acids.

In various embodiments, the CD8 binding agent comprises a targeting moiety comprising a sequence that is at least 60% identical to any one of SEQ ID NOs: 12-24. For example, the CD8 binding agent may comprise a targeting moiety comprising a sequence that is at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to SEQ ID NOs: 12-24 (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or

about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, about 99% or about 100% sequence identity to any one of SEQ ID NOs: 12-24).

In various embodiments, the CD8 binding agent comprises a targeting moiety comprising an amino acid sequence having one or more amino acid mutations with respect to SEQ ID NOs: 12-24. In various embodiments, the CD8 binding agent comprises a targeting moiety comprising an amino acid sequence having one, or two, or three, or four, or five, or six, or seen, or eight, or nine, or ten, or fifteen, or twenty amino acid mutations with respect to SEQ ID NOs: 12-24. In some embodiments, the one or more amino acid mutations may be independently selected from substitutions, insertions, deletions, and truncations.

In some embodiments, the amino acid mutations are amino acid substitutions, and may include conservative and/or non-conservative substitutions.

"Conservative substitutions" may be made, for instance, on the basis of similarity in polarity, charge, size, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the amino acid residues involved. The 20 naturally occurring amino acids can be grouped into the following six standard amino acid groups: (1) hydrophobic: Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic: Cys, Ser, Thr; Asn, Gln; (3) acidic: Asp, Glu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; and (6) aromatic: Trp, Tyr, Phe.

As used herein, "conservative substitutions" are defined as exchanges of an amino acid by another amino acid listed within the same group of the six standard amino acid groups shown above. For example, the exchange of Asp by Glu retains one negative charge in the so modified polypeptide. In addition, glycine and proline may be substituted for one another based on their ability to disrupt α-helices.

As used herein, "non-conservative substitutions" are defined as exchanges of an amino acid by another amino acid listed in a different group of the six standard amino acid groups (1) to (6) shown above.

In various embodiments, the substitutions may also include non-classical amino acids (*e.g.* selenocysteine, pyrrolysine, *N*-formylmethionine β-alanine, GABA and δ-Aminolevulinic acid, 4-aminobenzoic acid (PABA), D-isomers of the common amino acids, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosme, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β methyl amino acids, C α-methyl amino acids, N α-methyl amino acids, and amino acid analogs in general).

In various embodiments, the amino acid mutation may be in the CDRs of the targeting moiety (*e.g.*, the CDR1, CDR2 or CDR3 regions). In another embodiment, amino acid alteration may be in the framework regions (FRs) of the targeting moiety (*e.g.*, the FR1, FR2, FR3, or FR4 regions).

Modification of the amino acid sequences may be achieved using any known technique in the art *e.g.*, site-directed mutagenesis or PCR based mutagenesis. Such techniques are described, for example, in Sambrook *et*

al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y., 1989 and Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., 1989.

In various embodiments, the mutations do not substantially reduce the present CD8 binding agent's capability to specifically bind to CD8. In various embodiments, the mutations do not substantially reduce the present CD8 binding agent's capability to specifically bind to CD8 without functionally modulating CD8.

In various embodiments, the binding affinity of the CD8 binding agent of the invention for the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants (including monomeric, dimeric, heterodimeric, multimeric and/or associated forms) of human CD8 α and/or β chains may be described by the equilibrium dissociation constant (K_D). In various embodiments, the CD8 binding agent comprises a targeting moiety that binds to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants (including monomeric, dimeric, heterodimeric, multimeric and/or associated forms) of human CD8 α and/or β chains with a K_D of less than about 1 uM, about 900 nM, about 800 nM, about 700 nM, about 600 nM, about 500 nM, about 400 nM, about 300 nM, about 200 nM, about 100 nM, about 90 nM, about 80 nM, about 70 nM, about 60 nM, about 50 nM, about 40 nM, about 30 nM, about 20 nM, about 10 nM, or about 5 nM, or about 1 nM.

In various embodiments, the CD8 binding agent comprises a targeting moiety that binds but does not functionally modulate the antigen of interest, *i.e.*, CD8. For instance, in various embodiments, the targeting moiety of the CD8 binding agent simply targets the antigen but does not substantially functionally modulate the antigen, *e.g.*it does not substantially inhibit, reduce or neutralize a biological effect that the antigen has. In various embodiments, the targeting moiety of the CD8 binding agent binds an epitope that is physically separate from an antigen site that is important for its biological activity (*e.g.* an antigen's active site).

Such non-functionally modulating (*e.g.* non-neutralizing) binding finds use in various embodiments of the present invention, including methods in which the present CD8 binding agent is used to directly or indirectly recruit active immune cells to a site of need via an effector antigen. For example, in various embodiments, the present CD8 binding agent may be used to directly or indirectly recruit cytotoxic T cells via CD8 to a tumor cell in a method of reducing or eliminating a tumor (*e.g.* the CD8 binding agent may comprise a targeting moiety having an anti-CD8 antigen recognition domain and a targeting moiety having a recognition domain (*e.g.* an antigen recognition domain) directed against a tumor antigen or receptor). In such embodiments, it is desirable to directly or indirectly recruit CD8-expressing cytotoxic T cells but not to neutralize the CD8 activity. In these embodiments, CD8 signaling is an important piece of the tumor reducing or eliminating effect.

Therapeutic Agents Comprising the Present CD8 Binding Agents

Chimeras and Fusions with Signaling Agents

In various embodiments, the CD8 binding agent of the invention is part of a chimera or fusion with one or more signaling agents. Accordingly, the present invention provides for chimeric or fusion proteins that include, for example, a targeting moiety against CD8 and one or more signaling agents.

In various embodiments, the signaling agent is modified to have reduced affinity or activity for one or more of its receptors, which allows for attenuation of activity (inclusive of agonism or antagonism) and/or prevents non-specific signaling or undesirable sequestration of the chimeric or fusion protein.In various embodiments, the signaling agent is antagonistic in its wild type form and bears one or more mutations that attenuate its antagonistic activity. In various embodiments, the signaling agent is antagonistic due to one or more mutations, *e.g.* an agonistic signaling agent is converted to an antagonistic signaling agent and, such a converted signaling agent, optionally, also bears one or more mutations that attenuate its antagonistic activity (*e.g.* as described in WO 2015/007520, the entire contents of which are hereby incorporated by reference).

Accordingly, in various embodiments, the signaling agent is a modified (*e.g.* mutant) form of the signaling agent having one or more modifications (*e.g.* mutations). In various embodiments, the mutations allow for the modified signaling agent to have one or more of attenuated activity such as one or more of reduced binding affinity, reduced endogenous activity, and reduced specific bioactivity relative to unmutated, *i.e.* the wild type form of the signaling agent (*e.g.* comparing the same signaling agent in a wild type form versus a modified (*e.g.* mutant) form). In some embodiments, the mutations which attenuate or reduce binding or affinity include those mutations which substantially reduce or ablate binding or activity. In some embodiments, the mutations which attenuate or reduce binding or affinity are different than those mutations which substantially reduce or ablate binding or activity. Consequentially, in various embodiments, the mutations allow for the signaling agent to have improved safety, *e.g.* reduced systemic toxicity, reduced side effects, and reduced off-target effects relative to unmutated, *i.e.* wild type, signaling agent (*e.g.* comparing the same signaling agent in a wild type form versus a modified (*e.g.* mutant) form).

As described herein, the agent may have improved safety due to one of more modifications, *e.g.* mutations. In various embodiments, improved safety means that the present chimeric protein provides lower toxicity (*e.g.* systemic toxicity and/or tissue/organ-associated toxicities); and/or lessened or substantially eliminated side effects; and/or increased tolerability, lessened or substantially eliminated adverse events; and/or reduced or substantially eliminated off-target effects; and/or an increased therapeutic window.

In various embodiments, the signaling agent is modified to have one or more mutations that reduce its binding affinity or activity for one or more of its receptors. In some embodiments, the signaling agent is modified to have one or more mutations that substantially reduce or ablate binding affinity or activity for the receptors. In some embodiments, the activity provided by the wild type signaling agent is agonism at the receptor (*e.g.* activation of a cellular effect at a site of therapy). For example, the wild type signaling agent may activate its receptor. In such embodiments, the mutations result in the modified signaling agent to have reduced or ablated activating activity at the receptor. For example, the mutations may result in the modified signaling agent to deliver a reduced

activating signal to a target cell or the activating signal could be ablated. In some embodiments, the activity provided by the wild type signaling agent is antagonism at the receptor (*e.g.* blocking or dampening of a cellular effect at a site of therapy). For example, the wild type signaling agent may antagonize or inhibit the receptor. In these embodiments, the mutations result in the modified signaling agent to have a reduced or ablated antagonizing activity at the receptor. For example, the mutations may result in the modified signaling agent to deliver a reduced inhibitory signal to a target cell or the inhibitory signal could be ablated. In various embodiments, the signaling agent is antagonistic due to one or more mutations, *e.g.* an agonistic signaling agent is converted to an antagonistic signaling agent (*e.g.* as described in WO 2015/007520, the entire contents of which are hereby incorporated by reference) and, such a converted signaling agent, optionally, also bears one or mutations that reduce its binding affinity or activity for one or more of its receptors or that substantially reduce or ablate binding affinity or activity for one or more of its receptors.

In some embodiments, the reduced affinity or activity at the receptor is restorable by attachment with one or more of the targeting moieties as described herein (*e.g.*, targeting moiety against CD8). In other embodiments, the reduced affinity or activity at the receptor is not substantially restorable by the activity of one or more of the targeting moieties.

In various embodiments, the chimeric proteins of the present invention reduce off-target effects because their signaling agents have mutations that weaken or ablate binding affinity or activity at a receptor. In various embodiments, this reduction in side effects is observed relative with, for example, the wild type signaling agents. In various embodiments, the signaling agent is active on target cells because the targeting moiety(ies) compensates for the missing/insufficient binding (*e.g.*, without limitation and/or avidity) required for substantial activation. In various embodiments, the modified signaling agent is substantially inactive *en route* to the site of therapeutic activity and has its effect substantially on specifically targeted cell types which greatly reduces undesired side effects.

In some embodiments, the signaling agent may include one or more mutations that attenuate or reduce binding or affinity for one receptor (*i.e.*, a therapeutic receptor) and one or more mutations that substantially reduce or ablate binding or activity at a second receptor. In such embodiments, these mutations may be at the same or at different positions (*i.e.*, the same mutation or multiple mutations). In some embodiments, the mutation(s) that reduce binding and/or activity at one receptor is different than the mutation(s) that substantially reduce or ablate at another receptor. In some embodiments, the mutation(s) that reduce binding and/or activity at one receptor is the same as the mutation(s) that substantially reduce or ablate at another receptor. In some embodiments, the present chimeric proteins have a modified signaling agent that has both mutations that attenuate binding and/or activity at a therapeutic receptor and therefore allow for a more controlled, on-target therapeutic effect (*e.g.* relative wild type signaling agent) and mutations that substantially reduce or ablate binding and/or activity at another receptor and therefore reduce side effects (*e.g.* relative to wild type signaling agent).

In some embodiments, the substantial reduction or ablation of binding or activity is not substantially restorable with a targeting moiety (*e.g.*, a targeting moiety against CD8 or any other targeting moiety described herein). In some embodiments, the substantial reduction or ablation of binding or activity is restorable with a targeting moiety. In various embodiments, substantially reducing or ablating binding or activity at a second receptor also may prevent deleterious effects that are mediated by the other receptor. Alternatively, or in addition, substantially reducing or ablating binding or activity at the other receptor causes the therapeutic effect to improve as there is a reduced or eliminated sequestration of the therapeutic chimeric proteins away from the site of therapeutic action. For instance, in some embodiments, this obviates the need of high doses of the present chimeric proteins that compensate for loss at the other receptor. Such ability to reduce dose further provides a lower likelihood of side effects.

In various embodiments, the modified signaling agent comprises one or more mutations that cause the signaling agent to have reduced, substantially reduced, or ablated affinity, *e.g.* binding (*e.g.* K_D) and/or activation (for instance, when the modified signaling agent is an agonist of its receptor, measurable as, for example, K_A and/or EC_{50}) and/or inhibition (for instance, when the modified signaling agent is an antagonist of its receptor, measurable as, for example, K_I and/or IC_{50}), for one or more of its receptors. In various embodiments, the reduced affinity at the immumodulating agent's receptor allows for attenuation of activity (inclusive of agonism or antagonism). In such embodiments, the modified signaling agent has about 1%, or about 3%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 10%-20%, about 20%-40%, about 50%, about 40%-60%, about 60%-80%, about 80%-100% of the affinity for the receptor relative to the wild type signaling agent. In some embodiments, the binding affinity is at least about 2-fold lower, about 3-fold lower, about 4-fold lower, about 5-fold lower, about 6-fold lower, about 7-fold lower, about 8-fold lower, about 9-fold lower, at least about 10-fold lower, at least about 15-fold lower, at least about 20-fold lower, at least about 25-fold lower, at least about 30-fold lower, at least about 35-fold lower, at least about 40-fold lower, at least about 45-fold lower, at least about 50-fold lower, at least about 100-fold lower, at least about 150-fold lower, or about 10-50-fold lower, about 50-100-fold lower, about 100-150-fold lower, about 150-200-fold lower, or more than 200-fold lower relative to the wild type signaling agent.

In embodiments wherein the chimeric protein has mutations that reduce binding at one receptor and substantially reduce or ablate binding at a second receptor, the attenuation or reduction in binding affinity of a modified signaling agent for one receptor is less than the substantial reduction or ablation in affinity for the other receptor. In some embodiments, the attenuation or reduction in binding affinity of a modified signaling agent for one receptor is less than the substantial reduction or ablation in affinity for the other receptor by about 1%, or about 3%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In various embodiments, substantial reduction or ablation refers to a greater reduction in binding affinity and/or activity than attenuation or reduction.

In various embodiments, the modified signaling agent comprises one or more mutations that reduce the endogenous activity of the signaling agent to about 75%, or about 70%, or about 60%, or about 50%, or about 40%, or about 30%, or about 25%, or about 20%, or about 10%, or about 5%, or about 3%, or about 1%, *e.g.*, relative to the wild type signaling agent.

In some embodiments, the modified signaling agent comprises one or more mutations that cause the signaling agent to have reduced affinity for its receptor that is lower than the binding affinity of the targeting moiety(ies) for its(their) receptor(s). In some embodiments, this binding affinity differential is between signaling agent/receptor and targeting moiety/receptor on the same cell. In some embodiments, this binding affinity differential allows for the signaling agent, *e.g.* mutated signaling agent, to have localized, on-target effects and to minimize off-target effects that underlie side effects that are observed with wild type signaling agent. In some embodiments, this binding affinity is at least about 2-fold, or at least about 5-fold, or at least about 10-fold, or at least about 15-fold lower, or at least about 25-fold, or at least about 50-fold lower, or at least about 100-fold, or at least about 150-fold.

Receptor binding activity may be measured using methods known in the art. For example, affinity and/or binding activity may be assessed by Scatchard plot analysis and computer-fitting of binding data (*e.g.* Scatchard, 1949) or by reflectometric interference spectroscopy under flow through conditions, as described by Brecht *et al.* (1993), the entire contents of all of which are hereby incorporated by reference.

In various embodiments, the signaling agent is an immune-modulating agent, *e.g.* one or more of an interleukin, interferon, and tumor necrosis factor.

In some embodiments, the signaling agent is an interleukin or a modified interleukin, including for example IL-1; IL-2; IL-3; IL-4; IL-5; IL-6; IL-7; IL-8; IL-9; IL-10; IL-11; IL-12; IL-13; IL-14; IL-15; IL-16; IL-17; IL-18; IL-19; IL-20; IL-21; IL-22; IL-23; IL-24; IL-25; IL-26; IL-27; IL-28; IL-29; IL-30; IL-31; IL-32; IL-33; IL-35; IL-36 or a fragment, variant, analogue, or family-member thereof. Interleukins are a group of multi- functional cytokines synthesized by lymphocytes, monocytes, and macrophages. Known functions include stimulating proliferation of immune cells (*e.g.*, T helper cells, B cells, eosinophils, and lymphocytes), chemotaxis of neutrophils and T lymphocytes, and/or inhibition of interferons. Interleukin activity can be determined using assays known in the art: Matthews *et al.*, in *Lymphokines and Interferens: A Practical Approach*, Clemens *et al.*, eds, IRL Press, Washington, D.C. 1987, pp. 221-225; and Orencole & Dinarello (1989) Cytokine 1, 14-20.

In some embodiments, the signaling agent is an interferon or a modified version of an interferon such as interferon types I, II, and III. Illustrative interferons, including for example, interferon-α-1, 2, 4, 5, 6, 7, 8, 10, 13, 14, 16, 17, and 21, interferon-β and interferon-γ, interferon κ, interferon ε, interferon τ, and interferon ϖ.

In some embodiments, the signaling agent is a tumor necrosis factor (TNF) or a modified version of a tumor necrosis factor (TNF) or a protein in the TNF family, including but not limited to, TNF-α, TNF-β, LT-β, CD40L, CD27L, CD30L, FASL, 4-1BBL, OX40L, and TRAIL.

The amino acid sequences of the wild type signaling agents described herein are well known in the art. Accordingly, in various embodiments the modified signaling agent comprises an amino acid sequence that has at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity with the known wild type amino acid sequences of the signaling agents described herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% sequence identity).

In various embodiments the modified signaling agent comprises an amino acid sequence that has at least about 60%, or at least about 61%, or at least about 62%, or at least about 63%, or at least about 64%, or at least about 65%, or at least about 66%, or at least about 67%, or at least about 68%, or at least about 69%, or at least about 70%, or at least about 71%, or at least about 72%, or at least about 73%, or at least about 74%, or at least about 75%, or at least about 76%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 81%, or at least about 82%, or at least about 83%, or at least about 84%, or at least about 85%, or at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 90%, or at least about 91%, or at least about 92%, or at least about 93%, or at least about 94%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% sequence identity with any amino acid sequences of the signaling agents described herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, or about 99% sequence identity).

In various embodiments, the modified signaling agent comprises an amino acid sequence having one or more amino acid mutations. In some embodiments, the one or more amino acid mutations may be independently selected from substitutions, insertions, deletions, and truncations. In some embodiments, the amino acid

mutations are amino acid substitutions, and may include conservative and/or non-conservative substitutions, as described elsewhere herein.

In various embodiments, the substitutions may also include non-classical amino acids as described elsewhere herein.

As described herein, the modified signaling agents bear mutations that affect affinity and/or activity at one or more receptors. The receptors of any signaling agents, as described herein, are known in the art.

Illustrative mutations which provide reduced affinity and/or activity (*e.g.* agonistic) at a receptor are found in WO 2013/107791 (*e.g.* with regard to interferons), WO 2015/007542 (*e.g.* with regard to interleukins), and WO 2015/007903 (*e.g.* with regard to TNF), the entire contents of each of which are hereby incorporated by reference. Illustrative mutations which provide reduced affinity and/or activity (*e.g.* antagonistic) at a receptor are found in WO 2015/007520, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is interferon α. In such embodiments, the modified IFN-α agent has reduced affinity and/or activity for the IFN-α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains. In some embodiments, the modified IFN-α agent has substantially reduced or ablated affinity and/or activity for the IFN-α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains.

Mutant forms of interferon α are known to the person skilled in the art. In an illustrative embodiment, the modified signaling agent is the allelic form IFN-α2a having the amino acid sequence of:

IFN-α2a (SEQ ID NO:25):

CDLPQTHSLGSRRTLMLLAQMRKISLFSCLKDRHDFGFPQEEFGNQFQKAETIPVLH
EMIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVIQGVGVTETPLMKED
SILAVRKYFQRITLYLKEKKYSPCAWEVVRAEIMRSFSLSTNLQESLRSKE.

In an illustrative embodiment, the modified signaling agent is the allelic form IFN-α2b having the amino acid sequence of (which differs from IFN-α2a at amino acid position 23):

IFN-α2b (SEQ ID NO:26):

CDLPQTHSLGSRRTLMLLAQMRRISLFSCLKDRHDFGFPQEEFGNQFQKAETIPVLH
EMIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVIQGVGVTETPLMKED
SILAVRKYFQRITLYLKEKKYSPCAWEVVRAEIMRSFSLSTNLQESLRSKE.

In some embodiments, said IFN-α2 mutant (IFN-α2a or IFN-α2b) is mutated at one or more amino acids at positions 144-154, such as amino acid positions 148, 149 and/or 153. In some embodiments, the IFN-α2 mutant comprises one or more mutations selected from L153A, R149A, and M148A. Such mutants are described, for example, in WO2013/107791 and Piehler *et al.*, (2000) J. Biol. Chem, 275:40425-33, the entire contents of all of which are hereby incorporated by reference.

In some embodiments, the IFN-α2 mutants have reduced affinity and/or activity for IFNAR1. In some embodiments, the IFN-α2 mutant comprises one or more mutations selected from F64A, N65A, T69A, L80A, Y85A, and Y89A, as described in WO2010/030671, the entire contents of which is hereby incorporated by reference.

In some embodiments, the IFN-α2 mutant comprises one or more mutations selected from K133A, R144A, R149A, and L153A as described in WO2008/124086, the entire contents of which is hereby incorporated by reference.

In some embodiments, the IFN-α2 mutant comprises one or more mutations selected from R120E and R120E/K121E, as described in WO2015/007520 and WO2010/030671, the entire contents of which are hereby incorporated by reference. In such embodiments, said IFN-α2 mutant antagonizes wildtype IFN-α2 activity. In such embodiments, said mutant IFN-α2 has reduced affinity and/or activity for IFNAR1 while affinity and/or activity of IFNAR2 is retained.

In some embodiments, the human IFN-α2 mutant comprises (1) one or more mutations selected from R120E and R120E/K121E, which, without wishing to be bound by theory, create an antagonistic effect and (2) one or more mutations selected from K133A, R144A, R149A, and L153A, which, without wishing to be bound by theory, allow for an attenuated effect at, for example, IFNAR2. In an embodiment, the human IFN-α2 mutant comprises R120E and L153A.

In some embodiments, the human IFN-α2 mutant comprises one or more mutations selected from, L15A, A19W, R22A, R23A, L26A, F27A, L30A, L30V, K31A, D32A, R33K, R33A, R33Q, H34A, D35A, Q40A, D114R, L117A, R120A, R125A , K134A, R144A, A145G, A145M, M148A, R149A, S152A, L153A, and N156A as disclosed in WO 2013/059885, the entire disclosures of which are hereby incorporated by reference. In some embodiments, the human IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or L30A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or R33A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or M148A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations H57Y, E58N, Q61S, and/or L153A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations N65A, L80A, Y85A, and/or Y89A as disclosed in WO 2013/059885. In some embodiments, the human IFN-α2 mutant comprises the mutations N65A, L80A, Y85A, Y89A, and/or D114A as disclosed in WO 2013/059885.

In an embodiment, the modified signaling agent is interferon β. In such embodiments, the modified interferon β agent has reduced affinity and/or activity for the IFN-α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains. In some embodiments, the modified interferon β agent has substantially reduced or ablated affinity and/or activity for the IFN-α/β receptor (IFNAR), *i.e.*, IFNAR1 and/or IFNAR2 chains.

In an embodiment, the modified signaling agent is interferon γ. In such embodiments, the modified interferon γ agent has reduced affinity and/or activity for the interferon-gamma receptor (IFNGR), *i.e.*, IFNGR1 and IFNGR2

chains. In some embodiments, the modified interferon γ agent has substantially reduced or ablated affinity and/or activity for the interferon-gamma receptor (IFNGR), *i.e.*, IFNGR1 and/or IFNGR2 chains.

In an embodiment, the modified signaling agent is TNF-α. TNF is a pleiotropic cytokine with many diverse functions, including regulation of cell growth, differentiation, apoptosis, tumorigenesis, viral replication, autoimmunity, immune cell functions and trafficking, inflammation, and septic shock. It binds to two distinct membrane receptors on target cells: TNFR1 (p55) and TNFR2 (p75). TNFR1 exhibits a very broad expression pattern whereas TNFR2 is expressed preferentially on certain populations of lymphocytes, Tregs, endothelial cells, certain neurons, microglia, cardiac myocytes and mesenchymal stem cells. Very distinct biological pathways are activated in response to receptor activation, although there is also some overlap. As a general rule, without wishing to be bound by theory, TNFR1 signaling is associated with induction of apoptosis (cell death) and TNFR2 signaling is associated with activation of cell survival signals (*e.g.* activation of NFkB pathway). Administration of TNF is systemically toxic, and this is largely due to TNFR1 engagement. However, it should be noted that activation of TNFR2 is also associated with a broad range of activities and, as with TNFR1, in the context of developing TNF based therapeutics, control over TNF targeting and activity is important.

In some embodiments, the modified signaling agent has reduced affinity and/or activity for TNFR1 and/or TNFR2. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for TNFR1 and/or TNFR2. TNFR1 is expressed in most tissues, and is involved in cell death signaling while, by contrast, TNFR2 is involved in cell survival signaling. Accordingly, in embodiments directed to methods of treating cancer, the modified signaling agent has reduced affinity and/or activity for TNFR1 and/or substantially reduced or ablated affinity and/or activity for TNFR2. In these embodiments, the chimeric proteins may be targeted to a cell for which apoptosis is desired, *e.g.* a tumor cell or a tumor vasculature endothelial cell. In embodiments directed to methods of promoting cell survival, for example, in neurogenesis for the treatment of neurodegenerative disorders, the modified signaling agent has reduced affinity and/or activity for TNFR2 and/or substantially reduced or ablated affinity and/or activity for TNFR1. Stated another way, the present chimeric proteins, in some embodiments, comprise modified TNF-α agent that allows of favoring either death or survival signals.

In some embodiments, the chimeric protein has a modified TNF having reduced affinity and/or activity for TNFR1 and/or substantially reduced or ablated affinity and/or activity for TNFR2. Such a chimera, in some embodiments, is a more potent inducer of apoptosis as compared to a wild type TNF and/or a chimera bearing only mutation(s) causing reduced affinity and/or activity for TNFR1. Such a chimera, in some embodiments, finds use in inducing tumor cell death or a tumor vasculature endothelial cell death (*e.g.* in the treatment of cancers). Also, in some embodiments, these chimeras avoid or reduce activation of T_{reg} cells via TNFR2, for example, thus further supporting TNFR1-mediated antitumor activity *in vivo*.

In some embodiments, the chimeric protein has a modified TNF having reduced affinity and/or activity for TNFR2 and/or substantially reduced or ablated affinity and/or activity for TNFR1. Such a chimera, in some embodiments,

is a more potent activator of cell survival in some cell types, which may be a specific therapeutic objective in various disease settings, including without limitation, stimulation of neurogenesis. In addition, such a TNFR2-favoring chimeras also are useful in the treatment of autoimmune diseases (*e.g.* Crohn's, diabetes, MS, colitis *etc.* and many others described herein). In some embodiments, the chimera is targeted to auto-reactive T cells. In some embodiments, the chimera promotes T_{reg} cell activation and indirect suppression of cytotoxic T cells.

In some embodiments, the chimera causes the death of auto-reactive T cells, *e.g.* by activation of TNFR2 and/or avoidance of TNFR1 (*e.g.* a modified TNF having reduced affinity and/or activity for TNFR2 and/or substantially reduced or ablated affinity and/or activity for TNFR1). Without wishing to be bound by theory these auto-reactive T cells, have their apoptosis/survival signals altered *e.g.* by NFkB pathway activity/signaling alterations. In some embodiments, the chimera causes the death of autoreactive T cells having lesions or modifications in the NFkB pathway, which underlie an imbalance of their cell death (apoptosis)/survival signaling properties and, optionally, altered susceptibility to certain death-inducing signals (*e.g.*, TNFR2 activation).

In some embodiments, a TNFR2 based chimera has additional therapeutic applications in diseases, including various autoimmune diseases, heart disease, de-myelinating and neurodegenerative disorders, and infectious disease, among others.

In an embodiment, the wild type TNF-α has the amino acid sequence of:

TNF-α (SEQ ID NO:27)

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLV
VPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQ
RETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYF
GIIAL.

In such embodiments, the modified TNF-α agent has mutations at one or more amino acid positions 29, 31, 32, 84, 85, 86, 87, 88, 89, 145, 146 and 147 which produces a modified TNF-α with reduced receptor binding affinity. *See*, for example, U.S. Patent No. 7,993,636, the entire contents of which are hereby incorporated by reference.

In some embodiments, the modified human TNF-α moiety has mutations at one or more amino acid positions R32, N34, Q67, H73, L75, T77, S86, Y87, V91 , I97, T105, P106, A109, P113, Y115, E127, N137, D143, and A145, as described, for example, in WO/2015/007903, the entire contents of which is hereby incorporated by reference (numbering according to the human TNF sequence, Genbank accession number BAG70306, version BAG70306.1 GI: 197692685). In some embodiments, the modified human TNF-α moiety has substitution mutations selected from R32G, N34G, Q67G, H73G, L75G, L75A, L75S, T77A, S86G, Y87Q, Y87L, Y87A, Y87F, V91G, V91A, I97A, I97Q, I97S, T105G, P106G, A109Y, P113G, Y115G, Y115A, E127G, N137G, D143N, A145G and A145T. In an embodiment, the human TNF-α moiety has a mutation selected from Y87Q, Y87L, Y87A, and Y87F. In another embodiment, the human TNF-α moiety has a mutation selected from I97A, I97Q, and I97S. In a further embodiment, the human TNF-α moiety has a mutation selected from Y115A and Y115G.

In some embodiments, the modified TNF-α agent has one or more mutations selected from N39Y, S147Y, and Y87H, as described in WO2008/124086, the entire contents of which is hereby incorporated by reference.

In some embodiments, the modified human TNF-α moiety has mutations that provide receptor selectivity as described in PCT/IB2016/001668, the entire contents of which are hereby incorporated by reference. In some embodiments, the mutations to TNF are TNF-R1 selective. In some embodiments, the mutations to TNF which are TNF-R1 selective are at one or more of positions R32, S86, and E146. In some embodiments, the mutations to TNF which are TNF-R1 selective are one or more of R32W, S86T, and E146K. In some embodiments, the mutations to TNF which are TNF-R1 selective are one or more of R32W, R32W/S86T, R32W/E146K and E146K. In some embodiments, the mutations to TNF are TNF-R2 selective. In some embodiments, the mutations to TNF which are TNF-R2 selective are at one or more of positions A145, E146, and S147. In some embodiments, the mutations to TNF which are TNF-R2 selective are one or more of A145T, A145R, E146D, and S147D. In some embodiments, the mutations to TNF which are TNF-R2 selective are one or more of A145R, A145T/S147D, and A145T/E146D/S147D.

In an embodiment, the modified signaling agent is TNF-β. TNF-β can form a homotrimer or a heterotrimer with LT-β (LT-α1β2). In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for TNFR1 and/or TNFR2 and/or herpes virus entry mediator (HEVM) and/or LT-βR.

In an embodiment, the wild type TNF-β has the amino acid sequence of:

TNF-beta (SEQ ID NO:28)

LPGVGLTPSAAQTARQHPKMHLAHSNLKPAAHLIGDPSKQNSLLWRANTD
RAFLQDGFSLSNNSLLVPTSGIYFVYSQVVFSGKAYSPKATSSPLYLAHEV
QLFSSQYPFHVPLLSSQKMVYPGLQEPWLHSMYHGAAFQLTQGDQLSTH
TDGIPHLVLSPSTVFFGAFAL.

In such embodiments, the modified TNF-β agent may comprise mutations at one or more amino acids at positions 106-113, which produce a modified TNF-β with reduced receptor binding affinity to TNFR2. In an embodiment, the modified signaling agent has one or more substitution mutations at amino acid positions 106-113. In illustrative embodiments, the substitution mutations are selected from Q107E, Q107D, S106E, S106D, Q107R, Q107N, Q107E/S106E, Q107E/S106D, Q107D/S106E, and Q107D/S106D. In another embodiment, the modified signaling agent has an insertion of about 1 to about 3 amino acids at positions 106-113.

In some embodiments, the modified agent is a TNF family member (*e.g.* TNF-alpha, TNF-beta) which can be a single chain trimeric version as described in WO 2015/007903, the entire contents of which are incorporated by reference.

In some embodiments, the modified agent is a TNF family member (*e.g.* TNF-alpha, TNF-beta) which has reduced affinity and/or activity, *i.e.* antagonistic activity (*e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby

incorporated by reference) at TNFR1. In these embodiments, the modified agent is a TNF family member (*e.g.* TNF-alpha, TNF-beta) which also, optionally, has substantially reduced or ablated affinity and/or activity for TNFR2. In some embodiments, the modified agent is a TNF family member (*e.g.* TNF-alpha, TNF-beta) which has reduced affinity and/or activity, *i.e.* antagonistic activity (*e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) at TNFR2. In these embodiments, the modified agent is a TNF family member (*e.g.* TNF-alpha, TNF-beta) which also, optionally, has substantially reduced or ablated affinity and/or activity for TNFR1. The constructs of such embodiments find use in, for example, methods of dampening TNF response in a cell specific manner. In some embodiments, the antagonistic TNF family member (*e.g.* TNF-alpha, TNF-beta) is a single chain trimeric version as described in WO 2015/007903.

In an embodiment, the modified signaling agent is TRAIL. In some embodiments, the modified TRAIL agent has reduced affinity and/or activity for DR4 (TRAIL-RI) and/or DR5 (TRAIL-RII) and/or DcR1 and/or DcR2. In some embodiments, the modified TRAIL agent has substantially reduced or ablated affinity and/or activity for DR4 (TRAIL-RI) and/or DR5 (TRAIL-RII) and/or DcR1 and/or DcR2.

In an embodiment, the wild type TRAIL has the amino acid sequence of:

TRAIL (SEQ ID NO:29)

MAMMEVQGGPSLGQTCVLIVIFTVLLQSLCVAVTYVYFTNELKQMQDKYSK
SGIACFLKEDDSYWDPNDEESMNSPCWQVKWQLRQLVRKMILRTSEETIS
TVQEKQQNISPLVRERGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRKIN
SWESSRSGHSFLSNLHLRNGELVIHEKGFYYIYSQTYFRFQEEIKENTKND
KQMVQYIYKYTSYPDPILLMKSARNSCWSKDAEYGLYSIYQGGIFELKEND
RIFVSVTNEHLIDMDHEASFFGAFLVG.

In such embodiments, the modified TRAIL agent may comprise a mutation at amino acid positions T127-R132, E144-R149, E155-H161, Y189-Y209, T214-1220,K224-A226, W231, E236-L239, E249-K251, T261-H264 and H270-E271 (Numbering based on the human sequence, Genbank accession number NP _003801, version 10 NP _003801.1, GI: 4507593; see above).

In an embodiment, the modified signaling agent is an interleukin. In an embodiment, the modified signaling agent is IL-1. In an embodiment, the modified signaling agent is IL-1α or IL-1β. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-1R1 and/or IL-1RAcP. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-1R1 and/or IL-1RAcP. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-1R2. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-1R2. For instance, in some embodiments, the present modified IL-1 agents avoid interaction at IL-1R2 and therefore substantially reduce its function as a decoy and/or sink for therapeutic agents.

In an embodiment, the wild type IL-1β has the amino acid sequence of:

IL-1 beta (mature form, wild type) (SEQ ID NO:30)

APVRSLNCTLRDSQQKSLVMSGPYELKALHLQGQDMEQQVVFSMSFVQG
EESNDKIPVALGLKEKNLYLSCVLKDDKPTLQLESVDPKNYPKKKMEKRFV
FNKIEINNKLEFESAQFPNWYISTSQAENMPVFLGGTKGGQDITDFTMQFV
SS.

IL-1 is a proinflammatory cytokine and an important immune system regulator. It is a potent activator of CD4 T cell responses, increases proportion of Th17 cells and expansion of IFNγ and IL-4 producing cells. IL-1 is also a potent regulator of $CD8^+$ T cells, enhancing antigen-specific $CD8^+$ T cell expansion, differentiation, migration to periphery and memory. IL-1 receptors comprise IL-1R1 and IL-1R2. Binding to and signaling through the IL-1R1 constitutes the mechanism whereby IL-1 mediates many of its biological (and pathological) activities. IL1-R2 can function as a decoy receptor, thereby reducing IL-1 availability for interaction and signaling through the IL-1R1.

In some embodiments, the modified IL-1 has reduced affinity and/or activity (*e.g.* agonistic activity) for IL-1R1. In some embodiments, the modified IL-1 has substantially reduced or ablated affinity and/or activity for IL-1R2. In such embodiments, there is restorable IL-1/ IL-1R1 signaling and prevention of loss of therapeutic chimeras at IL-R2 and therefore a reduction in dose of IL-1 that is required (*e.g.* relative to wild type or a chimera bearing only an attenuation mutation for IL-R1). Such constructs find use in, for example, methods of treating cancer, including, for example, stimulating the immune system to mount an anti-cancer response.

In some embodiments, the modified IL-1 has reduced affinity and/or activity (*e.g.* antagonistic activity, *e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) for IL-1R1. In some embodiments, the modified IL-1 has substantially reduced or ablated affinity and/or activity for IL-1R2. In such embodiments, there is the IL-1/ IL-1R1 signaling is not restorable and prevention of loss of therapeutic chimeras at IL-R2 and therefore a reduction in dose of IL-1 that is required (*e.g.* relative to wild type or a chimera bearing only an attenuation mutation for IL-R1). Such constructs find use in, for example, methods of treating autoimmune diseases, including, for example, suppressing the immune system.

In such embodiments, the modified signaling agent has a deletion of amino acids 52-54 which produces a modified human IL-1β with reduced binding affinity for type I IL-1R and reduced biological activity. See, for example, WO 1994/000491, the entire contents of which are hereby incorporated by reference. In some embodiments, the modified human IL-1β has one or more substitution mutations selected from A117G/P118G, R120X, L122A, T125G/L126G, R127G, Q130X, Q131G, K132A, S137G/Q138Y, L145G, H146X, L145A/L147A, Q148X, Q148G/Q150G, Q150G/D151A, M152G, F162A, F162A/Q164E, F166A, Q164E/E167K, N169G/D170G, I172A, V174A, K208E, K209X, K209A/K210A, K219X, E221X, E221 S/N224A, N224S/K225S, E244K, N245Q (where X can be any change in amino acid, *e.g.*, a non-conservative change), which exhibit reduced binding to IL-1R, as described, for example, in WO2015/007542 and WO/2015/007536, the entire contents of which is

hereby incorporated by reference (numbering base on the human IL-1 β sequence, Genbank accession number NP_000567, version NP-000567.1 , GI: 10835145). In some embodiments, the modified human IL-1β may have one or more mutations selected from R120A, R120G, Q130A, Q130W, H146A, H146G, H146E, H146N, H146R, Q148E, Q148G, Q148L, K209A, K209D, K219S, K219Q, E221S and E221K. In an embodiment, the modified human IL-1β comprises the mutations Q131G and Q148G. In an embodiment, the modified human IL-1β comprises the mutations Q148G and K208E. In an embodiment, the modified human IL-1β comprises the mutations R120G and Q131G. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146A. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146N. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146R. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146E. In an embodiment, the modified human IL-1β comprises the mutations R120G and H146G. In an embodiment, the modified human IL-1β comprises the mutations R120G and K208E. In an embodiment, the modified human IL-1β comprises the mutations R120G, F162A, and Q164E.

In an embodiment, the modified signaling agent is IL-2. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-2Rα and/or IL-2Rβ and/or IL-2Rγ. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-2Rβ and/or IL-2Rγ. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-2Rα. Such embodiments may be relevant for treatment of cancer, for instance when the modified IL-2 is agonistic at IL-2Rβ and/or IL-2Rγ. For instance, the present constructs may favor attenuated activation of $CD8^+$ T cells (which can provide an anti-tumor effect), which have IL2 receptors β and γ and disfavor T_{regs} (which can provide an immune suppressive, pro-tumor effect), which have IL2 receptors α, β, and γ. Further, in some embodiments, the preferences for IL-2Rβ and/or IL-2Rγ over IL-2Rα avoid IL-2 side effects such as pulmonary edema. Also, IL-2-based chimeras are useful for the treatment of autoimmune diseases, for instance when the modified IL-2 is antagonistic (*e.g.* natural antagonistic activity or antagonistic activity that is the result of one or more mutations, *see, e.g.,* WO 2015/007520, the entire contents of which are hereby incorporated by reference) at IL-2Rβ and/or IL-2Rγ. For instance, the present constructs may favor attenuated suppression of $CD8^+$ T cells (and therefore dampen the immune response), which have IL2 receptors β and γ and disfavor T_{regs} which have IL2 receptors α, β, and γ. Alternatively, in some embodiments, the chimeras bearing IL-2 favor the activation of T_{regs}, and therefore immune suppression, and activation of disfavor of $CD8^+$ T cells. For instance, these constructs find use in the treatment of diseases or diseases that would benefit from immune suppression, *e.g.* autoimmune disorders.

In some embodiments, the chimeric protein has targeting moieties as described herein directed to $CD8^+$ T cells as well as a modified IL-2 agent having reduced affinity and/or activity for IL-2Rβ and/or IL-2Rγ and/or substantially reduced or ablated affinity and/or activity for IL-2Rα. In some embodiments, these constructs provide targeted $CD8^+$ T cell activity and are generally inactive (or have substantially reduced activity) towards T_{reg} cells. In some embodiments, such constructs have enhanced immune stimulatory effect compared to wild

type IL-2 (*e.g.*, without wishing to be bound by theory, by not stimulating Tregs), whilst eliminating or reducing the systemic toxicity associated with IL-2.

In an embodiment, the wild type IL-2 has the amino acid sequence of:

IL-2 (mature form, wild type) (SEQ ID NO:31)

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATE
LKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMC
EYADETATIVEFLNRWITFCQSIISTLT.

In such embodiments, the modified IL-2 agent has one or more mutations at amino acids L72 (L72G, L72A, L72S, L72T, L72Q, L72E, L72N, L72D, L72R, or L72K), F42 (F42A, F42G, F42S, F42T, F42Q, F42E, F42N, F42D, F42R, or F42K) and Y45 (Y45A, Y45G, Y45S, Y45T, Y45Q, Y45E, Y45N, Y45D, Y45R or Y45K). Without wishing to be bound by theory, it is believed that these modified IL-2 agents have reduced affinity for the high-affinity IL-2 receptor and preserves affinity to the intermediate-affinity IL-2 receptor, as compared to the wild-type IL-2. See, for example, US Patent Publication No. 2012/0244112, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-3. In some embodiments, the modified signaling agent has reduced affinity and/or activity for the IL-3 receptor, which is a heterodimer with a unique alpha chain paired with the common beta (beta c or CD131) subunit. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for the IL-3 receptor, which is a heterodimer with a unique alpha chain paired with the common beta (beta c or CD131) subunit.

In an embodiment, the modified signaling agent is IL-4. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for type 1 and/or type 2 IL-4 receptors. In such an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for type 1 and/or type 2 IL-4 receptors. Type 1 IL-4 receptors are composed of the IL-4Rα subunit with a common γ chain and specifically bind IL-4. Type 2 IL-4 receptors include an IL-4Rα subunit bound to a different subunit known as IL-13Rα1. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity the type 2 IL-4 receptors.

In an embodiment, the wild type IL-4 has the amino acid sequence of:

IL-4 (mature form, wild type) (SEQ ID NO:32)

HKCDITLQEIIKTLNSLTEQKTLCTELTVTDIFAASKNTTEKETFCRAATVLRQ
FYSHHEKDTRCLGATAQQFHRHKQLIRFLKRLDRNLWGLAGLNSCPVKEA
NQSTLENFLERLKTIMREKYSKCSS.

In such embodiments, the modified IL-4 agent has one or more mutations at amino acids R121 (R121A, R121D, R121E, R121F, R121H, R121I, R121K, R121N, R121P, R121T, R121W), E122 (E122F), Y124 (Y124A, Y124Q, Y124R, Y124S, Y124T) and S125 (S125A). Without wishing to be bound by theory, it is believed that these

modified IL-4 agents maintain the activity mediated by the type I receptor, but significantly reduces the biological activity mediated by the other receptors. See, for example, US Patent No. 6,433,157, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-6. IL-6 signals through a cell-surface type I cytokine receptor complex including the ligand-binding IL-6R chain (CD126), and the signal-transducing component gp130. IL-6 may also bind to a soluble form of IL-6R (sIL-6R), which is the extracellular portion of IL-6R. The sIL-6R/IL-6 complex may be involved in neurites outgrowth and survival of neurons and, hence, may be important in nerve regeneration through remyelination. Accordingly, in some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-6R/gp130 and/or sIL-6R. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-6R/gp130 and/or sIL-6R.

In an embodiment, the wild type IL-6 has the amino acid sequence of:

IL-6 (mature form, wild type) (SEQ ID NO:33)

APVPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCE
SSKEALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQN
RFESSEEQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLTTKLQAQN
QWLQDMTTHLILRSFKEFLQSSLRALRQM.

In such embodiments, the modified signaling agent has one or more mutations at amino acids 58, 160, 163, 171 or 177. Without wishing to be bound by theory, it is believed that these modified IL-6 agents exhibit reduced binding affinity to IL-6Ralpha and reduced biological activity. See, for example, WO 97/10338, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-10. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-10 receptor-1 and IL-10 receptor-2. In some embodiments, the modified signaling agent has substantially reduced affinity and/or activity for IL-10 receptor-1 and IL-10 receptor-2

In an embodiment, the modified signaling agent is IL-11. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-11Rα and/or IL-11Rβ and/or gp130. In such an embodiment, the modified signaling agent has substantially reduced affinity and/or activity for IL-11Rα and/or IL-11Rβ and/or gp130.

In an embodiment, the modified signaling agent is IL-12. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for IL-12Rβ1 and/or IL-12Rβ2. In such an embodiment, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-12Rβ1 and/or IL-12Rβ2.

In an embodiment, the modified signaling agent is IL-13. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for the IL-4 receptor (IL-4Rα) and IL-13Rα1. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-4 receptor (IL-4Rα) or IL-13Rα1.

In an embodiment, the wild type IL-13 has the amino acid sequence of:

IL-13 (mature form, wild type) (SEQ ID NO:34)

SPGPVPPSTALRELIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALES
LINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLH
LKKLFREGRFN.

In such embodiments, the modified IL-13 agent has one or more mutations at amino acids 13, 16, 17, 66, 69, 99, 102, 104, 105, 106, 107, 108, 109, 112, 113 and 114. Without wishing to be bound by theory, it is believed that these modified IL-13 agents exhibit reduced biological activity. See, for example, WO 2002/018422, the entire contents of which are hereby incorporated by reference.

In an embodiment, the modified signaling agent is IL-18. In some embodiments, the modified signaling agent has reduced affinity and/or activity for IL-18Rα and/or IL-18Rβ. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-18Rα and/or IL-18Rβ. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for IL-18Rα type II, which is an isoform of IL-18Rα that lacks the TIR domain required for signaling.

In an embodiment, the wild type IL-18 has the amino acid sequence of:

IL-18 (wild type) (SEQ ID NO:35)

MAAEPVEDNCINFVAMKFIDNTLYFIAEDDENLESDYFGKLESKLSVIRNLN
DQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFIISMYKDSQPRGMAVTISVKC
EKISTLSCENKIISFKEMNPPDNIKDTKSDIIFFQRSVPGHDNKMQFESSSYE
GYFLACEKERDLFKLILKKEDELGDRSIMFTVQNEDL.

In such embodiments, the modified IL-18 agent may comprise one or more mutations in amino acids or amino acid regions selected from Y37-K44, R49-Q54, D59-R63, E67-C74, R80, M87-A97, N 127-K129, Q139-M149, K165-K171, R183 and Q190-N191, as described in WO/2015/007542, the entire contents of which are hereby incorporated by reference (numbering based on the human IL-18 sequence, Genbank accession number AAV38697, version AAV38697.1, GI: 54696650).

In an embodiment, the modified signaling agent is IL-33. In such an embodiment, the modified signaling agent has reduced affinity and/or activity for the ST-2 receptor and IL-1RAcP. In some embodiments, the modified signaling agent has substantially reduced or ablated affinity and/or activity for the ST-2 receptor and IL-1RAcP.

In an embodiment, the wild type IL-33 has the amino acid sequence of:

MKPKMKYSTNKISTAKWKNTASKALCFKLGKSQQKAKEVCPMYFMKLRSG
LMIKKEACYFRRETTKRPSLKTGRKHKRHLVLAACQQQSTVECFAFGISGV
QKYTRALHDSSITGISPITEYLASLSTYNDQSITFALEDESYEIYVEDLKKDEK
KDKVLLSYYESQHPSNESGDGVDGKMLMVTLSPTKDFWLHANNKEHSVE

LHKCEKPLPDQAFFVLHNMHSNCVSFECKTDPGVFIGVKDNHLALIKVDSS
ENLCTENILFKLSET (SEQ ID NO:36).

In such embodiments, the modified IL-33 agent may comprise one or more mutations in amino acids or amino acid regions selected from I113-Y122, S127-E139, E144-D157, Y163-M183, E200, Q215, L220-C227 and T260-E269, as described in WO/2015/007542, the entire contents of which are hereby incorporated by reference (numbering based on the human sequence, Genbank accession number NP_254274, version NP_254274.1, GI:15559209).

In one embodiment, the present chimeric protein has (i) a CD8 binding agent and (ii) a targeting moiety which is directed against a tumor cell, along with any of the modified (*e.g.* mutant) form signaling agents described herein. In an embodiment, the present chimeric protein has a targeting moiety directed against CD8 on T cells and a second targeting moiety directed against PD-L1 or PD-L2 on tumor cells.

In various embodiments, the signaling agent is a toxin or toxic enzyme. In some embodiments, the toxin or toxic enzyme is derived from plants and bacteria. Illustrative toxins or toxic enzymes include, but are not limited to, the diphtheria toxin, Pseudomonas toxin, anthrax toxin, ribosome-inactivating proteins (RIPs) such as ricin and saporin, modeccin, abrin, gelonin, and poke weed antiviral protein. Additional toxins include those disclosed in Mathew et al., (2009) Cancer Sci 100(8): 1359-65, the entire disclosures are hereby incorporated by reference. In such embodiments, the chimeric proteins of the invention may be utilized to induce cell death in cell-type specific manner. In such embodiments, the toxin may be modified, *e.g.* mutated, to reduce affinity and/or activity of the toxin for an attenuated effect, as described with other signaling agents herein.

Multi-Specific Chimeras and Fusions with Signaling Agents

In various embodiments, the CD8 binding agent of the invention is part of a chimera or fusion with one or more signaling agents as described herein and/or one or more additional targeting moieties. Accordingly, the present invention provides for chimeric or fusion proteins that include one or more signaling agents and a targeting moiety against CD8 and/or one or more additional targeting moieties.

In various embodiments, the CD8 binding agent of the invention is multispecific, *i.e.*, the CD8 binding agent comprises two or more targeting moieties having recognition domains that recognize and bind two or more targets, *e.g.* antigens, or receptors, or epitopes. In such embodiments, the CD8 binding agent of the invention may comprise two more targeting moieties having recognition domains that recognize and bind two or more epitopes on the same antigen or on different antigens. In various embodiments, such multi-specific CD8 binding agents exhibit advantageous properties such as increased avidity and/or improved selectivity. In an embodiment, the CD8 binding agent of the invention comprises two targeting moieties and is bispecific, *i.e.*, binds and recognizes two epitopes on the same antigen or on different antigens.

In various embodiments, the multispecific CD8 binding agent of the invention comprises two or more targeting moieties with each targeting moiety being an antibody or an antibody derivative as described herein. In an

embodiment, the multispecific CD8 binding agent of the invention comprises at least one VHH comprising an antigen recognition domain against CD8 and one antibody or antibody derivative comprising an antigen recognition domain against a tumor antigen.

In various embodiments, the present multispecific CD8 binding agents have two or more targeting moieties that target different antigens or receptors, and one targeting moiety may be attenuated for its antigen or receptor, *e.g.* the targeting moiety binds its antigen or receptor with a low affinity or avidity (including, for example, at an affinity or avidity that is less than the affinity or avidity the other targeting moiety has for its for its antigen or receptor, for instance the difference between the binding affinities may be about 10-fold, or 25-fold, or 50-fold, or 100-fold, or 300-fold, or 500-fold, or 1000-fold, or 5000-fold; for instance the lower affinity or avidity targeting moiety may bind its antigen or receptor at a K_D in the mid- to high-nM or low- to mid-μM range while the higher affinity or avidity targeting moiety may bind its antigen or receptor at a K_D in the mid- to high-pM or low- to mid-nM range). For instance, in some embodiments, the present multispecific CD8 binding agents comprises an attenuated targeting moiety that is directed against a promiscuous antigen or receptor, which may improve targeting to a cell of interest (*e.g.* via the other targeting moiety) and prevent effects across multiple types of cells, including those not being targeted for therapy (*e.g.* by binding promiscuous antigen or receptor at a higher affinity than what is provided in these embodiments).

The multispecific CD8 binding agent of the invention may be constructed using methods known in the art, see for example, U.S. Patent No. 9,067,991, U.S. Patent Publication No. 20110262348 and WO 2004/041862, the entire contents of which are hereby incorporated by reference. In an illustrative embodiment, the multispecific CD8 binding agent of the invention comprising two or more targeting moieties may be constructed by chemical crosslinking, for example, by reacting amino acid residues with an organic derivatizing agent as described by Blattler *et al.*, Biochemistry 24,1517-1524 and EP294703, the entire contents of which are hereby incorporated by reference. In another illustrative embodiment, the multispecific CD8 binding agent comprising two or more targeting moieties is constructed by genetic fusion, *i.e.*, constructing a single polypeptide which includes the polypeptides of the individual targeting moieties. For example, a single polypeptide construct may be formed which encodes a first VHH with an antigen recognition domain against CD8 and a second antibody or antibody derivative with an antigen recognition domain against a tumor antigen. A method for producing bivalent or multivalent VHH polypeptide constructs is disclosed in PCT patent application WO 96/34103, the entire contents of which is hereby incorporated by reference. In a further illustrative embodiment, the multispecific CD8 binding agent of the invention may be constructed by using linkers. For example, the carboxy-terminus of a first VHH with an antigen recognition domain against CD8 may be linked to the amino-terminus of a second antibody or antibody derivative with an antigen recognition domain against a tumor antigen (or vice versa). Exemplary linkers that may be used are described herein. In some embodiments, the components of the multispecific CD8 binding agent of the invention are directly linked to each other without the use of linkers.

In various embodiments, the multi-specific CD8 binding agent of the invention recognizes and binds to CD8 and one or more antigens found on one or more immune cells, which can include, without limitation, megakaryocytes,

thrombocytes, erythrocytes, mast cells, basophils, neutrophils, eosinophils, monocytes, macrophages, natural killer cells, T lymphocytes (*e.g.*, cytotoxic T lymphocytes, T helper cells, natural killer T cells), B lymphocytes, plasma cells, dendritic cells, or subsets thereof. In some embodiments, the CD8 binding agent specifically binds to an antigen of interest and effectively directly or indirectly recruits one of more immune cells.

In various embodiments, the multi-specific CD8 binding agent of the invention recognizes and binds to CD8 and one or more antigens found on tumor cells. In these embodiments, the present CD8 binding agents may directly or indirectly recruit an immune cell to a tumor cell or the tumor microenvironment. In some embodiments, the present CD8 binding agents may directly or indirectly recruit an immune cell, *e.g.* an immune cell that can kill and/or suppress a tumor cell (*e.g.*, a CTL), to a site of action (such as, by way of non-limiting example, the tumor microenvironment).

In some embodiments, the present CD8 binding agents are capable of, or find use in methods involving, shifting the balance of immune cells in favor of immune attack of a tumor. For instance, the present CD8 binding agents can shift the ratio of immune cells at a site of clinical importance in favor of cells that can kill and/or suppress a tumor (*e.g.* T cells, cytotoxic T lymphocytes, T helper cells, natural killer (NK) cells, natural killer T (NKT) cells, anti-tumor macrophages (*e.g.* M1 macrophages), neutrophils, B cells, dendritic cells or subsets thereof and in opposition to cells that protect tumors (*e.g.* myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs); tumor associated neutrophils (TANs), M2 macrophages, tumor associated macrophages (TAMs), or subsets thereof). In some embodiments, the present CD8 binding agent is capable of increasing a ratio of effector T cells to regulatory T cells.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to an antigen associated with tumor cells. In some embodiments, the targeting moiety directly or indirectly recruits tumor cells. For instance, in some embodiments, the recruitment of the tumor cell is to one or more effector cell (*e.g.* an immune cell as described herein) that can kill and/or suppress the tumor cell. In some embodiments, the targeting moiety directly or indirectly recruits T cells to a tumor cell, for example, by virtue of the two targeting moieties interacting with their respective antigens on a tumor and CD8-positive immune cell (*e.g.* T cell).

Tumor cells, or cancer cells refer to an uncontrolled growth of cells or tissues and/or an abnormal increased in cell survival and/or inhibition of apoptosis which interferes with the normal functioning of bodily organs and systems. For example, tumor cells include benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases. Illustrative tumor cells include, but are not limited to cells of: basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (*e.g.*, small-cell

lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (*e.g.* that associated with brain tumors), and Meigs' syndrome.

Tumor cells, or cancer cells also include, but are not limited to, carcinomas, *e.g.* various subtypes, including, for example, adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma), sarcomas (including, for example, bone and soft tissue), leukemias (including, for example, acute myeloid, acute lymphoblastic, chronic myeloid, chronic lymphocytic, and hairy cell), lymphomas and myelomas (including, for example, Hodgkin and non-Hodgkin lymphomas, light chain, non-secretory, MGUS, and plasmacytomas), and central nervous system cancers (including, for example, brain (*e.g.* gliomas (*e.g.* astrocytoma, oligodendroglioma, and ependymoma), meningioma, pituitary adenoma, and neuromas, and spinal cord tumors (*e.g.* meningiomas and neurofibroma).

Illustrative tumor antigens include, but are not limited to, MART-1/Melan-A, gp100, Dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, Colorectal associated antigen (CRC)-0017-1A/GA733, Carcinoembryonic Antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, Prostate Specific Antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens (*e.g.*, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5), GAGE-family of tumor antigens (*e.g.*, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, α-fetoprotein, E-cadherin, α-catenin, β-catenin and γ-catenin, p120ctn, gp100 Pmel117, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, viral products such as human papilloma virus proteins, Smad family of tumor antigens, Imp-1, NA, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 CT-7, c-erbB-2, CD19, CD20, CD22, CD30, CD33, CD37, CD56, CD70, CD74, CD138, AGS16, MUC1, GPNMB, Ep-CAM, PD-L1, PD-

L2, PMSA, and BCMA (TNFRSF17).. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these tumor antigens.

In some embodiments, the present multi-specific CD8 binding agent recognizes and binds to CD8 as well as an antigen on a tumor cell. In some embodiments, the multi-specific CD8 binding agent directly or indirectly recruits CTLs to the tumor cell or tumor microenvironment.

In various embodiments, the present multi-specific CD8 binding agent has targeting moieties which target two different cells (*e.g.* to make a synapse) or the same cell (*e.g.* to get a more concentrated signaling agent effect).

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with T cells. In some embodiments, the targeting moiety recruits directly or indirectly T cells. In an embodiment, the antigen recognition domains specifically bind to effector T cells. In some embodiments, the antigen recognition domain directly or indirectly recruits effector T cells, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative effector T cells include cytotoxic T cells (*e.g.* αβ TCR, $CD3^+$, $CD8^+$, $CD45RO^+$); $CD4^+$ effector T cells (*e.g.* αβ TCR, $CD3^+$, $CD4^+$, $CCR7^+$, CD62Lhi, $IL\text{-}7R/CD127^+$); $CD8^+$ effector T cells (*e.g.* αβ TCR, $CD3^+$, $CD8^+$, $CCR7^+$, CD62Lhi, $IL\text{-}7R/CD127^+$); effector memory T cells (*e.g.* CD62Llow, $CD44^+$, TCR, $CD3^+$, $IL\text{-}7R/CD127^+$, $IL\text{-}15R^+$, CCR7low); central memory T cells (*e.g.* $CCR7^+$, $CD62L^+$, $CD27^+$; or CCR7hi, $CD44^+$, CD62Lhi, TCR, $CD3^+$, IL-7R/CD127$^+$, $IL\text{-}15R^+$); $CD62L^+$ effector T cells; $CD8^+$ effector memory T cells (TEM) including early effector memory T cells ($CD27^+$ $CD62L^-$) and late effector memory T cells ($CD27^-$ $CD62L^-$) (TemE and TemL, respectively); CD127($^+$)CD25(low/-) effector T cells; CD127($^-$)CD25($^-$) effector T cells; $CD8^+$ stem cell memory effector cells (TSCM) (*e.g.* CD44(low)CD62L(high)CD122(high)sca($^+$)); TH1 effector T-cells (*e.g.* $CXCR3^+$, $CXCR6^+$ and $CCR5^+$; or αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}12R^+$, $IFN\gamma R^+$, $CXCR3^+$), TH2 effector T cells (*e.g.* $CCR3^+$, $CCR4^+$ and $CCR8^+$; or αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}4R^+$, $IL\text{-}33R^+$, $CCR4^+$, $IL\text{-}17RB^+$, $CRTH2^+$); TH9 effector T cells (*e.g.* αβ TCR, $CD3^+$, $CD4^+$); TH17 effector T cells (*e.g.* αβ TCR, $CD3^+$, $CD4^+$, $IL\text{-}23R^+$, $CCR6^+$, $IL\text{-}1R^+$); $CD4^+CD45RO^+CCR7^+$ effector T cells, $ICOS^+$ effector T cells; $CD4^+CD45RO^+CCR7(^-)$ effector T cells; and effector T cells secreting IL-2, IL-4 and/or IFN-γ.

Illustrative T cell antigens of interest include, for example (and inclusive of the extracellular domains, where applicable): CD8, CD3, SLAMF4, IL-2Rα, 4-1BB/TNFRSF9, IL-2 R β, ALCAM, B7-1, IL-4 R, B7-H3, BLAME/SLAMFS, CEACAM1, IL-6 R, CCR3, IL-7 Rα, CCR4, CXCRl/IL-S RA, CCR5, CCR6, IL-10R α, CCR 7, IL-l 0 R β, CCRS, IL-12 R β 1, CCR9, IL-12 R β 2, CD2, IL-13 R α 1, IL-13, CD3, CD4, ILT2/CDS5j, ILT3/CDS5k, ILT4/CDS5d, ILT5/CDS5a, lutegrin α 4/CD49d, CDS, Integrin α E/CD103, CD6, Integrin α M/CD 11 b, CDS, Integrin α X/CD11c, Integrin β 2/CDIS, KIR/CD15S, CD27/TNFRSF7, KIR2DL1, CD2S, KIR2DL3, CD30/TNFRSFS, KIR2DL4/CD15Sd, CD31/PECAM-1, KIR2DS4, CD40 Ligand/TNFSF5, LAG-3, CD43, LAIR1, CD45, LAIR2, CDS3, Leukotriene B4-R1, CDS4/SLAMF5, NCAM-L1, CD94, NKG2A, CD97, NKG2C, CD229/SLAMF3, NKG2D, CD2F-10/SLAMF9, NT-4, CD69, NTB-A/SLAMF6, Common γ Chain/IL-2 R γ,

Osteopontin, CRACC/SLAMF7, PD-1, CRTAM, PSGL-1, CTLA-4, RANK/TNFRSF11A, CX3CR1, CX3CL1, L-Selectin, CXCR3, SIRP β 1, CXCR4, SLAM, CXCR6, TCCR/WSX-1, DNAM-1, Thymopoietin, EMMPRIN/CD147, TIM-1, EphB6, TIM-2, Fas/TNFRSF6, TIM-3, Fas Ligand/TNFSF6, TIM-4, Fcγ RIII/CD16, TIM-6, TNFR1/TNFRSF1A, Granulysin, TNF RIII/TNFRSF1B, TRAIL Rl/TNFRSFlOA, ICAM-1/CD54, TRAIL R2/TNFRSF10B, ICAM-2/CD102, TRAILR3/TNFRSF10C,IFN-γR1, TRAILR4/TNFRSF10D, IFN-γ R2, TSLP, IL-1 R1 and TSLP R. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these illustrative T cell antigens.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with B cells. In some embodiments, the targeting moiety directly or indirectly recruits B cells, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative B cell antigens of interest include, for example, CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD37, CD38, CD39, CD40, CD70, CD72, CD73, CD74, CDw75, CDw76, CD77, CD78, CD79a/b, CD80, CD81, CD82, CD83, CD84, CD85, CD86, CD89, CD98, CD126, CD127, CDw130, CD138, CDw150, and B-cell maturation antigen (BCMA). In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these illustrative B cell antigens.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically bind to a target (*e.g.* antigen, receptor) associated with Natural Killer cells. In some embodiments, the targeting moiety directly or indirectly recruits Natural Killer cells, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative Natural Killer cell antigens of interest include, for example TIGIT, 2B4/SLAMF4, KIR2DS4, CD155/PVR, KIR3DL1, CD94, LMIR1/CD300A, CD69, LMIR2/CD300c, CRACC/SLAMF7, LMIR3/CD300LF, Kir1alpha, DNAM-1, LMIR5/CD300LB, Fc-epsilon RII, LMIR6/CD300LE, Fc-γ Rl/CD64, MICA, Fc-γ RIIB/CD32b, MICB, Fc-γ RIIC/CD32c, MULT-1, Fc-γ RIIA/CD32a, Nectin-2/CD112, Fc-γ RIII/CD16, NKG2A, FcRH1/IRTA5, NKG2C, FcRH2/IRTA4, NKG2D, FcRH4/IRTA1, NKp30, FcRH5/IRTA2, NKp44, Fc-Receptor-like 3/CD16-2, NKp46/NCR1, NKp80/KLRF1, NTB-A/SLAMF6, Rae-1, Rae-1 α, Rae-1 β, Rae-1 delta, H60, Rae-1 epsilon, ILT2/CD85j, Rae-1 γ, ILT3/CD85k, TREM-1, ILT4/CD85d, TREM-2, ILT5/CD85a, TREM-3, KIR/CD158, TREML1/TLT-1, KIR2DL1, ULBP-1, KIR2DL3, ULBP-2, KIR2DL4/CD158d and ULBP-3. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these illustrative NK cell antigens.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with macrophages/monocytes. In some embodiments, the targeting moiety directly or indirectly recruits macrophages/monocytes, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative macrophages/monocyte antigens of interest include, for example SIRP1a, B7-1/CD80, ILT4/CD85d, B7-H1, ILT5/CD85a, Common β Chain, Integrin

α 4/CD49d, BLAME/SLAMF8, Integrin α X/CDIIc, CCL6/C10, Integrin β 2/CD18, CD155/PVR, Integrin β 3/CD61, CD31/PECAM-1, Latexin, CD36/SR-B3, Leukotriene B4 R1, CD40/TNFRSF5, LIMPIIISR-B2, CD43, LMIR1/CD300A, CD45, LMIR2/CD300c, CD68, LMIR3/CD300LF, CD84/SLAMF5, LMIR5/CD300LB, CD97, LMIR6/CD300LE, CD163, LRP-1, CD2F-10/SLAMF9, MARCO, CRACC/SLAMF7, MD-1, ECF-L, MD-2, EMMPRIN/CD147, MGL2, Endoglin/CD105, Osteoactivin/GPNMB, Fc-γ RI/CD64, Osteopontin, Fc-γ RIIB/CD32b, PD-L2, Fc-γ RIIC/CD32c, Siglec-3/CD33, Fc-γ RIIA/CD32a, SIGNR1/CD209, Fc-γ RIII/CD16, SLAM, GM-CSF R α, TCCR/WSX-1, ICAM-2/CD102, TLR3, IFN-γ RI, TLR4, IFN-gannna R2, TREM-I, IL-I RII, TREM-2, ILT2/CD85j, TREM-3, ILT3/CD85k, TREML1/TLT-1, 2B4/SLAMF 4, IL-10 R α, ALCAM, IL-10 R β, AminopeptidaseN/ANPEP, ILT2/CD85j, Common β Chain, ILT3/CD85k, Clq R1/CD93, ILT4/CD85d, CCR1, ILT5/CD85a, CCR2, CD206, Integrin α 4/CD49d, CCR5, Integrin α M/CDll b, CCR8, Integrin α X/CDIIc, CD155/PVR, Integrin β 2/CD18, CD14, Integrin β 3/CD61, CD36/SR-B3, LAIR1, CD43, LAIR2, CD45, Leukotriene B4-R1, CD68, LIMPIIISR-B2, CD84/SLAMF5, LMIR1/CD300A, CD97, LMIR2/CD300c, CD163, LMIR3/CD300LF, Coagulation Factor III/Tissue Factor, LMIR5/CD300LB, CX3CR1, CX3CL1, LMIR6/CD300LE, CXCR4, LRP-1, CXCR6, M-CSF R, DEP-1/CD148, MD-1, DNAM-1, MD-2, EMMPRIN/CD147, MMR, Endoglin/CD105, NCAM-L1, Fc-γ RI/CD64, PSGL-1, Fc-γ RIIIICD16, RP105, G-CSF R, L-Selectin, GM-CSF R α, Siglec-3/CD33, HVEM/TNFRSF14, SLAM, ICAM-1/CD54, TCCR/WSX-1, ICAM-2/CD102, TREM-I, IL-6 R, TREM-2, CXCRl/IL-8 RA, TREM-3 and TREMLI/TLT-1. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these illustrative macrophage/monocyte antigens.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with dendritic cells. In some embodiments, the targeting moiety directly or indirectly recruits dendritic cells, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect). Illustrative dendritic cell antigens of interest include, for example, CLEC9A, XCR1, RANK, CD36/SRB3, LOX-1/SR-E1, CD68, MARCO, CD163, SR-A1/MSR, CD5L, SREC-1, CL-PI/COLEC12, SREC-II, LIMPIIISRB2, RP105, TLR4, TLR1, TLR5, TLR2, TLR6, TLR3, TLR9, 4-IBB Ligand/TNFSF9, IL-12/IL-23 p40, 4-Amino-1,8-naphthalimide, ILT2/CD85j, CCL21/6Ckine, ILT3/CD85k, 8-oxo-dG, ILT4/CD85d, 8D6A, ILT5/CD85a, A2B5, lutegrin α 4/CD49d, Aag, Integrin β 2/CD18, AMICA, Langerin, B7-2/CD86, Leukotriene B4 RI, B7-H3, LMIR1/CD300A, BLAME/SLAMF8, LMIR2/CD300c, Clq R1/CD93, LMIR3/CD300LF, CCR6, LMIR5/CD300LB CCR7, LMIR6/CD300LE, CD40/TNFRSF5, MAG/Siglec-4-a, CD43, MCAM, CD45, MD-1, CD68, MD-2, CD83, MDL-1/CLEC5A, CD84/SLAMF5, MMR, CD97, NCAMLI, CD2F-10/SLAMF9, Osteoactivin GPNMB, Chern 23, PD-L2, CLEC-1, RP105, CLEC-2, CLEC-8, Siglec-2/CD22, CRACC/SLAMF7, Siglec-3/CD33, DC-SIGN, DEC-205, Siglec-5, DC-SIGNR/CD299, Siglec-6, DCAR, Siglec-7, DCIR/CLEC4A, Siglec-9, DEC-205, Siglec-10, Dectin-1/CLEC7A, Siglec-F, Dectin-2/CLEC6A, SIGNR1/CD209, DEP-1/CD148, SIGNR4, DLEC, SLAM, EMMPRIN/CD147, TCCR/WSX-1, Fc-γ R1/CD64, TLR3, Fc-γ RIIB/CD32b, TREM-1, Fc-γ RIIC/CD32c, TREM-2, Fc-γ RIIA/CD32a, TREM-3, Fc-γ RIII/CD16, TREML1/TLT-1, ICAM-2/CD102, DEC205, and Vanilloid R1. In

various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these illustrative DC antigens.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds a target (*e.g.* antigen, receptor) on immune cells selected from, but not limited to, megakaryocytes, thrombocytes, erythrocytes, mast cells, basophils, neutrophils, and eosinophils. In some embodiments, the antigen recognition domains directly or indirectly recruit megakaryocytes, thrombocytes, erythrocytes, mast cells, basophils, neutrophils, and eosinophil, *e.g.*, in some embodiments, to a therapeutic site (*e.g.* a locus with one or more disease cell or cell to be modulated for a therapeutic effect).

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with megakaryocytes and/or thrombocytes. Illustrative megakaryocyte and/or thrombocyte antigens of interest include, for example, GP IIb/IIIa, GPIb, vWF, PF4, and TSP. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these illustrative megakaryocyte and/or thrombocyte antigens.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with erythrocytes. Illustrative erythrocyte antigens of interest include, for example, CD34, CD36, CD38, CD41a (platelet glycoprotein IIb/IIIa), CD41b (GPIIb), CD71 (transferrin receptor), CD105, glycophorin A, glycophorin C, c-kit, HLA-DR, H2 (MHC-II), and Rhesus antigens. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these illustrative erythrocyte antigens.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with mast cells. Illustrative mast cells antigens of interest include, for example, SCFR/CD117, $Fc_{\varepsilon}RI$, CD2, CD25, CD35, CD88, CD203c, C5R1, CMAI, FCERIA, FCER2, TPSABI. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these mast cell antigens.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with basophils. Illustrative basophils antigens of interest include, for example, $Fc_{\varepsilon}RI$, CD203c, CD123, CD13, CD107a, CD107b, and CD164. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these basophil antigens.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with neutrophils. Illustrative neutrophils antigens of interest include, for example, 7D5, CD10/CALLA, CD13, CD16 (FcRIII), CD18 proteins (LFA-1, CR3, and p150, 95), CD45, CD67, and CD177. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these neutrophil antigens.

In some embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a target (*e.g.* antigen, receptor) associated with eosinophils. Illustrative eosinophils antigens of interest include, for example, CD35, CD44 and CD69. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these eosinophil antigens.

In various embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to any appropriate antigen or receptor or cell surface markers known by the skilled artisan. In some embodiments, the antigen or cell surface marker is a tissue-specific marker. Illustrative tissue-specific markers include, but are not limited to, endothelial cell surface markers such as ACE, CD14, CD34, CDH5, ENG, ICAM2, MCAM, NOS3, PECAMI, PROCR, SELE, SELP, TEK, THBD, VCAMI, VWF; smooth muscle cell surface markers such as ACTA2, MYHIO, MYHI 1, MYH9, MYOCD; fibroblast (stromal) cell surface markers such as ALCAM, CD34, COLIAI, COL1A2, COL3A1, FAP, PH-4; epithelial cell surface markers such as CDID, K6IRS2, KRTIO, KRT13, KRT17, KRT18, KRT19, KRT4, KRT5, KRT8, MUCI, TACSTDI; neovasculature markers such as CD13, TFNA, Alpha-v beta-3 ($\alpha_V\beta_3$), E-selectin; and adipocyte surface markers such as ADIPOQ, FABP4, and RETN. In various embodiments, the CD8 binding agent comprises a targeting moiety that binds one or more of these antigens.

In various embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a checkpoint marker expressed on a T cell, *e.g.* one or more of PD-1, CD28, CTLA4, ICOS, BTLA, KIR, LAG3, CD137, OX40, CD27, CD40L, TIM3, and A2aR.

In various embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to a checkpoint marker, *e.g.* one or more of PD-1/PD-L1 or PD-L2, CD28/CD80 or CD86, CTLA4/ CD80 or CD86, ICOS/ICOSL or B7RP1, BTLA/HVEM, KIR, LAG3, CD137/CD137L, OX40/OX40L, CD27, CD40L, TIM3/Gal9, and A2aR.

By way of non-limiting example, in various embodiments, the present multispecific CD8 binding agent comprises a targeting moiety directed against (i) CD8; (ii) a checkpoint marker expressed on a T cell, *e.g.* one or more of PD-1, CD28, CTLA4, ICOS, BTLA, KIR, LAG3, CD137, OX40, Cd27, CD40L, TIM3, and A2aR and/or (iii) a targeting moiety is directed against a tumor cell, along with any of the modified (*e.g.* mutant) signaling agents described herein.

In various embodiments, the present multi-specific CD8 binding agent has one or more targeting moieties directed against PD-1. In some embodiments, the CD8 binding agent has one or more targeting moieties which selectively bind a PD-1 polypeptide. In some embodiments, the CD8 binding agent comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind a PD-1 polypeptide.

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody pembrolizumab (aka MK-3475, KEYTRUDA), or fragments thereof. Pembrolizumab and other humanized anti-PD-1 antibodies are disclosed in

Hamid, et al. (2013) New England Journal of Medicine 369 (2): 134-44, US 8,354,509, and WO 2009/114335, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, pembrolizumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

QVQLVQSGVEVKKPGASVKVSCKASGYTFTNYYMYWVRQAPGQGLEWMGGINPSNGGTNF
NEKFKNRVTLTTDSSTTTAYMELKSLQFDDTAVYYCARRDYRFDMGFDYWGQGTTVTVSS
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPEFLGGPSV
FLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEG
NVFSCSVMHEALHNHYTQKSLSLSLGK (SEQ ID NO:37);

and/or a light chain comprising the amino acid sequence (of:

EIVLTQSPATLSLSPGERATLSCRASKGVSTSGYSYLHWYQQKPGQAPRLLIYLASYLES
GVPARFSGSGSGTDFTLTISSLEPEDFAVYYCQHSRDLPLTFGGGTKVEIKRTVAAPSVF
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC SEQ ID NO:38).

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody, nivolumab (aka BMS-936558, MDX-1106, ONO-4538, OPDIVO), or fragments thereof. Nivolumab (clone 5C4) and other human monoclonal antibodies that specifically bind to PD-1 are disclosed in US 8,008,449 and WO 2006/121168, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, nivolumab or an antigen-binding fragment thereof comprises a heavy chain comprising the amino acid sequence of:

QVQLVESGGG VVQPGRSLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRYY
ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTLVT VSSASTKGPS
VFPLAPCSRS TSESTAALGC LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS
VVTVPSSSLG TKTYTCNVDH KPSNTKVDKR VESKYGPPCP PCPAPEFLGG PSVFLFPPKP
KDTLMISRTP EVTCVVVDVS QEDPEVQFNW YVDGVEVHNA KTKPREEQFN STYRVVSVLT
VLHQDWLNGK EYKCKVSNKG LPSSIEKTIS KAKGQPREPQ VYTLPPSQEE MTKNQVSLTC
LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SRLTVDKSRW QEGNVFSCSV
MHEALHNHYT QKSLSLSLGK (SEQ ID NO:39);

and/or a light chain comprising the amino acid sequence of:

EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA
RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ SSNWPRTFGQ GTKVEIKRTV AAPSVFIFPP
SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT
LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC (SEQ ID NO:40).

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody pidilizumab (aka CT-011, hBAT or hBAT-1), or fragments thereof. Pidilizumab and other humanized anti-PD-I monoclonal antibodies are disclosed in US 2008/0025980 and WO 2009/101611, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the anti-PD-1 antibody or an antigen-binding fragment thereof for use in

the methods provided herein comprises a light chain variable regions comprising an amino acid sequence selected from SEQ ID NOS: 15-18 of US 2008/0025980:

SEQ ID No: 15 of US 2008/0025980 (SEQ ID NO:41):

EIVLTQSPSSLSASVGDRVTITCSARSSVSYMHWYQQKPGKAPKLLIYRTSNLASGVPSR
FSGSGSGTDFTLTINSLQPEDFATYYCQQRSSFPLTFGGGTKLEIK;

SEQ ID No: 16 of US 2008/0025980 (SEQ ID NO:42):

EIVLTQSPSSLSASVGDRVTITCSARSSVSYMHWFQQKPGKAPKLWIYRTSNLASGVPSR
FSGSGSGTDYTLTINSLQPEDFATYYCQQRSSFPLTFGGGTKLEIK;

SEQ ID No: 17 of US 2008/0025980 (SEQ ID NO:43):

EIVLTQSPSSLSASVGDRVTITCSARSSVSYMHWFQQKPGKAPKLWIYRTSNLASGVPSR
FSGSGSGTDYCLTINSLQPEDFATYYCQQRSSFPLTFGGGTKLEIK;

SEQ ID No: 18 of US 2008/0025980 (SEQ ID NO:44):

EIVLTQSPSSLSASVGDRVTITCSARSSVSYMHWFQQKPGKAPKLWIYRTSNLASGVPSR
FSGSGSGTSYCLTINSLQPEDFATYYCQQRSSFPLTFGGGTKLEIK;

and/or a heavy chain comprising an amino acid sequence selected from SEQ ID NOS: 20-24 of US 2008/0025980:

SEQ ID No: 20 of US 2008/0025980 (SEQ ID NO:45):

QVQLVQSGSELKKPGASVKISCKASGYSFSNYGMNWVRQAPGQGLQWMGWINTDSGESTY
AEEFKGRFVFSLDTSVSTAYLQITSLTAEDTGMYFCAKVGYDALDYWGQGTLVTVSS;

SEQ ID No: 21 of US 2008/0025980 (SEQ ID NO:46):

QVQLVQSGSELKKPGASVKISCKASGYTFTNYGMNWVRQAPGQGLQWMGWINTDSGESTY
AEEFKGRFVFSLDTSVSTAYLQITSLTAEDTGMYFCAKVGYDALDYWGQGTLVTVSS;

SEQ ID No: 22 of US 2008/0025980 (SEQ ID NO:47):

QVQLVQSGSELKKPGASVKISCKASGYTFTNYGMNWVRQAPGQGLQWMGWINTDSGESTY
AEEFKGRFVFSLDTSVNTAYLQITSLTAEDTGMYFCVRVGYDALDYWGQGTLVTVSS;

SEQ ID No: 23 of US 2008/0025980 (SEQ ID NO:48):

QIQLVQSGSELKKPGASVKISCKASGYTFTNYGMNWVRQAPGQGLQWMGWINTDSGESTY
AEEFKGRFVFSLDTSVNTAYLQITSLTAEDTGMYFCVRVGYDALDYWGQGTLVTVSS;

SEQ ID No: 24 of US 2008/0025980 (SEQ ID NO:49):

QIQLVQSGSELKKPGASVKISCKASGYTFTNYGMNWVKQAPGQGLKWMGWINTDSGESTY

AEEFKGRFAFSLDTSVNTAYLQITSLNAEDTGMYFCVRVGYDALDYWGQGTLVTVSS.

In an embodiment, the targeting moiety comprises a light chain comprising SEQ ID NO:18 of US 2008/0025980 and a heavy chain comprising SEQ ID NO:22 of US 2008/0025980.

In an embodiment, the targeting moiety comprises AMP-514 (aka MEDI-0680).

In an embodiment, the targeting moiety comprises the PD-L2-Fc fusion protein AMP-224, which is disclosed in WO2010/027827 and WO 2011/066342, the entire disclosures of which are hereby incorporated by reference. In such an embodiment, the targeting moiety may include a targeting domain which comprises SEQ ID NO:4 of WO2010/027827 (SEQ ID NO:50):

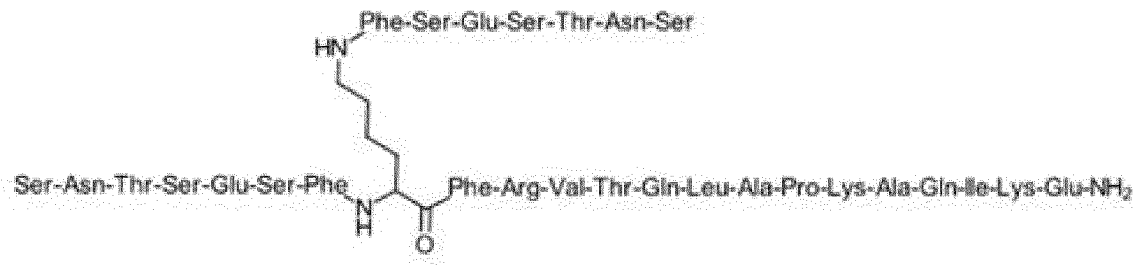
LFTVTVPKELYIIEHGSNVTLECNFDTGSHVNLGAITASLQKVENDTSPHRERATLLEEQ
LPLGKASFHIPQVQVRDEGQYQCIIIYGVAWDYKYLTLKVKASYRKINTHILKVPETDEV
ELTCQATGYPLAEVSWPNVSVPANTSHSRTPEGLYQVTSVLRLKPPPGRNFSCVFWNTHV
RELTLASIDLQSQMEPRTHPTWLLHIFIPFCIIAFIFIATVIALRKQLCQKLYSSKDTTK
RPVTTTKREVNSAI

and/or the B7-DC fusion protein which comprises SEQ ID NO:83 of WO2010/027827 (SEQ ID NO:51):

MIFLLLMLSLELQLHQIAALFTVTVPKELYIIEHGSNVTLECNFDTGSHVNLGAITASLQ
KVENDTSPHRERATLLEEQLPLGKASFHIPQVQVRDEGQYQCIIIYGVAWDYKYLTLKVK
ASYRKINTHILKVPETDEVELTCQATGYPLAEVSWPNVSVPANTSHSRTPEGLYQVTSVL
RLKPPPGRNFSCVFWNTHVRELTLASIDLQSQMEPRTHPTWEPKSCDKTHTCPPCPAPEL
LGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
RDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

In an embodiment, the targeting moiety comprises the peptide AUNP 12 or any of the other peptides disclosed in US 2011/0318373 or 8,907,053. For example, the targeting moiety may comprise AUNP 12 (*i.e.*, Compound 8 or SEQ ID NO:49 of US 2011/0318373) which has the sequence of SEQ ID NO:52:

SNTSESFK(SNTSESF)FRVTQLAPKAQIKE-NH2



or: SNTSESF-NH
|
SNTSESFKFRVTQLAPKAQIKE-NH2

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody 1E3, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1E3 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLQQSGPV LVKPGASVKM SCKASGYTFT DYYMNWVKQS HGKSLEWIGN INPYNGGTTY NQKFKGKATL TVDKSSRTAY MEINSLTSED SAVYYCARGR IYDGSLDYWG QGTALTVSS (SEQ ID NO:53);

and/or a light chain variable region comprising the amino acid sequence of:

DIQMTQFPSS LCASQGGKVT VTCKASQDIN NYMAWYQHKP GKGPRLLIHY TSTLLSGIPS RFSGSGSGRD YSFSISNLEP EDIATYYCLQ YDNLWTFGGG TKLEIK (SEQ ID NO:54).

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody 1E8, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1E8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLQQSGAE LAKPGASVRL SCKASGYTFT NYWMHWVKQR PGQGLEWIGH INPSSGFTTY NQNFKDKATL TADKSSNTAY MQLSSLTYED SAVYFCARED YDVDYWGQGT TLTVSS (SEQ ID NO:55);

and/or a light chain variable region comprising the amino acid sequence of:

DIVMTQSQKF MSTSVGDRVS VTCKASQSVD TNVAWYQQKP GQSPKALIFS ASYRYSGVPD RFTGSGSGTD FTLTINSVQS EDLAEYFCQQ YNSYPYTFGS GTKLEIK (SEQ ID NO:56).

In an embodiment, the targeting moiety comprises the anti-PD-1 antibody 1H3, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1H3 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLVESGGG LVKPGGSLKL SCAASGFTFS DYGMHWVRQA PEKGLEWVAY ISSGSYTIYY TDTVKGRFTI SRDNAKNTLF LQMTSLRSED TAMYYCARRG YGSFYEYYFD YWGQGTTLTV SS (SEQ ID NO:57);

and/or light chain variable region comprising the amino acid sequence of:

QIVLTQSPAL MSASPGEKVT MTCSASSSVS YMYWYQQKPR SSPKPWIYLT SNLASGVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQQW SSNPFTFGSG TKLEIK (SEQ ID NO:58).

In an embodiment, the targeting moiety comprises a VHH directed against PD-1 as disclosed, for example, in US 8,907,065 and WO 2008/071447, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the VHHs against PD-1 comprise SEQ ID NOS: 347-351 of US 8,907,065:

SEQ ID No: 347 of US 8,907,065 (SEQ ID NO:59):

EVQLVESGGGLVQAGKSLRLSCAASGSIFSIHAMGWFRQAPGKEREFVAA
ITWSGGITYYEDSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCAADR
AESSWYDYWGQGTQVTVSS;

SEQ ID No: 348 of US 8,907,065 (SEQ ID NO:60):

EVQLVESGGGLVQAGGSLRLSCAASGSIASIHAMGWFRQAPGKEREFVAV
ITWSGGITYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCAGDK
HQSSWYDYWGQGTQVTVSS;

SEQ ID No: 349 of US 8,907,065 (SEQ ID NO:61):

EVQLVESGGGLVQAGGSLRLSCAASGSISSIHAMGWFRQAPGKEREFVAA
ITWSGGITYYADSLKGRFTISRDNAKNTGYLQMNSLKPEDTAIYYCAADR
AQSSWYDYWGQGTQVTVSS;

SEQ ID No: 350 of US 8,907,065 (SEQ ID NO:62):

EVQLVESGGGLVQAGGSLGLSCAASGSIFSINAMAWFRQAPGKEREFVAL
ISWSGGSTYYEDSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCAADR
VDSNWYDYWGQGTQVTVSS;

SEQ ID No: 351 of US 8,907,065 (SEQ ID NO:63):

EVQLVESGGGLVQAGGSLRLSCAASGRAFSSGTMGWFRRAPGKEREFVA
SIPWSGGRIYYADSVKGRFTISRDNAQNTVYLQMNSLKPEDTAVYYCAVK
ERSTGWDFASWGQCTQVTVSS.

In an embodiment, the targeting moiety comprises any one of the anti-PD-1 antibodies, or fragments thereof, as disclosed in US2011/0271358 and WO2010/036959, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID NOS: 25-29 of US2011/0271358:

SEQ ID No: 25 of US2011/0271358 (SEQ ID NO:64):

QVQLVQSGAELKQPGASVKMSCKASGYSFTSSWIHWVKQAPGQGLEWIGYIYPSTGFTEY
NQKFKDRATLTADKSTSTAYMELSSLRSEDSAVYYCARWRDSSGYHAMDYWGQGTSVTVS
S;

SEQ ID No: 26 of US2011/0271358 (SEQ ID NO:65):

QVQLVQSGAEVKQPGASVKMSCKASGYSFTSSWIHWVKQAPGQGLEWIGYIYPSTGFTEY
NQKFKDRATLTADKSTSTAYMELSSLRSEDTAVYY3/d10CARWRDSSGYHAMDYWGQGTSVTVS
S;

SEQ ID No: 27 of US2011/0271358 (SEQ ID NO:66):

QVQLVQSGHEVKQPGASVKMSCKASGYSFTSSWIHWVKQAPGQGLEWIGYIYPSTGFTEY
NQKFKDRATLTADKSTSTAYMELSSLRSEDTAVYYCARWRDSSGYHAMDYWGQGTLVTVS
S;

SEQ ID No: 28 of US2011/0271358 (SEQ ID NO:67):

QVQLVQSGHEVKQPGASVKMSCKASGYSFTSSWIHWVRQAPGQGLEWIGYIYPSTGFTEY
NQKFKDRATLTADKSTSTAYMELSSLRSEDTAVYYCARWRDSSGYHAMDYWGQGTLVTVS
S;

SEQ ID No: 29 of US2011/0271358 (SEQ ID NO:68):

QVQLVQSGHEVKQPGASVKVSCKASGYSFTSSWIHWVRQAPGQGLEWIGYIYPSTGFTEY
NQKFKDRATITADKSTSTAYMELSSLRSEDTAVYYCARWRDSSGYHAMDYWGQGTLVTVS
S;

and/or a light chain comprising an amino acid sequence selected from SEQ ID NOS: 30-33 of US2011/0271358:

SEQ ID No: 30 of US2011/0271358 (SEQ ID NO:69):

DIVLTQSPASLTLSPGQRLTISCRASQSVSTSGYSYMHWYQQKPDQSPKLLIKFGSNLES
GIPARFSGSGSGTDFTLTISSLEEEDFATYYCQHSWEIPYTFGQGTKLEIK;

SEQ ID No: 31 of US2011/0271358 (SEQ ID NO:70):

DIVLTQSPATLSLSPGQRLTISCRASQSVSTSGYSYMHWYQQKPDQSPKLLIKFGSNLES
GIPARFSGSGSGTDFTLTISSLEPEDFATYYCQHSWEIPYTFGQGTKLEIK;

SEQ ID No: 32 of US2011/0271358 (SEQ ID NO:71):

EIVLTQSPATLSLSPGQRLTISCRASQSVSTSGYSYMHWYQQKPDQSPKLLIKFGSNLES
GIPARFSGSGSGTDFTLTISSLEPEDFATYYCQHSWEIPYTFGQGTKLEIK;

SEQ ID No: 33 of US2011/0271358 (SEQ ID NO:72):

DIVLTQSPATLSLSPGQRLTISCRASQSVSTSGYSYMHWYQQKPDQSPKLLIKFGSNLES
GIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQHSWEIPYTFGQGTKLEIK.

In various embodiments, the present multi-specific CD8 binding agent comprises one or more antibodies directed against PD-1, or antibody fragments thereof, selected from TSR-042 (Tesaro, Inc.), REGN2810 (Regeneron Pharmaceuticals, Inc.), PDR001 (Novartis Pharmaceuticals), and BGB-A317 (BeiGene Ltd.)

In various embodiments, the present multi-specific CD8 binding agent has one or more targeting moieties directed against PD-L1. In some embodiments, the CD8 binding agent has one or more targeting moieties which

selectively bind a PD-L1 polypeptide. In some embodiments, the CD8 binding agent comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind a PD-L1 polypeptide.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody MEDI4736 (aka durvalumab), or fragments thereof. MEDI4736 is selective for PD-L1 and blocks the binding of PD-L1 to the PD-1 and CD80 receptors. MEDI4736 and antigen-binding fragments thereof for use in the methods provided herein comprises a heavy chain and a light chain or a heavy chain variable region and a light chain variable region. The sequence of MEDI4736 is disclosed in WO/2016/06272, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, MEDI4736 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

EVQLVESGGG LVQPGGSLRL SCAASGFTFS RYWMSWVRQA PGKGLEWVAN
IKQDGSEKYY VDSVKGRFTI SRDNAKNSLY LQMNSLRAED TAVYYCAREG
GWFGELAFDY WGQGTLVTVS SASTKGPSVF PLAPSSKSTS GGTAALGCLV
KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTQ
TYICNVNHKP SNTKVDKRVE PKSCDKTHTC PPCPAPEFEG GPSVFLFPPK
PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY
NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPASIEKTI SKAKGQPREP
QVYTLPPSRE EMTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP
VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG
K (SEQ ID NO:73);

and/or a light chain comprising the amino acid sequence of:

EIVLTQSPGT LSLSPGERAT LSCRASQRVS SSYLAWYQQK PGQAPRLLIY
DASSRATGIP DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSLPWTFG
QGTKVEIKRT VAAPSVFIFP PSDEQLKSGT ASVVCLLNNF YPREAKVQWK
VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH KVYACEVTHQ
GLSSPVTKSF NRGEC (SEQ ID NO:74).

In illustrative embodiments, the MEDI4736 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:4 of WO/2016/06272 (SEQ ID NO:75):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYWMSWVRQAPGKGLEWVANIKQDGSEKYY
VDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAREGGWFGELAFDYWGQGTLVTVS
S;

and/or a light chain variable region comprising the amino acid sequence of SEQ ID NO:3 of WO/2016/06272 (SEQ ID NO:76):

EIVLTQSPGTLSLSPGERATLSCRASQRVSSSYLAWYQQKPGQAPRLLIYDASSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSLPWTFGQGTKVEIK

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody atezolizumab (aka MPDL3280A, RG7446), or fragments thereof. In illustrative embodiments, atezolizumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQAPGKGLEWVAWISPYGGSTYYADSVKGRF
TISADTSKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSSASTKGPSVFPLAPSSKST
SGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVN
HKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY
SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:77);

and/or a light chain comprising the amino acid sequence of:

DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSGSG
TDFTLTISSLQPEDFATYYCQQYLYHPATFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLL
NNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSP
VTKSFNRGEC (SEQ ID NO:78).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody avelumab (aka MSB0010718C), or fragments thereof. In illustrative embodiments, avelumab or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising the amino acid sequence of:

EVQLLESGGG LVQPGGSLRL SCAASGFTFS SYIMMWVRQA PGKGLEWVSS
IYPSGGITFY ADTVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARIK
LGTVTTVDYW GQGTLVTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK
DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT
YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP
KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN
STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV
LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK (SEQ ID NO:79);

and/or a light chain comprising the amino acid sequence of:

QSALTQPASV SGSPGQSITI SCTGTSSDVG GYNYVSWYQQ HPGKAPKLMI
YDVSNRPSGV SNRFSGSKSG NTASLTISGL QAEDEADYYC SSYTSSSTRV
FGTGTKVTVL GQPKANPTVT LFPPSSEELQ ANKATLVCLI SDFYPGAVTV
AWKADGSPVK AGVETTKPSK QSNNKYAASS YLSLTPEQWK SHRSYSCQVT
HEGSTVEKTV APTECS (SEQ ID NO:80).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody BMS-936559 (aka 12A4, MDX-1105), or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, BMS-936559 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGSSVKVSCKTSGDTFSTYAISWVRQAPGQGLEWMGGIIPIFGKAHY

AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYFCARKFHFVSGSPFGMDVWGQGTTVT
VSS (SEQ ID NO:81);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGQGTKVEIK (SEQ ID NO:82).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3G10, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3G10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGASVKVSCKASGYTFTDYGFSWVRQAPGQGLEWMGWITAYNGNTNY
AQKLQGRVTMTTDTSTSTVYMELRSLRSDDTAVYYCARDYFYGMDVWGQGTTVTVSS (SEQ ID NO:83);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLVWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPRTFGQGTKVEIK (SEQ ID NO:84).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 10A5, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 10A5 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYDVHWVRQAPGQRLEWMGWLHADTGITKF
SQKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYCARERIQLWFDYWGQGTLVTVSS (SEQ ID NO:85);

and/or a light chain variable region comprising the amino acid sequence of:

DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK (SEQ ID NO:86).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 5F8, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 5F8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGSSVKVSCKVSGGIFSTYAINWVRQAPGQGLEWMGGIIPIFGTANH
AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARDQGIAAALFDYWGQGTLVTVSS (SEQ ID NO:87);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQGTKVEIK (SEQ ID NO:88).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 10H10, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 10H10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLVESGGGLVQPGRSLRLSCAVSGFTFDDYVVHWVRQAPGKGLEWVSGISGNSGNIGY
ADSVKGRFTISRDNAKNSLYLQMNSLRAEDTALYYCAVPFDYWGQGTLVTVSS (SEQ ID NO:89);

and/or a light chain variable region comprising the amino acid sequence of:

DIQMTQSPSSLSASVGDRVTITCRASQGISSWLAWYQQKPEKAPKSLIYAASSLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPYTFGQGTKLEIK (SEQ ID NO:90).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 1B12, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1B12 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGSSVKVSCKTSGDTFSSYAISWVRQAPGQGLEWMGGIIPIFGRAHY
AQKFQGRVTITADESTSTAYMELSSLRSEDTAVYFCARKFHFVSGSPFGMDVWGQGTTVT
VSS (SEQ ID NO:91);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGQGTKVEIK (SEQ ID NO:92).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 7H1, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 7H1 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGSSVKVSCKTSGGTFSSYAISWVRQAPGQGLEWMGGIIPIFGKAHY
AQKFQGRVTITADESTTTAYMELSSLRSEDTAVYYCARKYDYVSGSPFGMDVWGQGTTVT
VSS (SEQ ID NO:93);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGQGTKVEIK (SEQ ID NO:94).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 11E6, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by

reference. In illustrative embodiments, 11E6 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAINWVRQAPGQGLEWMGGIIPIFGSANY
AQKFQDRVTITADESTSAAYMELSSLRSEDTAVYYCARDSSGWSRYYMDVWGQGTTVTVS
S (SEQ ID NO:95);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPFGGGTKVEIK (SEQ ID NO:96).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 12B7, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 12B7 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

QVQLVQSGAEVKEPGSSVKVSCKASGGTFNSYAISWVRQAPGQGLEWMGGIIPLFGIAHY
AQKFQGRVTITADESTNTAYMDLSSLRSEDTAVYYCARKYSYVSGSPFGMDVWGQGTTVT
VSS (SEQ ID NO:97);

and/or a light chain variable region comprising the amino acid sequence of:

EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRATGIPA
RFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPTFGQGTRLEIK (SEQ ID NO:98).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 13G4, or fragments thereof, as disclosed in US 2013/0309250 and WO2007/005874, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 13G4 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLVESGGGLVQPGRSLRLSCAASGITFDDYGMHWVRQAPGKGLEWVSGISWNRGRIEY
ADSVKGRFTISRDNAKNSLYLQMNSLRAEDTALYYCAKGRFRYFDWFLDYWGQGTLVTVS
S (SEQ ID NO:99);

and/or a light chain variable region comprising the amino acid sequence of:

AIQLTQSPSSLSASVGDRVTITCRASQGISSALAWYQQKPGKAPKLLIYDASSLESGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQFNSYPFTFGPGTKVDIK (SEQ ID NO:100).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 1E12, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1E12 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVKLQESGPS LVKPSQTLSL TCSVTGYSIT SDYWNWIRKF PGNKLEYVGY
ISYTGSTYYN PSLKSRISIT RDTSKNQYYL QLNSVTSEDT ATYYCARYGG

WLSPFDYWGQ GTTLTVSS (SEQ ID NO:101);

and/or a light chain variable region comprising the amino acid sequence of:

DIVMTQSHKL MSTSVGDRVS ITCKASQDVG TAVAWYQQKP GQSPKLLIYW
ASTRHTGVPD RFTGSGSGTD FTLTISNVQS EDLADYFCQQ DSSYPLTFGA
GTKVELK (SEQ ID NO:102).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 1F4, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 1F4 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLQESGPG LVAPSQSLSI TCTVSGFSLT TYSINWIRQP PGKGLEWLGV
MWAGGGTNSN SVLKSRLIIS KDNSKSQVFL KMNSLQTDDT ARYYCARYYG
NSPYYAIDYW GQGTSVTVSS (SEQ ID NO:103);

and/or a light chain variable region comprising the amino acid sequence of:

DIVTTQSHKL MSTSVGDRVS ITCKASQDVG TAVAWYQQKP GQSPKLLIYW
ASTRHTGVPD RFTGSGSGTD FTLTISNVQS EDLADYFCQQ DSSYPLTFGA
GTKVELK (SEQ ID NO:104).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2G11, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2G11 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVKLQESGPS LVKPSQTLSL TCSVTGYSII SDYWNWIRKF PGNKLEYLGY
ISYTGSTYYN PSLKSRISIT RDTSKNQYYL QLNSVTTEDT ATYYCARRGG
WLLPFDYWGQ GTTLTVSS (SEQ ID NO:105);

and/or a light chain variable region comprising the amino acid sequence of:

DIVMTQSPSS LAVSVGEKVS MGCKSSQSLL YSSNQKNSLA WYQQKPGQSP
KLLIDWASTR ESGVPDRFTG SGSGTDFTLT ISSVKAEDLA VYYCQQYYGY
PLTFGAGTKL ELK (SEQ ID NO:106).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3B6, or fragments thereof, as disclosed in US 2014/0044738, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3B6 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVKLQESGPS LVKPGASVKL SCKASGYTFT SYDINWVKQR PGQGLEWIGW
IFPRDNNTKY NENFKGKATL TVDTSSTTAY MELHSLTSED SAVYFCTKEN
WVGDFDYWGQ GTTLTLSS (SEQ ID NO:107);

and/or a light chain variable region comprising the amino acid sequence of:

DIVMTQSPAI MSASPGEKVT MTCSASSSIR YMHWYQQKPG TSPKRWISDT
SKLTSGVPAR FSGSGSGTSY ALTISSMEAE DAATYYCHQR SSYPWTFGGG
TKLEIK (SEQ ID NO:108).

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3D10, or fragments thereof, as disclosed in US 2014/0044738 and WO2012/145493, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3D10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

EVQLQQSGPD LVTPGASVRI SCQASGYTFP DYYMNWVKQS HGKSLEWIGD
IDPNYGGTTY NQKFKGKAIL TVDRSSSTAY MELRSLTSED SAVYYCARGA
LTDWGQGTSL TVSS (SEQ ID NO:109);

and/or a light chain variable region comprising the amino acid sequence of:

QIVLSQSPAI LSASPGEKVT MTCRASSSVS YIYWFQQKPG SSPKPWIYAT
FNLASGVPAR FSGSGSGTSY SLTISRVETE DAATYYCQQW SNNPLTFGAG
TKLELK (SEQ ID NO:110).

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in US2011/0271358 and WO2010/036959, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 34-38 of US2011/0271358:

SEQ ID No: 34 of US2011/0271358 (SEQ ID NO:111):

EVQLVQSGPELKKPGASVKMSCKASGYTFTSYVMHWVKQAPGQRLEWIGYVNPFNDGTKY
NEMFKGRATLTSDKSTSTAYMELSSLRSEDSAVYYCARQAWGYPWGQGTLVTVSS;

SEQ ID No: 35 of US2011/0271358 (SEQ ID NO:112):

EVQLVQSGAEVKKPGASVKMSCKASGYTFTSYVMHWVKQAPGQRLEWIGYVNPFNDGTKY
NEMFKGRATLTSDKSTSTAYMELSSLRSEDTAVYYCARQAWGYPWGQGTLVTVSS;

SEQ ID No: 36 of US2011/0271358 (SEQ ID NO:113):

EVQLVQSGAEVKKPGASVKMSCKASGYTFTSYVMHWVRQAPGQRLEWIGYVNPFNDGTKY
NEMFKGRATLTSDKSTSTAYMELSSLRSEDTAVYYCARQAWGYPWGQGTLVTVSS;

SEQ ID No: 37 of US2011/0271358 (SEQ ID NO:114):

EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYVMHWVRQAPGQRLEWIGYVNPFNDGTKY
NEMFKGRATLTSDKSTSTAYMELSSLRSEDTAVYYCARQAWGYPWGQGTLVTVSS;

SEQ ID No: 38 of US2011/0271358 (SEQ ID NO:115):

EVQLVQSGAEVKKPGASVKVSCKASGYTFTSYVMHWVRQAPGQRLEWIGYVNPFNDGTKY
NEMFKGRATITSDKSTSTAYMELSSLRSEDTAVYYCARQAWGYPWGQGTLVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 39-42 of US2011/0271358:

SEQ ID No: 39 of US2011/0271358 (SEQ ID NO:116):

DIVLTQSPASLALSPGERATLSCRATESVEYYGTSLVQWYQQKPGQPPKLLIYAASSVDS
GVPSRFSGSGSGTDFTLTINSLEEEDAAMYFCQQSRRVPYTFGQGTKLEIK;

SEQ ID No: 40 of US2011/0271358 (SEQ ID NO:117):

DIVLTQSPATLSLSPGERATLSCRATESVEYYGTSLVQWYQQKPGQPPKLLIYAASSVDS
GVPSRFSGSGSGTDFTLTINSLEAEDAAMYFCQQSRRVPYTFGQGTKLEIK;

SEQ ID No: 41 of US2011/0271358 (SEQ ID NO:118):

EIVLTQSPATLSLSPGERATLSCRATESVEYYGTSLVQWYQQKPGQPPKLLIYAASSVDS
GVPSRFSGSGSGTDFTLTINSLEAEDAAMYFCQQSRRVPYTFGQGTKLEIK;

SEQ ID No: 42 of US2011/0271358 (SEQ ID NO:119):

DIVLTQSPATLSLSPGERATLSCRATESVEYYGTSLVQWYQQKPGQPPKLLIYAASSVDS
GVPSRFSGSGSGTDFTLTINSLEAEDAATYFCQQSRRVPYTFGQGTKLEIK.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.7A4, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.7A4 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 2 of WO 2011/066389 (SEQ ID NO:120):

EVQLVESGGGLVKPGGSLRLSCAASGFTFSTYSMNWVRQAPGKGLEWVSSISSSGDYIYY
ADSVKGRFTISRDNAKNSLFLQMNSLKAEDTAVYYCARDLVTSMVAFDYWGQGTLVTVSS;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 7 of WO 2011/066389 (SEQ ID NO:121):

SYELTQPPSVSVSPGQAARITCSGDALPQKYVFWYQQKSGQAPVLVIYEDSKRPSGIPER
FSGSSSGTMATLTISGAQVEDEADYYCYSTDRSGNHRVFGGGTRLTVL.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.9D10, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.9D10 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 12 of WO 2011/066389 (SEQ ID NO:122):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVANIKQDGGEQYY
VDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDWNYGYYDMDVWGQGTTVTVSS;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 17 of WO 2011/066389 (SEQ ID NO:123):

EIVLTQSPGTLSLSPGERATLSCRASQSVSSNYLAWFQQKPGQAPRLLIFGTSSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSIFTFGPGTKVDIK.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.14H9, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.14H9 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 22 of WO 2011/066389 (SEQ ID NO:124):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYWMSWVRQAPGKGLEWVANIKQDGSEKYY
VDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAREGGWFGELAFDYWGQGTLVTVS
S;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 27 of WO 2011/066389 (SEQ ID NO:125):

EIVLTQSPGTLSLSPGERATLSCRASQRVSSSYLAWYQQKPGQAPRLLIYDASSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSLPWTFGQGTEVEIK.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.20A8, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.20A8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 32 of WO 2011/066389 (SEQ ID NO:126):

EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYAMSWVRQAPGKGLEWVSAIRGSGGSTYY
ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKDLHYDSSGYLDYWGQGTLVTVS
S;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 37 of WO 2011/066389 (SEQ ID NO:127):

DIQMTQSPSSVSASVGDRVTITCRASQGIRSWLAWYQQKPGKAPKLLIYAISRLQSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQANSFPLTFGGGTKVEIK.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3.15G8, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby

incorporated by reference. In illustrative embodiments, 3.15G8 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 42 of WO 2011/066389 (SEQ ID NO:128):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYWMSWVRQAPGKGLEWVANIKQDGGEKYY
VDSVKGRFTISRDNAKNSLFLQMNSLRAEDTAVYYCARVQLYSDYFDYWGQGTLVTVSS;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 47 of WO 2011/066389 (SEQ ID NO:129):

DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQQKSGKAPKLLIYAASGLQSGVPS
RFSGSGSGTDFTLTISSLQPEDLATYYCQQSHSLPPTFGQGTKVEIK.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 3.18G1, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 3.18G1 or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 52 of WO 2011/066389 (SEQ ID NO:130):

EVQLLESGGDLVQPGGSLRLSCAASGFTFNSYAMSWVRQAPGKGLEWVSTISGSGGFTFS
ADSVKGRFTISRDNSKNTLFLQMNSLRVEDSAVYSCAKVLVGFNNGCWDYWGQGTLVTVS
S;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 57 of WO 2011/066389 (SEQ ID NO:131):

SYVLTQPPSVSVAPGQTARITCGGNNIGSKSVHWYQQKPGQAPVLVVYDDSDRPSGIPER
FSGSNSGNTATLTISRVEAGDEADYYCQVWDSSNDHVVFGGGTKLTVL.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.7A4OPT, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.7A4OPT or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 62 of WO 2011/066389 (SEQ ID NO:132):

EVQLVESGGGLVKPGGSLRLSCAASGFTFSTYSMNWVRQAPGKGLEWVSSISSSGDYIYY
ADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARDLVTSMVAFDYWGQGTLVTVSS;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 67 of WO 2011/066389 (SEQ ID NO:133):

SYELTQPPSVSVSPGQTARITCSGDALPQKYVFWYQQKSGQAPVLVIYEDSKRPSGIPER
FSGSSSGTMATLTISGAQVEDEADYYCYSTDRSGNHRVFGGGTKLTVL.

In an embodiment, the targeting moiety comprises the anti-PD-L1 antibody 2.14H9OPT, or fragments thereof, as disclosed in WO 2011/066389, US8,779,108, and US2014/0356353, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, 2.14H9OPT or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain variable region comprising the amino acid sequence of:

SEQ ID No: 72 of WO 2011/066389 (SEQ ID NO:134):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSRYWMSWVRQAPGKGLEWVANIKQDGSEKYY
VDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAREGGWFGELAFDYWGQGTLVTVS
S;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 77 of WO 2011/066389 (SEQ ID NO:135):

EIVLTQSPGTLSLSPGERATLSCRASQRVSSSYLAWYQQKPGQAPRLLIYDASSRATGIP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSLPWTFGQGTKVEIK.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO2016/061142, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 18, 30, 38, 46, 50, 54, 62, 70, and 78 of WO2016/061142:

SEQ ID No: 18 of WO2016/061142 (SEQ ID NO:136):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMYWVRQATGQGLEWMGRIDPNSGSTKY
NEKFKNRFTISRDDSKNTAYLQMNSLKTEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 30 of WO2016/061142 (SEQ ID NO:137):

EVQLVQSGAEVKKPGATVKISCKVSGYTFTSYWMYWVRQATGQGLEWMGRIDPNSGSTKY
NEKFKNRVTITADKSTSTAYMELSSLRSEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 38 of WO2016/061142 (SEQ ID NO:138):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTSYWMYWVRQAPGQGLEWMGRIDPNSGSTKY
NEKFKNRVTISVDTSKNQFSLKLSSVTAADTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 46 of WO2016/061142(SEQ ID NO:139):

EVQLVQSGAEVKKPGATVKISCKVSGYTFTSYWMYWIRQSPSRGLEWLGRIDPNSGSTKY
NEKFKNRLTISKDTSKNQVVLTMTNMDPVDTATYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 50 of WO2016/061142 (SEQ ID NO:140):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTSYWMYWIRQPPGKGLEWIGRIDPNSGSTKY
NEKFKNRVTITADKSTSTAYMELSSLRSEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 54 of WO2016/061142 (SEQ ID NO:141):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMYWIRQSPSRGLEWLGRIDPNSGSTKY
NEKFKNRFTISRDDSKNTAYLQMNSLKTEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 62 of WO2016/061142 (SEQ ID NO:142):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTSYWMYWVRQARGQRLEWIGRIDPNSGSTKY
NEKFKNRLTISKDTSKNQVVLTMTNMDPVDTATYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 70 of WO2016/061142 (SEQ ID NO:143):

QITLKESGPTLVKPTQTLTLTCTFSGYTFTSYWMYWVRQAPGKGLEWVSRIDPNSGSTKY
NEKFKNRVTITADKSTSTAYMELSSLRSEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

SEQ ID No: 78 of WO2016/061142 (SEQ ID NO:144):

EVQLVQSGAEVKKPGATVKISCKVSGYTFTSYWMYWVRQARGQRLEWIGRIDPNSGSTKY
NEKFKNRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDYRKGLYAMDYWGQGTTVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 22, 26, 34, 42, 58, 66, 74, 82, and 86 of WO2016/061142:

SEQ ID No: 22 of WO2016/061142 (SEQ ID NO:145):

DIVMTQTPLSLPVTPGEPASISCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGIPA
RFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 26 of WO2016/061142 (SEQ ID NO:146):

DIQMTQSPSSLSASVGDRVTITCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 34 of WO2016/061142 (SEQ ID NO:147):

EIVLTQSPDFQSVTPKEKVTITCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGVPD
RFSGSGSGTDFTLKISRVEAEDVGVYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 42 of WO2016/061142 (SEQ ID NO:148):

EIVLTQSPDFQSVTPKEKVTITCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGVPS
RFSGSGSGTDFTFTISSLQPEDIATYYCQQYNSYPLTFGQGTKVEIK.

SEQ ID No: 58 of WO2016/061142 (SEQ ID NO:149):

EIVLTQSPATLSLSPGERATLSCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGIPP

RFSGSGYGTDFTLTINNIESEDAAYYFCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 66 of WO2016/061142 (SEQ ID NO:150):

DVVMTQSPLSLPVTLGQPASISCKASQDVGTAVAWYQQKPGQAPRLLIYWASTRHTGVPS
RFSGSGSGTEFTLTISSLQPDDFATYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 74 of WO2016/061142 (SEQ ID NO:151):

DIQMTQSPSSLSASVGDRVTITCKASQDVGTAVAWYQQKPGQAPRLLIYWASTRHTGVPS
RFSGSGSGTDFTFTISSLQPEDIATYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 82 of WO2016/061142 (SEQ ID NO:152):

AIQLTQSPSSLSASVGDRVTITCKASQDVGTAVAWYLQKPGQSPQLLIYWASTRHTGVPS
RFSGSGSGTDFTFTISSLEAEDAATYYCQQYNSYPLTFGQGTKVEIK;

SEQ ID No: 86 of WO2016/061142 (SEQ ID NO:153):

EIVLTQSPDFQSVTPKEKVTITCKASQDVGTAVAWYQQKPGQAPRLLIYWASTRHTGVPS
RFSGSGSGTEFTLTISSLQPDDFATYYCQQYNSYPLTFGQGTKVEIK.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO2016/022630, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 2, 6, 10, 14, 18, 22, 26, 30, 34, 38, 42, and 46 of WO2016/022630:

SEQ ID No: 2 of WO2016/022630 (SEQ ID NO:154):

EVKLVESGGGLVKPGGSLKLSCAASGFIFRSYGMSWVRQTPEKRLEWVASISSGGSTYYP
DSVKGRFTISRDNARNILYLQMSSLRSEDTAMYDCARGYDSGFAYWGQGTLVTVSE;

SEQ ID No: 6 of WO2016/022630 (SEQ ID NO:155):

EVKLVESGGGLVKPGGSLKLSCAASGFTFRSYGMSWVRQTPEKRLEWVASISSGGTTYYP
DSVKGRFIISRDNARNILYLQMSSLRSEDTAMYYCAKGYDSGFAYWGQGTLVIVSA;

SEQ ID No: 10 of WO2016/022630 (SEQ ID NO:156):

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTTYGVHWVRQSPGKGLEWLGVIWRGVTTDYN
AAFMSRLTITKDNSKSQVFFKMNSLQANDTAIYYCARLGFYAMDYWGQGTSVTVSS;

SEQ ID No: 14 of WO2016/022630 (SEQ ID NO:157):

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTSYGVHWVRQSPGKGLEWLGVIWSGGVTDYN
AAFISRLSISKDNSKSQVFFKMNSLQANDTAIYYCARLGFYAMDYWGQGTSVTVSS;

SEQ ID No: 18 of WO2016/022630 (SEQ ID NO:158):

EVKLFESGGGLVQPGGSLKLSCVASGFDFSTYWMHWVRQAPGQGLEWIGQINPDSTTINY
APSLKDRFIISRDNAKNTLFLQMSKVRSEDTALYYCAKPGDYGYDFDCWGQGTTLTVSS;

SEQ ID No: 22 of WO2016/022630 (SEQ ID NO:159):

EVQLQESGPSLVKPSQTLSLTCSVTGDSITSGYWNWIRKFPGNKLEYMGYISYSGSTYYN
PSLKSRISITRDTSKNQYYLQLNSVTTEDTATYYCARSLLWFSTGFAYWGQGTLVTVSA;

SEQ ID No: 26 of WO2016/022630 (SEQ ID NO:160):

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTSYGVHWVRQSPGKGLEWLGVIWSGGITDYN
AAFKSRLSISKDNSKSQVFFKMNSLQANDTAIYFCARLGFYAMDYWGQGTSVTVSS;

SEQ ID No: 30 of WO2016/022630 (SEQ ID NO:161):

EVKLVESGGGLVKPGGSLKLSCAASGFTFRSYGMSWARQIPEKRLEWVASISSGGTTYYL
GSVQGRFTISRDNARNILYLQMSSLRSEDTAMYYCARGYDAGFAYWGQGTLVSVSE;

SEQ ID No: 34 of WO2016/022630 (SEQ ID NO:162):

EVQLQESGPSLVKPSQTLSLTCSVTGDSITSGYWTWIRKFPGNKLEYMGYISYTGSTYYN
PSLKSRISISRDTSKSQYYLQLNSVTTEDTATYYCARQRDWLGFAYWGQGTLVTVSA;

SEQ ID No: 38 of WO2016/022630 (SEQ ID NO:163):

EEKLVESGGGLVKPGGSLKLSCAASGFSFSSYGMSWVRQTPEKRLEWVASISSGGSIYYP
DSVKGRFTISRDNARNILYLQMSSLRSEDTAMYYCARGYDAGFAFWGQGTLVTASA;

SEQ ID No: 42 of WO2016/022630 (SEQ ID NO:164):

QITLKESGPTLVKPTQTLTLTCTVSGFSLSTYGVHWIRQPPGKALEWLGVIWRGVTTDYN
AAFMSRLTITKDNSKNQVVLTMNNMDPVDTATYYCARLGFYAMDYWGQGTLVTVSS;

SEQ ID No: 46 of WO2016/022630 (SEQ ID NO:165):

EVQLVESGGGLVKPGGSLRLSCAASGFIFRSYGMSWVRQAPGKGLEWVASISSGGSTYYP
DSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYDCARGYDSGFAYWGQGTLVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 of WO2016/022630:

SEQ ID No: 4 of WO2016/022630 (SEQ ID NO:166):

DIVLTQSPASLAVSLGQRATISCRASQSVSTSSSSFMHWYQQKPGQPPKLLIKYASNLES
GVPARFSGSGSGTDFTLNIHPVEEEDTATYYCQHSWEIPYTFGGGTKLEIKR;

SEQ ID No: 8 of WO2016/022630 (SEQ ID NO:167):

DIVLTQSPPSLAVSLGQRATISCRASQSVSTSSSSYMHWYQQKPGQPPKLLIKYASNLES
GVPARFSGSGSGTDFTLNIHPVEEEDTATYYCQHSWEIPYTFGGGTKLEIK;

SEQ ID No: 12 of WO2016/022630 (SEQ ID NO:168):

SIVMTQTPKFLLVSAGDRVTITCKASQSVSNDVAWYQQKPGQSPKLLIYYAANRYTGVPD
RFTGSGYGTDFTFTISIVQAEDLAVYFCQQDYTSPYTFGGGTKLEIK;

SEQ ID No: 16 of WO2016/022630 (SEQ ID NO:169):

SIVMTQTPKFLLVSAGDRVTITCKASQSVSNDVGWYQQKPGQSPKLLIYYASNRYSGVPD
RFTGSGYGTDFTFTISTVQAEDLAVYFCQQDYTSPYTFGGGTKLEIK;

SEQ ID No: 20 of WO2016/022630 (SEQ ID NO:170):

DVLMTQTPLYLPVSLGDQASISCRSSQIIVHSNANTYLEWFLQKPGQSPKLLIYKVSNRF
SGVPDRFSGSGSGTDFTLKISRVEAEDLGVYYCFQGSHVPYTFGGGTKLEIK;

SEQ ID No: 24 of WO2016/022630 (SEQ ID NO:171):

QIVLTQSPAIMSASPGEKVTLTCSASSSVSSSYLYWNQQKPGSSPKVWIYNTSNLASGVP
ARFSGSGSGTSYSLTISSMEAEDAASYFCHQWRSYPPTLGAGTKLELK;

SEQ ID No: 28 of WO2016/022630 (SEQ ID NO:172):

QIVLTQSPAIMSASPGEKVTMTCSANSSVSYMHWYQQKSGTSPKRWIYDTSKLASGVPAR
FSGSGSGTSYSLTISSMGAEDAATYYCQQWSSNPWTFGGGTKLEIK;

SEQ ID No: 32 of WO2016/022630 (SEQ ID NO:173):

DIVLTQSPASLAVSLGQRATISCRASQSVSTSSYSYMHWYQQKPGQPPKLLIKYASNLES
GVPARFSGSGSGTDFTLNIHPVEEEDTATYYCQNSWEIPYTFGGGTKLEIK;

SEQ ID No: 36 of WO2016/022630 (SEQ ID NO:174):

DIVMTQTPSSLAVSLGEKVTMSCKSSQSLLYSSNQKNSLAWYQQKPGQSPKLLIYWASNR
ESGVPDRFTGSSSGTDFTLTISSVKAEDLAVYYCQQYYSYPLTFGAGTKLELK;

SEQ ID No: 40 of WO2016/022630 (SEQ ID NO:175):

DIVLTQSPASLAVSLGQRATISCRASQSVSTSSYSYVHWYQQKPGQPPKLLIKYASNLES
GVPARFSGSGSGTDFTLNIHPVEEEDTATYYCQHSWEIPYTFGGGTKLEIK;

SEQ ID No: 44 of WO2016/022630 (SEQ ID NO:176):

DIQMTQSPSSLSASVGDRVTITCKASQSVSNDVAWYQQKPGKAPKLLIYYAANRYTGVPD
RFSGSGYGTDFTFTISSLQPEDIATYFCQQDYTSPYTFGQGTKLEIK;

SEQ ID No: 48 of WO2016/022630 (SEQ ID NO:177):

DIVLTQSPASLAVSPGQRATITCRASQSVSTSSSSFMHWYQQKPGQPPKLLIKYASNLES
GVPARFSGSGSGTDFTLTINPVEANDTANYYCQHSWEIPYTFGQGTKLEIK.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO2015/112900, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 38, 50, 82, and 86 of WO 2015/112900:

SEQ ID No: 38 of WO2015/112900 (SEQ ID NO:178):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTTYWMHWVRQATGQGLEWMGNIYPGTGGSNF
DEKFKNRVTITADKSTSTAYMELSSLRSEDTAVYYCTRWTTGTGAYWGQGTTVTVSS;

SEQ ID No: 50 of WO 2015/112900 (SEQ ID NO:179):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTTYWMHWIRQSPSRGLEWLGNIYPGTGGSNF
DEKFKNRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTRWTTGTGAYWGQGTTVTVSS;

SEQ ID No: 82 of WO 2015/112900 (SEQ ID NO:180):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTTYWMHWIRQSPSRGLEWLGNIYPGTGGSNF
DEKFKNRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTRWTTGTGAYWGQGTTVTVSS;

SEQ ID No: 86 of WO 2015/112900 (SEQ ID NO:181):

EVQLVQSGAEVKKPGESLRISCKGSGYTFTTYWMHWVRQAPGQGLEWMGNIYPGTGGSNF
DEKFKNRFTISRDNSKNTLYLQMNSLRAEDTAVYYCTRWTTGTGAYWGQGTTVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 42, 46, 54, 58, 62, 66, 70, 74, and 78 of WO 2015/112900:

SEQ ID No: 42 of WO2015/112900 (SEQ ID NO:182):

EIVLTQSPATLSLSPGERATLSCKSSQSLLDSGNQKNFLTWYQQKPGQAPRLLIYWASTR
ESGVPSRFSGSGSGTEFTLTISSLQPDDFATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 46 of WO 2015/112900 (SEQ ID NO:183):

DIQMTQSPSSLSASVGDRVTITCKSSQSLLDSGNQKNFLTWYQQKPGQAPRLLIYWASTR
ESGIPPRFSGSGYGTDFTLTINNIESEDAAYYFCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 54 of WO 2015/112900 (SEQ ID NO:184):

EIVLTQSPATLSLSPGERATLSCKSSQSLLDSGNQKNFLTWYQQKPGKAPKLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 58 of WO 2015/112900 (SEQ ID NO:185):

DIVMTQTPLSLPVTPGEPASISCKSSQSLLDSGNQKNFLTWYQQKPGQAPRLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 62 of WO 2015/112900 (SEQ ID NO:186):

EIVLTQSPATLSLSPGERATLSCKSSQSLLDSGNQKNFLTWYQQKPGKAPKLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 66 of WO 2015/112900 (SEQ ID NO:187):

EIVLTQSPDFQSVTPKEKVTITCKSSQSLLDSGNQKNFLTWYQQKPGQAPRLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 70 of WO 2015/112900 (SEQ ID NO:188):

EIVLTQSPATLSLSPGERATLSCKSSQSLLDSGNQKNFLTWYQQKPGQAPRLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 74 of WO 2015/112900 (SEQ ID NO:189):

DIQMTQSPSSLSASVGDRVTITCKSSQSLLDSGNQKNFLTWYLQKPGQSPQLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK;

SEQ ID No: 78 of WO 2015/112900 (SEQ ID NO:190):

DVVMTQSPLSLPVTLGQPASISCKSSQSLLDSGNQKNFLTWYQQKPGKAPKLLIYWASTR
ESGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQNDYSYPYTFGQGTKVEIK.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies disclosed in WO 2010/077634 and US 8,217,149, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the anti-PD-L1 antibody or an antigen-binding fragment thereof for use in the methods provided herein comprises a heavy chain region comprising the amino acid sequence of:

SEQ ID No: 20 of WO 2010/077634 (SEQ ID NO:191):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDSWIHWVRQAPGKGLEWVAWISPYGGSTYY
ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCARRHWPGGFDYWGQGTLVTVSA;

and/or a light chain variable region comprising the amino acid sequence of:

SEQ ID No: 21 of WO 2010/077634 (SEQ ID NO:192):

DIQMTQSPSSLSASVGDRVTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYSGVPS
RFSGSGSGTDFTLTISSLQPEDFATYYCQQYLYHPATFGQGTKVEIKR.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L1 antibodies obtainable from the hybridoma accessible under CNCM deposit numbers CNCM I-4122, CNCM I-4080 and CNCM I-4081 as disclosed in US 20120039906, the entire disclosures of which are hereby incorporated by reference.

In an embodiment, the targeting moiety comprises a VHH directed against PD-L1 as disclosed, for example, in US 8,907,065 and WO 2008/071447, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the VHHs against PD-L1 comprise SEQ ID NOS: 394-399 of US 8,907,065:

SEQ ID No: 394 of US 8,907,065 (SEQ ID NO:193):

EVQLVESGGGLVQPGGSLRLSCAASGFTLDYYAIGWFRQAPGKEREWASS
ISSSDGSTYYADSVKGRFTISRDNAKNTVFLQMNSLKPEDTAVYSCAASQ
APITIATMMKPFYDYWGQGTQVTVSS;

SEQ ID No: 395 of US 8,907,065 (SEQ ID NO:194):

EVQLVESGGGLVQPGGSLRLSCAASGFTLDYYAKCWFRQAPGKEREWVSC
ISSSDGSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYFCAARH
GGPLTVEYFFDYWGQGTQVTVSS:

SEQ ID No: 396 of US 8,907,065 (SEQ ID NO:195):

EVQLVESGGGLVQPGGSLRLSCAASGFTFDYYAIGWFRQAPGKAREGVSC
ISGGDNSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCATGG
WKYCSGYDPEYIYWGQGTQVTVSS;

SEQ ID No: 397 of US 8,907,065 (SEQ ID NO:196):

EVQLVESGGGLVQAGGSLRLSCAASGSTFSQYDVGWYRQAPGKQRELVA
FSSSGGRTIYPDSVKGRFTFSRDNTKNTVYLQMTSLKPEDTAVYYCKIDW
YLNSYWGQGTQVTVSS;

SEQ ID No: 398 of US 8,907,065 (SEQ ID NO:197):

EVQLVESGGGLVQAGGSLRLSCAASGVDASNSAMGWYRQAPGKQREWVAR
ITGGGLIAYTDSVKGRFTISRDNAKSTVYLQMNSLEPEDTAVYYCNTINS
RDGWGQGTQVTVSS;

SEQ ID No: 399 of US 8,907,065 (SEQ ID NO:198):

EVQLVESGGGLVQAGGSLTISCAASGITFSDSIVSWYRRARGKQREWVAG
ISNGGTTKYAESVLGRFTISRDNAKNNVYLQMNGLNPEDTAVYLCKVRQY
WGQGTQVTVSS.

In various embodiments, the present multi-specific CD8 binding agent has one or more targeting moieties directed against PD-L2. In some embodiments, the CD8 binding agent has one or more targeting moieties which selectively bind a PD-L2 polypeptide. In some embodiments, the CD8 binding agent comprises one or more antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind a PD-L2 polypeptide.

In an embodiment, the targeting moiety comprises a VHH directed against PD-L2 as disclosed, for example, in US 8,907,065 and WO 2008/071447, the entire disclosures of which are hereby incorporated by reference. In illustrative embodiments, the VHHs against PD-1 comprise SEQ ID Nos: 449-455 of US 8,907,065:

SEQ ID No: 449 of US 8,907,065 (SEQ ID NO:199):

EVQLVESGGGLVQAGGSLRLSCAASESTVLINAMGWYRQAPGKQRELVAS
ISSGGSTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNADVY
PQDYGLGYVEGKVYYGHDYWGTGTLVTVSS;

SEQ ID No: 450 of US 8,907,065 (SEQ ID NO:200):

EVQLVESGGGLVQAGGSLRLSCAASGSTFSNYVSNYAMGWGRQAPGTQ
RELVASISNGDTTNYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYY
CFEHQVAGLTWGQGTQVTVSS;

SEQ ID No: 451 of US 8,907,065 (SEQ ID NO:201):

EVQLVESGGGLVQAGGSLRLSCVASGXALKIXVMGWYRQAPGKQRELV
AAITSGGRTNYSDSVKGRFTISGDNAXNTVYLQMNSLKSEDTAVYYCRE
WNSGYPPVDYWGQGTQVTVSS;

SEQ ID No: 452 of US 8,907,065 (SEQ ID NO:202):

EVQLVESGGGLVQAGGSLRLSCAASGRTFSSGTMGWFRRAPGKEREFV
ASIPWSGGRTYYADSVKDRFTISRDNAQNTVFLQMNSLKPEDTAVYYCAF
KERSTGWDFASWGQGIQVTVSS;

SEQ ID No: 453 of US 8,907,065 (SEQ ID NO:203):

EVQLVESGGGLVQTGGSLRLSCAASGFTLDYYGIGWFRQAPGKEREGVS
FISGSDGSTYYAESVKGRFTISRDKAKNTVYLQMNSLKPEDTAVYYCAAD
PWGPPSIATMTSYEYKHWGQGTQVTVSS;

SEQ ID No: 454 of US 8,907,065 (SEQ ID NO:204):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYTMIWLRRAPGKGFEWV
STIDKDGNTNYVDSVKGRFAVSRDNTKNTLYLQMNSLKPEDTAMYYCTK
HGSSARGQGTRVTVSS;

SEQ ID No: 455 of US 8,907,065 (SEQ ID NO:205):

EVQLVESGGGLVEPGGSLRLSCVASGFTFSSYDMSWVRQAPGKGLE
WVSTINSGGGITYRGSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVYY
CENGGSSYRRGQGTQVTVSS.

In an embodiment, the targeting moiety comprises any one of the anti-PD-L2 antibodies disclosed in US2011/0271358 and WO2010/036959, the entire contents of which are hereby incorporated by reference. In illustrative embodiments, the antibody or an antigen-binding fragment thereof for use in the methods provided

herein comprises a heavy chain comprising an amino acid sequence selected from SEQ ID Nos: 43-47 of US2011/0271358:

SEQ ID No: 43 of US2011/0271358 (SEQ ID NO:206):

QVQLVQSGAELKKPGASVKMSCKASGYTFTGYTMHWVKQAPGQGLEWIGYINPRSGYTEY
NQKFKDRTTLTADKSTSTAYMELSSLRSEDSAVYYCARPWFAYWGQGTLVTVSS;

SEQ ID No: 44 of US2011/0271358 (SEQ ID NO:207):

QVQLVQSGAEVKKPGASVKMSCKASGYTFTGYTMHWVKQAPGQGLEWIGYINPRSGYTEY
NQKFKDRTTLTADKSTSTAYMELSSLRSEDTAVYYCARPWFAYWGQGTLVTVSS;

SEQ ID No: 45 of US2011/0271358 (SEQ ID NO:208):

QVQLVQSGAEVKKPGASVKMSCKASGYTFTGYTMHWVRQAPGQGLEWIGYINPRSGYTEY
NQKFKDRTTLTADKSTSTAYMELSSLRSEDTAVYYCARPWFAYWGQGTLVTVSS;

SEQ ID No: 46 of US2011/0271358 (SEQ ID NO:209):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYTMHWVRQAPGQGLEWIGYINPRSGYTEY
NQKFKDRTTLTADKSTSTAYMELSSLRSEDTAVYYCARPWFAYWGQGTLVTVSS;

SEQ ID No: 47 of US2011/0271358 (SEQ ID NO:210):

QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYTMHWVRQAPGQGLEWIGYINPRSGYTEY
NQKFKDRTTITADKSTSTAYMELSSLRSEDTAVYYCARPWFAYWGQGTLVTVSS;

and/or a light chain comprising an amino acid sequence selected from SEQ ID Nos: 48-51 of US2011/0271358:

SEQ ID No: 48 of US2011/0271358 (SEQ ID NO:211):

DIVMTQSPASLTVTPGEKVTITCKSSQSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTR
ESGVPDRFTGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPLTFGQGTKLEIK;

SEQ ID No: 49 of US2011/0271358 (SEQ ID NO:212):

DIVMTQSPASLSVTPGEKVTITCKSSQSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTR
ESGVPDRFTGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPLTFGQGTKLEIK;

SEQ ID No: 50 of US2011/0271358 (SEQ ID NO:213):

DIVMTQSPAFLSVTPGEKVTITCKSSQSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTR
ESGVPDRFTGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPLTFGQGTKLEIK;

SEQ ID No: 51 of US2011/0271358 (SEQ ID NO:214):

DIVMTQSPAFLSVTPGEKVTITCKSSQSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTR
ESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQNDYSYPLTFGQGTKLEIK.

In various embodiments, the targeting moieties of the invention may comprise a sequence that targets PD-1, PD-L1, and/or PD-L2 which is at least about 60%, at least about 61%, at least about 62%, at least about 63%, at least about 64%, at least about 65%, at least about 66%, at least about 67%, at least about 68%, at least about 69%, at least about 70%, at least about 71%, at least about 72%, at least about 73%, at least about 74%, at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or 100% identical to any of the sequences disclosed herein (*e.g.* about 60%, or about 61%, or about 62%, or about 63%, or about 64%, or about 65%, or about 66%, or about 67%, or about 68%, or about 69%, or about 70%, or about 71%, or about 72%, or about 73%, or about 74%, or about 75%, or about 76%, or about 77%, or about 78%, or about 79%, or about 80%, or about 81%, or about 82%, or about 83%, or about 84%, or about 85%, or about 86%, or about 87%, or about 88%, or about 89%, or about 90%, or about 91%, or about 92%, or about 93%, or about 94%, or about 95%, or about 96%, or about 97%, or about 98%, about 99% or about 100% sequence identity with any of the sequences disclosed herein).

In various embodiments, the targeting moieties of the invention may comprise any combination of heavy chain, light chain, heavy chain variable region, light chain variable region, complementarity determining region (CDR), and framework region sequences that target PD-1, PD-L1, and/or PD-L2 as disclosed herein.

Additional antibodies, antibody derivatives or formats, peptides or polypeptides, or fusion proteins that selectively bind or target PD-1, PD-L1 and/or PD-L2 are disclosed in WO 2011/066389, US 2008/0025980, US 2013/0034559, US 8,779,108, US 2014/0356353, US 8,609,089, US 2010/028330, US 2012/0114649, WO 2010/027827, WO 2011,/066342, US 8,907,065, WO 2016/062722, WO 2009/101611, WO2010/027827, WO 2011/066342, WO 2007/005874 , WO 2001/014556, US2011/0271358, WO 2010/036959, WO 2010/077634, US 8,217,149, US 2012/0039906, WO 2012/145493, US 2011/0318373, U.S. Patent No. 8,779,108, US 20140044738, WO 2009/089149, WO 2007/00587, WO 2016061142, WO 2016,02263, WO 2010/077634, and WO 2015/112900, the entire disclosures of which are hereby incorporated by reference.

In various embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that specifically binds to XCR1, *e.g.* on DCs. In various embodiments, the multi-specific CD8 binding agent of the invention comprises a targeting moiety having an antigen recognition domain that comprise all of or part of XCL1.

In various embodiments, the multi-specific CD8 binding agents have targeting moieties having recognition domains which specifically bind to a target (*e.g.* antigen, receptor) which is part of a non-cellular structure. In some embodiments, the antigen or receptor is not an integral component of an intact cell or cellular structure. In

some embodiments, the antigen or receptor is an extracellular antigen or receptor. In some embodiments, the target is a non-proteinaceous, non-cellular marker, including, without limitation, nucleic acids, inclusive of DNA or RNA, such as, for example, DNA released from necrotic tumor cells or extracellular deposits such as cholesterol.

In some embodiments, the target (*e.g.* antigen, receptor) of interest is part of the non-cellular component of the stroma or the extracellular matrix (ECM) or the markers associated therewith. As used herein, stroma refers to the connective and supportive framework of a tissue or organ. Stroma may include a compilation of cells such as fibroblasts/myofibroblasts, glial, epithelia, fat, immune, vascular, smooth muscle, and immune cells along with the extracellular matrix (ECM) and extracellular molecules. In various embodiments, the target (*e.g.* antigen, receptor) of interest is part of the non-cellular component of the stroma such as the extracellular matrix and extracellular molecules. As used herein, the ECM refers to the non-cellular components present within all tissues and organs. The ECM is composed of a large collection of biochemically distinct components including, without limitation, proteins, glycoproteins, proteoglycans, and polysaccharides. These components of the ECM are usually produced by adjacent cells and secreted into the ECM via exocytosis. Once secreted, the ECM components often aggregate to form a complex network of macromolecules. In various embodiments, the chimeric protein of the invention comprises a targeting moiety that recognizes a target (*e.g.*, an antigen or receptor or non-proteinaceous molecule) located on any component of the ECM. Illustrative components of the ECM include, without limitation, the proteoglycans, the non-proteoglycan polysaccharides, fibers, and other ECM proteins or ECM non-proteins, *e.g.* polysaccharides and/or lipids, or ECM associated molecules (*e.g.* proteins or non-proteins, *e.g.* polysaccharides, nucleic acids and/or lipids).

In some embodiments, the targeting moiety recognizes a target (*e.g.* antigen, receptor) on ECM proteoglycans. Proteoglycans are glycosylated proteins. The basic proteoglycan unit includes a core protein with one or more covalently attached glycosaminoglycan (GAG) chains. Proteoglycans have a net negative charge that attracts positively charged sodium ions (Na+), which attracts water molecules via osmosis, keeping the ECM and resident cells hydrated. Proteoglycans may also help to trap and store growth factors within the ECM. Illustrative proteoglycans that may be targeted by the chimeric proteins of the invention include, but are not limited to, heparan sulfate, chondroitin sulfate, and keratan sulfate. In an embodiment, the targeting moiety recognizes a target (*e.g.* antigen, receptor) on non-proteoglycan polysaccharides such as hyaluronic acid.

In some embodiments, the targeting moiety recognizes a target (*e.g.* antigen, receptor) on ECM fibers. ECM fibers include collagen fibers and elastin fibers. In some embodiments, the targeting moiety recognizes one or more epitopes on collagens or collagen fibers. Collagens are the most abundant proteins in the ECM. Collagens are present in the ECM as fibrillar proteins and provide structural support to resident cells. In one or more embodiments, the targeting moiety recognizes and binds to various types of collagens present within the ECM including, without limitation, fibrillar collagens (types I, II, III, V, XI), facit collagens (types IX, XII, XIV), short chain collagens (types VIII, X), basement membrane collagens (type IV), and/or collagen types VI, VII, or XIII. Elastin fibers provide elasticity to tissues, allowing them to stretch when needed and then return to their original state. In some embodiments, the target moiety recognizes one or more epitopes on elastins or elastin fibers.

In some embodiments, the targeting moiety recognizes one or more ECM proteins including, but not limited to, a tenascin, a fibronectin, a fibrin, a laminin, or a nidogen/entactin.

In an embodiment, the targeting moiety recognizes and binds to tenascin. The tenascin (TN) family of glycoproteins includes at least four members, tenascin-C, tenascin-R, tenascin-X, and tenascin W. The primary structures of tenascin proteins include several common motifs ordered in the same consecutive sequence: amino-terminal heptad repeats, epidermal growth factor (EGF)-like repeats, fibronectin type III domain repeats, and a carboxyl-terminal fibrinogen-like globular domain. Each protein member is associated with typical variations in the number and nature of EGF-like and fibronectin type III repeats. Isoform variants also exist particularly with respect to tenascin-C. Over 27 splice variants and/or isoforms of tenascin-C are known. In a particular embodiment, the targeting moiety recognizes and binds to tenascin-CA1. Similarly, tenascin-R also has various splice variants and isoforms. Tenascin-R usually exists as dimers or trimers. Tenascin-X is the largest member of the tenascin family and is known to exist as trimers. Tenascin-W exists as trimers. In some embodiments, the targeting moiety recognizes one or more epitopes on a tenascin protein. In some embodiments, the targeting moiety recognizes the monomeric and/or the dimeric and/or the trimeric and/or the hexameric forms of a tenascin protein.

In an embodiment, the targeting moieties recognize and bind to fibronectin. Fibronectins are glycoproteins that connect cells with collagen fibers in the ECM, allowing cells to move through the ECM. Upon binding to integrins, fibronectins unfolds to form functional dimers. In some embodiments, the targeting moiety recognizes the monomeric and/or the dimeric forms of fibronectin. In some embodiments, the targeting moiety recognizes one or more epitopes on fibronectin. In illustrative embodiments, the targeting moiety recognizes fibronectin extracellular domain A (EDA) or fibronectin extracellular domain B (EDB). Elevated levels of EDA are associated with various diseases and disorders including psoriasis, rheumatoid arthritis, diabetes, and cancer. In some embodiments, the targeting moiety recognizes fibronectin that contains the EDA isoform and may be utilized to target the chimeric protein to diseased cells including cancer cells. In some embodiments, the targeting moiety recognizes fibronectin that contains the EDB isoform. In various embodiments, such targeting moieties may be utilized to target the chimeric protein to tumor cells including the tumor neovasculature.

In an embodiment, the targeting moiety recognizes and binds to fibrin. Fibrin is another protein substance often found in the matrix network of the ECM. Fibrin is formed by the action of the protease thrombin on fibrinogen which causes the fibrin to polymerize. In some embodiments, the targeting moiety recognizes one or more epitopes on fibrin. In some embodiments, the targeting moiety recognizes the monomeric as well as the polymerized forms of fibrin.

In an embodiment, the targeting moiety recognizes and binds to laminin. Laminin is a major component of the basal lamina, which is a protein network foundation for cells and organs. Laminins are heterotrimeric proteins that contain an α-chain, a β-chain, and a γ-chain. In some embodiments, the targeting moiety recognizes one or

more epitopes on laminin. In some embodiments, the targeting moiety recognizes the monomeric, the dimeric as well as the trimeric forms of laminin.

In an embodiment, the targeting moiety recognizes and binds to a nidogen or entactin. Nidogens/entactins are a family of highly conserved, sulfated glycoproteins. They make up the major structural component of the basement membranes and function to link laminin and collagen IV networks in basement membranes. Members of this family include nidogen-1 and nidogen-2. In various embodiments, the targeting moiety recognizes an epitope on nidogen-1 and/or nidogen-2.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes an epitope present on any of the targets (*e.g.*, ECM proteins) described herein. In an embodiment, the antigen-recognition domain recognizes one or more linear epitopes present on the protein. As used herein, a linear epitope refers to any continuous sequence of amino acids present on the protein. In another embodiment, the antigen-recognition domain recognizes one or more conformational epitopes present on the protein. As used herein, a conformation epitope refers to one or more sections of amino acids (which may be discontinuous) which form a three-dimensional surface with features and/or shapes and/or tertiary structures capable of being recognized by an antigen recognition domain.

In various embodiments, the targeting moiety may bind to the full-length and/or mature forms and/or isoforms and/or splice variants and/or fragments and/or any other naturally occurring or synthetic analogs, variants, or mutants of any of the targets (*e.g.*, ECM proteins) described herein. In various embodiments, the targeting moiety may bind to any forms of the proteins described herein, including monomeric, dimeric, trimeric, tetrameric, heterodimeric, multimeric and associated forms. In various embodiments, the targeting moiety may bind to any post-translationally modified forms of the proteins described herein, such as glycosylated and/or phosphorylated forms.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes extracellular molecules such as DNA. In some embodiments, the targeting moiety comprises an antigen recognition domain that recognizes DNA. In an embodiment, the DNA is shed into the extracellular space from necrotic or apoptotic tumor cells or other diseased cells.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes one or more non-cellular structures associated with atherosclerotic plaques. Two types of atherosclerotic plaques are known. The fibro-lipid (fibro-fatty) plaque is characterized by an accumulation of lipid-laden cells underneath the intima of the arteries. Beneath the endothelium there is a fibrous cap covering the atheromatous core of the plaque. The core includes lipid-laden cells (macrophages and smooth muscle cells) with elevated tissue cholesterol and cholesterol ester content, fibrin, proteoglycans, collagen, elastin, and cellular debris. In advanced plaques, the central core of the plaque usually contains extracellular cholesterol deposits (released from dead cells), which form areas of cholesterol crystals with empty, needle-like clefts. At the periphery of the plaque are younger foamy cells and capillaries. A fibrous plaque is also localized under the intima, within the wall of the

artery resulting in thickening and expansion of the wall and, sometimes, spotty localized narrowing of the lumen with some atrophy of the muscular layer. The fibrous plaque contains collagen fibers (eosinophilic), precipitates of calcium (hematoxylinophilic) and lipid-laden cells. In some embodiments, the targeting moiety recognizes and binds to one or more of the non-cellular components of these plaques such as the fibrin, proteoglycans, collagen, elastin, cellular debris, and calcium or other mineral deposits or precipitates. In some embodiments, the cellular debris is a nucleic acid, *e.g.* DNA or RNA, released from dead cells.

In various embodiments, the targeting moiety comprises an antigen recognition domain that recognizes one or more non-cellular structures found in the brain plaques associated with neurodegenerative diseases. In some embodiments, the targeting moiety recognizes and binds to one or more non-cellular structures located in the amyloid plaques found in the brains of patients with Alzheimer's disease. For example, the targeting moiety may recognize and bind to the peptide amyloid beta, which is a major component of the amyloid plaques. In some embodiments, the targeting moiety recognizes and binds to one or more non-cellular structures located in the brains plaques found in patients with Huntington's disease. In various embodiments, the targeting moiety recognizes and binds to one or more non-cellular structures found in plaques associated with other neurodegenerative or musculoskeletal diseases such as Lewy body dementia and inclusion body myositis.

Linkers and Functional Groups

In various embodiments, the CD8 binding agent may include one or more functional groups, residues, or moieties. In various embodiments, the one or more functional groups, residues, or moieties are attached or genetically fused to any of the signaling agents or targeting moieties described herein. In some embodiments, such functional groups, residues or moieties confer one or more desired properties or functionalities to the CD8 binding agent of the invention. Examples of such functional groups and of techniques for introducing them into the CD8 binding agent are known in the art, for example, see *Remington's Pharmaceutical Sciences,* 16th ed., Mack Publishing Co., Easton, Pa. (1980).

In various embodiments, the CD8 binding agent may by conjugated and/or fused with another agent to extend half-life or otherwise improve pharmacodynamic and pharmacokinetic properties. In some embodiments, the CD8 binding agent may be fused or conjugated with one or more of PEG, XTEN (*e.g.*, as rPEG), polysialic acid (POLYXEN), albumin (*e.g.*, human serum albumin or HAS), elastin-like protein (ELP), PAS, HAP, GLK, CTP, transferrin, and the like. In some embodiments, the CD8 binding agent may be fused or conjugated with an antibody or an antibody fragment such as an Fc fragment. For example, the chimeric protein may be fused to either the N-terminus or the C-terminus of the Fc domain of human immunoglobulin (Ig) G. In various embodiments, each of the individual chimeric proteins is fused to one or more of the agents described in BioDrugs (2015) 29:215–239, the entire contents of which are hereby incorporated by reference.

In some embodiments, the functional groups, residues, or moieties comprise a suitable pharmacologically acceptable polymer, such as poly(ethyleneglycol) (PEG) or derivatives thereof (such as methoxypoly(ethyleneglycol) or mPEG). In some embodiments, attachment of the PEG moiety increases the

half-life and/or reduces the immunogenecity of the CD8 binding protein. Generally, any suitable form of pegylation can be used, such as the pegylation used in the art for antibodies and antibody fragments (including but not limited to single domain antibodies such as VHHs); see, for example, Chapman, *Nat. Biotechnol.,* 54, 531-545 (2002); by Veronese and Harris, *Adv. Drug Deliv. Rev.* 54, 453-456 (2003), by Harris and Chess, *Nat. Rev. Drug. Discov.,* 2, (2003) and in WO 04060965, the entire contents of which are hereby incorporated by reference. Various reagents for pegylation of proteins are also commercially available, for example, from Nektar Therapeutics, USA. In some embodiments, site-directed pegylation is used, in particular via a cysteine-residue (see, for example, Yang *et al.*, Protein Engineering, 16, 10, 761-770 (2003), the entire contents of which is hereby incorporated by reference). For example, for this purpose, PEG may be attached to a cysteine residue that naturally occurs in the CD8 binding agent of the invention. In some embodiments, the CD8 binding agent of the invention is modified so as to suitably introduce one or more cysteine residues for attachment of PEG, or an amino acid sequence comprising one or more cysteine residues for attachment of PEG may be fused to the amino- and/or carboxy-terminus of the CD8 binding agent, using techniques known in the art. In some embodiments, the functional groups, residues, or moieties comprise N-linked or O-linked glycosylation. In some embodiments, the N-linked or O-linked glycosylation is introduced as part of a co-translational and/or post-translational modification.

In some embodiments, the functional groups, residues, or moieties comprise one or more detectable labels or other signal-generating groups or moieties. Suitable labels and techniques for attaching, using and detecting them are known in the art and, include, but are not limited to, fluorescent labels (such as fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, and fluorescamine and fluorescent metals such as Eu or others metals from the lanthanide series), phosphorescent labels, chemiluminescent labels or bioluminescent labels (such as luminal, isoluminol, theromatic acridinium ester, imidazole, acridinium salts, oxalate ester, dioxetane or GFP and its analogs), radio-isotopes, metals, metals chelates or metallic cations or other metals or metallic cations that are particularly suited for use in *in vivo*, *in vitro* or *in situ* diagnosis and imaging, as well as chromophores and enzymes (such as malate dehydrogenase, staphylococcal nuclease, delta- V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, biotinavidin peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase). Other suitable labels include moieties that can be detected using NMR or ESR spectroscopy. Such labeled VHHs and polypeptides of the invention may, for example, be used for *in vitro, in vivo or in situ* assays (including immunoassays known per se such as ELISA, RIA, EIA and other "sandwich assays," *etc.*) as well as *in vivo* diagnostic and imaging purposes, depending on the choice of the specific label.

In some embodiments, the functional groups, residues, or moieties comprise a tag that is attached or genetically fused to the CD8 binding agent. In some embodiments, the CD8 binding agent may include a single tag or multiple tags. The tag for example is a peptide, sugar, or DNA molecule that does not inhibit or prevent binding of

the CD8 binding agent to CD8 or any other antigen of interest such as tumor antigens. In various embodiments, the tag is at least about: three to five amino acids long, five to eight amino acids long, eight to twelve amino acids long, twelve to fifteen amino acids long, or fifteen to twenty amino acids long. Exemplary tags are described for example, in U.S. Patent Publication No. US2013/0058962. In some embodiment, the tag is an affinity tag such as glutathione-S-transferase (GST) and histidine (His) tag. In an embodiment, the CD8 binding agent comprises a His tag.

In some embodiments, the functional groups, residues, or moieties comprise a chelating group, for example, to chelate one of the metals or metallic cations. Suitable chelating groups, for example, include, without limitation, diethyl-enetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

In some embodiments, the functional groups, residues, or moieties comprise a functional group that is one part of a specific binding pair, such as the biotin-(strept)avidin binding pair. Such a functional group may be used to link the CD8 binding agent of the invention to another protein, polypeptide or chemical compound that is bound to the other half of the binding pair, *i.e.*, through formation of the binding pair. For example, a CD8 binding agent of the invention may be conjugated to biotin, and linked to another protein, polypeptide, compound or carrier conjugated to avidin or streptavidin. For example, such a conjugated CD8 binding agent may be used as a reporter, for example, in a diagnostic system where a detectable signal-producing agent is conjugated to avidin or streptavidin. Such binding pairs may, for example, also be used to bind the CD8 binding agent to a carrier, including carriers suitable for pharmaceutical purposes. One non-limiting example are the liposomal formulations described by Cao and Suresh, *Journal of Drug Targeting,* 8, 4, 257 (2000). Such binding pairs may also be used to link a therapeutically active agent to the CD8 binding agent of the invention.

In some embodiments, the present CD8 binding agent optionally comprises one or more linkers. In some embodiments, the present CD8 binding agent comprises a linker connecting the targeting moiety and the signaling agent. In some embodiments, the present chimeric protein comprises a linker within the signaling agent (*e.g.* in the case of single chain TNF, which can comprise two linkers to yield a trimer).

In some embodiments, the CD8 binding agent includes a linker that connects each binding region and/or targeting moieties. In some embodiments, the linker may be utilized to link various functional groups, residues, or moieties as described herein to the CD8 binding agent. In some embodiments, the linker is a single amino acid or a plurality of amino acids that does not affect or reduce the stability, orientation, binding, neutralization, and/or clearance characteristics of the binding regions and the binding protein. In various embodiments, the linker is selected from a peptide, a protein, a sugar, or a nucleic acid.

The invention contemplates the use of a variety of linker sequences. In various embodiments, the linker may be derived from naturally-occurring multi-domain proteins or are empirical linkers as described, for example, in Chichili *et al.*, (2013), Protein Sci. 22(2):153-167, Chen *et al.*, (2013), Adv Drug Deliv Rev. 65(10):1357-1369, the entire contents of which are hereby incorporated by reference. In some embodiments, the linker may be designed using linker designing databases and computer programs such as those described in Chen *et al.*,

(2013), Adv Drug Deliv Rev. 65(10):1357-1369 and Crasto *et al.*, (2000), Protein Eng. 13(5):309-312, the entire contents of which are hereby incorporated by reference. In various embodiments, the linker may be functional. For example, without limitation, the linker may function to improve the folding and/or stability, improve the expression, improve the pharmacokinetics, and/or improve the bioactivity of the present CD8 binding agent.

In some embodiments, the linker is a polypeptide. In some embodiments, the linker is less than about 100 amino acids long. For example, the linker may be less than about 100, about 95, about 90, about 85, about 80, about 75, about 70, about 65, about 60, about 55, about 50, about 45, about 40, about 35, about 30, about 25, about 20, about 19, about 18, about 17, about 16, about 15, about 14, about 13, about 12, about 11, about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, or about 2 amino acids long. In some embodiments, the linker is flexible. In another embodiment, the linker is rigid.

In some embodiments, the linker is a polypeptide. In some embodiments, the linker is greater than about 100 amino acids long. For example, the linker may be greater than about 100, about 95, about 90, about 85, about 80, about 75, about 70, about 65, about 60, about 55, about 50, about 45, about 40, about 35, about 30, about 25, about 20, about 19, about 18, about 17, about 16, about 15, about 14, about 13, about 12, about 11, about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, or about 2 amino acids long. In some embodiments, the linker is flexible. In another embodiment, the linker is rigid.

In various embodiments, the linker is substantially comprised of glycine and serine residues (*e.g.* about 30%, or about 40%, or about 50%, or about 60%, or about 70%, or about 80%, or about 90%, or about 95%, or about 97% glycines and serines). For example, in some embodiments, the linker is $(Gly_4Ser)_n$, where n is from about 1 to about 8, *e.g.* 1, 2, 3, 4, 5, 6, 7, or 8. In an embodiment, the linker sequence is GGSGGSGGGGSGGGGS (SEQ ID NO:215). Additional illustrative linkers include, but are not limited to, linkers having the sequence LE, GGGGS (SEQ ID NO:216), $(GGGGS)_n$ (n=1-4) (SEQ ID NO:217), $(Gly)_8$ (SEQ ID NO:218), $(Gly)_6$ (SEQ ID NO:219), $(EAAAK)_n$ (n=1-3) (SEQ ID NO:220), $A(EAAAK)_nA$ (n = 2-5) (SEQ ID NO:221), AEAAAKEAAAKA (SEQ ID NO:222), $A(EAAAK)_4ALEA(EAAAK)_4A$ (SEQ ID NO:223), PAPAP (SEQ ID NO:224), KESGSVSSEQLAQFRSLD (SEQ ID NO:225), EGKSSGSGSESKST (SEQ ID NO:226), GSAGSAAGSGEF (SEQ ID NO:227), and $(XP)_n$, with X designating any amino acid, *e.g.*, Ala, Lys, or Glu. In various embodiments, the linker is GGS.

In some embodiments, the linker is a hinge region of an antibody (*e.g.*, of IgG, IgA, IgD, and IgE, inclusive of subclasses (*e.g.* IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). In various embodiments, the linker is a hinge region of an antibody (*e.g.*, of IgG, IgA, IgD, and IgE, inclusive of subclasses (*e.g.* IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). The hinge region, found in IgG, IgA, IgD, and IgE class antibodies, acts as a flexible spacer, allowing the Fab portion to move freely in space. In contrast to the constant regions, the hinge domains are structurally diverse, varying in both sequence and length among immunoglobulin classes and subclasses. For example, the length and flexibility of the hinge region varies among the IgG subclasses. The hinge region of IgG1 encompasses amino acids 216-231 and, because it is freely flexible, the Fab fragments can rotate about

their axes of symmetry and move within a sphere centered at the first of two inter-heavy chain disulfide bridges. IgG2 has a shorter hinge than IgG1, with 12 amino acid residues and four disulfide bridges. The hinge region of IgG2 lacks a glycine residue, is relatively short, and contains a rigid poly-proline double helix, stabilized by extra inter-heavy chain disulfide bridges. These properties restrict the flexibility of the IgG2 molecule. IgG3 differs from the other subclasses by its unique extended hinge region (about four times as long as the IgG1 hinge), containing 62 amino acids (including 21 prolines and 11 cysteines), forming an inflexible poly-proline double helix. In IgG3, the Fab fragments are relatively far away from the Fc fragment, giving the molecule a greater flexibility. The elongated hinge in IgG3 is also responsible for its higher molecular weight compared to the other subclasses. The hinge region of IgG4 is shorter than that of IgG1 and its flexibility is intermediate between that of IgG1 and IgG2. The flexibility of the hinge regions reportedly decreases in the order IgG3>IgG1>IgG4>IgG2.

According to crystallographic studies, the immunoglobulin hinge region can be further subdivided functionally into three regions: the upper hinge region, the core region, and the lower hinge region. *See* Shin *et al.*, 1992 *Immunological Reviews* 130:87. The upper hinge region includes amino acids from the carboxyl end of C_{H1} to the first residue in the hinge that restricts motion, generally the first cysteine residue that forms an interchain disulfide bond between the two heavy chains. The length of the upper hinge region correlates with the segmental flexibility of the antibody. The core hinge region contains the inter-heavy chain disulfide bridges, and the lower hinge region joins the amino terminal end of the C_{H2} domain and includes residues in C_{H2}. *Id.* The core hinge region of wild-type human IgG1 contains the sequence Cys-Pro-Pro-Cys which, when dimerized by disulfide bond formation, results in a cyclic octapeptide believed to act as a pivot, thus conferring flexibility. In various embodiments, the present linker comprises, one, or two, or three of the upper hinge region, the core region, and the lower hinge region of any antibody (*e.g.*, of IgG, IgA, IgD, and IgE, inclusive of subclasses (*e.g.* IgG1, IgG2, IgG3, and IgG4, and IgA1 and IgA2)). The hinge region may also contain one or more glycosylation sites, which include a number of structurally distinct types of sites for carbohydrate attachment. For example, IgA1 contains five glycosylation sites within a 17-amino-acid segment of the hinge region, conferring resistance of the hinge region polypeptide to intestinal proteases, considered an advantageous property for a secretory immunoglobulin. In various embodiments, the linker of the present invention comprises one or more glycosylation sites. In various embodiments, the linker is a hinge-CH2-CH3 domain of a human IgG4 antibody.

If desired, the present CD8 binding agent can be linked to an antibody Fc region, comprising one or both of C_H2 and C_H3 domains, and optionally a hinge region. For example, vectors encoding the present CD8 binding agents linked as a single nucleotide sequence to an Fc region can be used to prepare such polypeptides.

In some embodiments, the linker is a synthetic linker such as PEG.

In various embodiments, the linker may be functional. For example, without limitation, the linker may function to improve the folding and/or stability, improve the expression, improve the pharmacokinetics, and/or improve the bioactivity of the present CD8 binding agent. In another example, the linker may function to target the CD8 binding agent to a particular cell type or location.

Modifications and Production of CD8 Binding Agents

In various embodiments, the CD8 binding agent comprises a targeting moiety that is a VHH. In various embodiments, the VHH is not limited to a specific biological source or to a specific method of preparation. For example, the VHH can generally be obtained: (1) by isolating the V_HH domain of a naturally occurring heavy chain antibody; (2) by expression of a nucleotide sequence encoding a naturally occurring V_HH domain; (3) by "humanization" of a naturally occurring V_HH domain or by expression of a nucleic acid encoding a such humanized V_HH domain; (4) by "camelization" of a naturally occurring VH domain from any animal species, such as from a mammalian species, such as from a human being, or by expression of a nucleic acid encoding such a camelized VH domain; (5) by "camelization" of a "domain antibody" or "Dab" as described in the art, or by expression of a nucleic acid encoding such a camelized VH domain; (6) by using synthetic or semi-synthetic techniques for preparing proteins, polypeptides or other amino acid sequences known in the art; (7) by preparing a nucleic acid encoding a VHH using techniques for nucleic acid synthesis known in the art, followed by expression of the nucleic acid thus obtained; and/or (8) by any combination of one or more of the foregoing.

In an embodiment, the CD8 binding agent comprises a VHH that corresponds to the V_HH domains of naturally occurring heavy chain antibodies directed against human CD8. In some embodiments, such V_HH sequences can generally be generated or obtained by suitably immunizing a species of Camelid with a CD8 molecule, (*i.e.*, so as to raise an immune response and/or heavy chain antibodies directed against CD8), by obtaining a suitable biological sample from the Camelid (such as a blood sample, or any sample of B-cells), and by generating V_HH sequences directed against CD8, starting from the sample, using any suitable known techniques. In some embodiments, naturally occurring V_HH domains against CD8 can be obtained from naive libraries of Camelid V_HH sequences, for example, by screening such a library using CD8 or at least one part, fragment, antigenic determinant or epitope thereof using one or more screening techniques known in the art. Such libraries and techniques are, for example, described in WO9937681, WO0190190, WO03025020 and WO03035694, the entire contents of which are hereby incorporated by reference. In some embodiments, improved synthetic or semi-synthetic libraries derived from naive V_HH libraries may be used, such as V_HH libraries obtained from naive V_HH libraries by techniques such as random mutagenesis and/or CDR shuffling, as for example, described in WO0043507, the entire contents of which are hereby incorporated by reference. In some embodiments, another technique for obtaining V_HH sequences directed against a CD8 involves suitably immunizing a transgenic mammal that is capable of expressing heavy chain antibodies (*i.e.*, so as to raise an immune response and/or heavy chain antibodies directed against CD8), obtaining a suitable biological sample from the transgenic mammal (such as a blood sample, or any sample of B-cells), and then generating V_HH sequences directed against CD8 starting from the sample, using any suitable known techniques. For example, for this purpose, the heavy chain antibody-expressing mice and the further methods and techniques described in WO02085945 and in WO04049794 (the entire contents of which are hereby incorporated by reference) can be used.

In an embodiment, the CD8 binding agent comprises a VHH that has been "humanized" *i.e.*, by replacing one or more amino acid residues in the amino acid sequence of the naturally occurring V_HH sequence (and in particular

in the framework sequences) by one or more of the amino acid residues that occur at the corresponding position(s) in a VH domain from a conventional 4-chain antibody from a human being. This can be performed using humanization techniques known in the art. In some embodiments, possible humanizing substitutions or combinations of humanizing substitutions may be determined by methods known in the art, for example, by a comparison between the sequence of a VHH and the sequence of a naturally occurring human VH domain. In some embodiments, the humanizing substitutions are chosen such that the resulting humanized VHHs still retain advantageous functional properties. Generally, as a result of humanization, the VHHs of the invention may become more "human-like," while still retaining favorable properties such as a reduced immunogenicity, compared to the corresponding naturally occurring V_HH domains. In various embodiments, the humanized VHHs of the invention can be obtained in any suitable manner known in the art and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring V_HH domain as a starting material.

In an embodiment, the CD8 binding agent comprises a VHH that has been "camelized," *i.e.*, by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring VH domain from a conventional 4-chain antibody by one or more of the amino acid residues that occur at the corresponding position(s) in a V_HH domain of a heavy chain antibody of a camelid. In some embodiments, such "camelizing" substitutions are inserted at amino acid positions that form and/or are present at the VH-VL interface, and/or at the so-called Camelidae hallmark residues (see, for example, WO9404678, the entire contents of which are hereby incorporated by reference). In some embodiments, the VH sequence that is used as a starting material or starting point for generating or designing the camelized VHH is a VH sequence from a mammal, for example, the VH sequence of a human being, such as a VH3 sequence. In various embodiments, the camelized VHHs can be obtained in any suitable manner known in the art (*i.e.*, as indicated under points (1)-(8) above) and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring VH domain as a starting material.

In various embodiments, both "humanization" and "camelization" can be performed by providing a nucleotide sequence that encodes a naturally occurring V_HH domain or VH domain, respectively, and then changing, in a manner known in the art, one or more codons in the nucleotide sequence in such a way that the new nucleotide sequence encodes a "humanized" or "camelized" VHH, respectively. This nucleic acid can then be expressed in a manner known in the art, so as to provide the desired VHH of the invention. Alternatively, based on the amino acid sequence of a naturally occurring V_HH domain or VH domain, respectively, the amino acid sequence of the desired humanized or camelized VHH of the invention, respectively, can be designed and then synthesized de novo using techniques for peptide synthesis known in the art. Also, based on the amino acid sequence or nucleotide sequence of a naturally occurring V_HH domain or VH domain, respectively, a nucleotide sequence encoding the desired humanized or camelized VHH, respectively, can be designed and then synthesized de novo using techniques for nucleic acid synthesis known in the art, after which the nucleic acid thus obtained can be expressed in a manner known in the art, so as to provide the desired VHH of the invention. Other suitable

methods and techniques for obtaining the VHHs of the invention and/or nucleic acids encoding the same, starting from naturally occurring VH sequences or V_HH sequences, are known in the art, and may, for example, comprise combining one or more parts of one or more naturally occurring VH sequences (such as one or more FR sequences and/or CDR sequences), one or more parts of one or more naturally occurring V_HH sequences (such as one or more FR sequences or CDR sequences), and/or one or more synthetic or semi-synthetic sequences, in a suitable manner, so as to provide a VHH of the invention or a nucleotide sequence or nucleic acid encoding the same.

Methods for producing the CD8 binding agents of the invention are described herein. For example, DNA sequences encoding the CD8 binding agents of the invention can be chemically synthesized using methods known in the art. Synthetic DNA sequences can be ligated to other appropriate nucleotide sequences, including, *e.g.*, expression control sequences, to produce gene expression constructs encoding the desired CD8 binding agents. Accordingly, in various embodiments, the present invention provides for isolated nucleic acids comprising a nucleotide sequence encoding the CD8 binding agent of the invention.

Nucleic acids encoding the CD8 binding agent of the invention can be incorporated (ligated) into expression vectors, which can be introduced into host cells through transfection, transformation, or transduction techniques. For example, nucleic acids encoding the CD8 binding agent of the invention can be introduced into host cells by retroviral transduction. Illustrative host cells are *E.coli* cells, Chinese hamster ovary (CHO) cells, human embryonic kidney 293 (HEK 293) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (*e.g.*, Hep G2), and myeloma cells. Transformed host cells can be grown under conditions that permit the host cells to express the genes that encode the CD8 binding agent of the invention. Accordingly, in various embodiments, the present invention provides expression vectors comprising nucleic acids that encode the CD8 binding agent of the invention. In various embodiments, the present invention additional provides host cells comprising such expression vectors.

Specific expression and purification conditions will vary depending upon the expression system employed. For example, if a gene is to be expressed in *E. coli*, it is first cloned into an expression vector by positioning the engineered gene downstream from a suitable bacterial promoter, *e.g.*, Trp or Tac, and a prokaryotic signal sequence. In another example, if the engineered gene is to be expressed in eukaryotic host cells, *e.g.*, CHO cells, it is first inserted into an expression vector containing for example, a suitable eukaryotic promoter, a secretion signal, enhancers, and various introns. The gene construct can be introduced into the host cells using transfection, transformation, or transduction techniques.

The CD8 binding agent of the invention can be produced by growing a host cell transfected with an expression vector encoding the CD8 binding agent under conditions that permit expression of the protein. Following expression, the protein can be harvested and purified using techniques well known in the art, *e.g.*, affinity tags such as glutathione-S-transferase (GST) and histidine (His) tags or by chromatography. In an embodiment, the

CD8 binding agent comprises a His tag (which is optionally cleavable via an engineered proteolytic cleavage site).

Accordingly, in various embodiments, the present invention provides for a nucleic acid encoding a CD8 binding agent of the present invention. In various embodiments, the present invention provides for a host cell comprising a nucleic acid encoding a CD8 binding agent of the present invention.

Pharmaceutically Acceptable Salts and Excipients

The CD8 binding agents described herein can possess a sufficiently basic functional group, which can react with an inorganic or organic acid, or a carboxyl group, which can react with an inorganic or organic base, to form a pharmaceutically acceptable salt. A pharmaceutically acceptable acid addition salt is formed from a pharmaceutically acceptable acid, as is well known in the art. Such salts include the pharmaceutically acceptable salts listed in, for example, *Journal of Pharmaceutical Science*, 66, 2-19 (1977) and *The Handbook of Pharmaceutical Salts; Properties, Selection, and Use*. P. H. Stahl and C. G. Wermuth (eds.), Verlag, Zurich (Switzerland) 2002, which are hereby incorporated by reference in their entirety.

Pharmaceutically acceptable salts include, by way of non-limiting example, sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, camphorsulfonate, pamoate, phenylacetate, trifluoroacetate, acrylate, chlorobenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, methylbenzoate, o-acetoxybenzoate, naphthalene-2-benzoate, isobutyrate, phenylbutyrate, α-hydroxybutyrate, butyne-1,4-dicarboxylate, hexyne-1,4-dicarboxylate, caprate, caprylate, cinnamate, glycollate, heptanoate, hippurate, malate, hydroxymaleate, malonate, mandelate, mesylate, nicotinate, phthalate, teraphthalate, propiolate, propionate, phenylpropionate, sebacate, suberate, p-bromobenzenesulfonate, chlorobenzenesulfonate, ethylsulfonate, 2-hydroxyethylsulfonate, methylsulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, naphthalene-1,5-sulfonate, xylenesulfonate, and tartarate salts.

The term “pharmaceutically acceptable salt” also refers to a salt of the compositions of the present invention having an acidic functional group, such as a carboxylic acid functional group, and a base. Suitable bases include, but are not limited to, hydroxides of alkali metals such as sodium, potassium, and lithium; hydroxides of alkaline earth metal such as calcium and magnesium; hydroxides of other metals, such as aluminum and zinc; ammonia, and organic amines, such as unsubstituted or hydroxy-substituted mono-, di-, or tri-alkylamines, dicyclohexylamine; tributyl amine; pyridine; N-methyl, N-ethylamine; diethylamine; triethylamine; mono-, bis-, or tris-(2-OH-lower alkylamines), such as mono-; bis-, or tris-(2-hydroxyethyl)amine, 2-hydroxy-tert-butylamine, or tris-(hydroxymethyl)methylamine, N,N-di-lower alkyl-N-(hydroxyl-lower alkyl)-amines, such as N,N-dimethyl-N-(2-hydroxyethyl)amine or tri-(2-hydroxyethyl)amine; N-methyl-D-glucamine; and amino acids such as arginine, lysine, and the like.

In some embodiments, the compositions described herein are in the form of a pharmaceutically acceptable salt.

Pharmaceutical Compositions and Formulations

In various embodiments, the present invention pertains to pharmaceutical compositions comprising the CD8 binding agents described herein and a pharmaceutically acceptable carrier or excipient. Any pharmaceutical compositions described herein can be administered to a subject as a component of a composition that comprises a pharmaceutically acceptable carrier or vehicle. Such compositions can optionally comprise a suitable amount of a pharmaceutically acceptable excipient so as to provide the form for proper administration.

In various embodiments, pharmaceutical excipients can be liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical excipients can be, for example, saline, gum acacia, gelatin, starch paste, talc, keratin, colloidal silica, urea and the like. In addition, auxiliary, stabilizing, thickening, lubricating, and coloring agents can be used. In one embodiment, the pharmaceutically acceptable excipients are sterile when administered to a subject. Water is a useful excipient when any agent described herein is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid excipients, specifically for injectable solutions. Suitable pharmaceutical excipients also include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Any agent described herein, if desired, can also comprise minor amounts of wetting or emulsifying agents, or pH buffering agents. Other examples of suitable pharmaceutical excipients are described in *Remington's Pharmaceutical Sciences* 1447-1676 (Alfonso R. Gennaro eds., 19th ed. 1995), incorporated herein by reference.

The present invention includes the described pharmaceutical compositions (and/or additional therapeutic agents) in various formulations. Any inventive pharmaceutical composition (and/or additional therapeutic agents) described herein can take the form of solutions, suspensions, emulsion, drops, tablets, pills, pellets, capsules, capsules containing liquids, gelatin capsules, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, lyophilized powder, frozen suspension, dessicated powder, or any other form suitable for use. In one embodiment, the composition is in the form of a capsule. In another embodiment, the composition is in the form of a tablet. In yet another embodiment, the pharmaceutical composition is formulated in the form of a soft-gel capsule. In a further embodiment, the pharmaceutical composition is formulated in the form of a gelatin capsule. In yet another embodiment, the pharmaceutical composition is formulated as a liquid.

Where necessary, the inventive pharmaceutical compositions (and/or additional agents) can also include a solubilizing agent. Also, the agents can be delivered with a suitable vehicle or delivery device as known in the art. Combination therapies outlined herein can be co-delivered in a single delivery vehicle or delivery device.

The formulations comprising the inventive pharmaceutical compositions (and/or additional agents) of the present invention may conveniently be presented in unit dosage forms and may be prepared by any of the methods well known in the art of pharmacy. Such methods generally include the step of bringing the therapeutic agents into

association with a carrier, which constitutes one or more accessory ingredients. Typically, the formulations are prepared by uniformly and intimately bringing the therapeutic agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into dosage forms of the desired formulation (*e.g.*, wet or dry granulation, powder blends, *etc.*, followed by tableting using conventional methods known in the art).

In various embodiments, any pharmaceutical compositions (and/or additional agents) described herein is formulated in accordance with routine procedures as a composition adapted for a mode of administration described herein.

Routes of administration include, for example: oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically. Administration can be local or systemic. In some embodiments, the administering is effected orally. In another embodiment, the administration is by parenteral injection. The mode of administration can be left to the discretion of the practitioner, and depends in-part upon the site of the medical condition. In most instances, administration results in the release of any agent described herein into the bloodstream.

In one embodiment, the CD8 binding agent described herein is formulated in accordance with routine procedures as a composition adapted for oral administration. Compositions for oral delivery can be in the form of tablets, lozenges, aqueous or oily suspensions, granules, powders, emulsions, capsules, syrups, or elixirs, for example. Orally administered compositions can comprise one or more agents, for example, sweetening agents such as fructose, aspartame or saccharin; flavoring agents such as peppermint, oil of wintergreen, or cherry; coloring agents; and preserving agents, to provide a pharmaceutically palatable preparation. Moreover, where in tablet or pill form, the compositions can be coated to delay disintegration and absorption in the gastrointestinal tract thereby providing a sustained action over an extended period of time. Selectively permeable membranes surrounding an osmotically active driving any CD8 binding agents described herein are also suitable for orally administered compositions. In these latter platforms, fluid from the environment surrounding the capsule is imbibed by the driving compound, which swells to displace the agent or agent composition through an aperture. These delivery platforms can provide an essentially zero order delivery profile as opposed to the spiked profiles of immediate release formulations. A time-delay material such as glycerol monostearate or glycerol stearate can also be useful. Oral compositions can include standard excipients such as mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, and magnesium carbonate. In one embodiment, the excipients are of pharmaceutical grade. Suspensions, in addition to the active compounds, may contain suspending agents such as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, tragacanth, *etc.*, and mixtures thereof.

Dosage forms suitable for parenteral administration (*e.g.* intravenous, intramuscular, intraperitoneal, subcutaneous and intra-articular injection and infusion) include, for example, solutions, suspensions, dispersions, emulsions, and the like. They may also be manufactured in the form of sterile solid compositions (*e.g.* lyophilized

composition), which can be dissolved or suspended in sterile injectable medium immediately before use. They may contain, for example, suspending or dispersing agents known in the art. Formulation components suitable for parenteral administration include a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as EDTA; buffers such as acetates, citrates or phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose.

For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). The carrier should be stable under the conditions of manufacture and storage, and should be preserved against microorganisms. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol), and suitable mixtures thereof.

The compositions provided herein, alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Any inventive pharmaceutical compositions (and/or additional agents) described herein can be administered by controlled-release or sustained-release means or by delivery devices that are well known to those of ordinary skill in the art. Examples include, but are not limited to, those described in U.S. Patent Nos. 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 5,674,533; 5,059,595; 5,591,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; and 5,733,556, each of which is incorporated herein by reference in its entirety. Such dosage forms can be useful for providing controlled- or sustained-release of one or more active ingredients using, for example, hydropropyl cellulose, hydropropylmethyl cellulose, polyvinylpyrrolidone, other polymer matrices, gels, permeable membranes, osmotic systems, multilayer coatings, microparticles, liposomes, microspheres, or a combination thereof to provide the desired release profile in varying proportions. Suitable controlled- or sustained-release formulations known to those skilled in the art, including those described herein, can be readily selected for use with the active ingredients of the agents described herein. The invention thus provides single unit dosage forms suitable for oral administration such as, but not limited to, tablets, capsules, gelcaps, and caplets that are adapted for controlled- or sustained-release.

Controlled- or sustained-release of an active ingredient can be stimulated by various conditions, including but not limited to, changes in pH, changes in temperature, stimulation by an appropriate wavelength of light, concentration or availability of enzymes, concentration or availability of water, or other physiological conditions or compounds.

In another embodiment, a controlled-release system can be placed in proximity of the target area to be treated, thus requiring only a fraction of the systemic dose (s*ee, e.g.,* Goodson, in *Medical Applications of Controlled*

Release, *supra*, vol. 2, pp. 115-138 (1984)). Other controlled-release systems discussed in the review by Langer, 1990, *Science* 249:1527-1533) may be used.

Pharmaceutical formulations preferably are sterile. Sterilization can be accomplished, for example, by filtration through sterile filtration membranes. Where the composition is lyophilized, filter sterilization can be conducted prior to or following lyophilization and reconstitution.

Administration and Dosage

It will be appreciated that the actual dose of the CD8 binding agent to be administered according to the present invention will vary according to the particular dosage form, and the mode of administration. Many factors that may modify the action of the CD8 binding agent (*e.g.*, body weight, gender, diet, time of administration, route of administration, rate of excretion, condition of the subject, drug combinations, genetic disposition and reaction sensitivities) can be taken into account by those skilled in the art. Administration can be carried out continuously or in one or more discrete doses within the maximum tolerated dose. Optimal administration rates for a given set of conditions can be ascertained by those skilled in the art using conventional dosage administration tests.

In some embodiments, a suitable dosage of the CD8 binding agent is in a range of about 0.01 mg/kg to about 10 g/kg of body weight of the subject, about 0.01 mg/kg to about 1 g/kg of body weight of the subject, about 0.01 mg/kg to about 100 mg/kg of body weight of the subject, about 0.01 mg/kg to about 10 mg/kg of body weight of the subject, for example, about 0.01 mg/kg, about 0.02 mg/kg, about 0.03 mg/kg, about 0.04 mg/kg, about 0.05 mg/kg, about 0.06 mg/kg, about 0.07 mg/kg, about 0.08 mg/kg, about 0.09 mg/kg, about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1 mg/kg, about 1.1 mg/kg, about 1.2 mg/kg, about 1.3 mg/kg, about 1.4 mg/kg, about 1.5 mg/kg, about 1.6 mg/kg, about 1.7 mg/kg, about 1.8 mg/kg, 1.9 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 6 mg/kg, about 7 mg/kg, about 8 mg/kg, about 9 mg/kg, about 10 mg/kg body weight, about 100 mg/kg body weight, about 1 g/kg of body weight, about 10 g/kg of body weight, inclusive of all values and ranges therebetween.

Individual doses of the CD8 binding agent can be administered in unit dosage forms containing, for example, from about 0.01 mg to about 100 g, from about 0.01 mg to about 75 g, from about 0.01 mg to about 50 g, from about 0.01 mg to about 25 g, about 0.01 mg to about 10 g, about 0.01 mg to about 7.5 g, about 0.01 mg to about 5 g, about 0.01 mg to about 2.5 g, about 0.01 mg to about 1 g, about 0.01 mg to about 100 mg, from about 0.1 mg to about 100 mg, from about 0.1 mg to about 90 mg, from about 0.1 mg to about 80 mg, from about 0.1 mg to about 70 mg, from about 0.1 mg to about 60 mg, from about 0.1 mg to about 50 mg, from about 0.1 mg to about 40 mg active ingredient, from about 0.1 mg to about 30 mg, from about 0.1 mg to about 20 mg, from about 0.1 mg to about 10 mg, from about 0.1 mg to about 5 mg, from about 0.1 mg to about 3 mg, from about 0.1 mg to about 1 mg per unit dosage form, or from about 5 mg to about 80 mg per unit dosage form. For example, a unit dosage form can be about 0.01 mg, about 0.02 mg, about 0.03 mg, about 0.04 mg, about 0.05 mg, about 0.06

mg, about 0.07 mg, about 0.08 mg, about 0.09 mg, about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.6 mg, about 0.7 mg, about 0.8 mg, about 0.9 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg about 10 mg, about 15 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg, about 200 mg, about 500 mg, about 1 g, about 2.5 g, about 5 g, about 10 g, about 25 g, about 50 g, about 75 g, about 100 g, inclusive of all values and ranges therebetween.

In one embodiment, the CD8 binding agent is administered at an amount of from about 0.01 mg to about 100 g daily, from about 0.01 mg to about 75 g daily, from about 0.01 mg to about 50 g daily, from about 0.01 mg to about 25 g daily, from about 0.01 mg to about 10 g daily, from about 0.01 mg to about 7.5 g daily, from about 0.01 mg to about 5 g daily, from about 0.01 mg to about 2.5 g daily, from about 0.01 mg to about 1 g daily, from about 0.01 mg to about 100 mg daily, from about 0.1 mg to about 100 mg daily, from about 0.1 mg to about 95 mg daily, from about 0.1 mg to about 90 mg daily, from about 0.1 mg to about 85 mg daily, from about 0.1 mg to about 80 mg daily, from about 0.1 mg to about 75 mg daily, from about 0.1 mg to about 70 mg daily, from about 0.1 mg to about 65 mg daily, from about 0.1 mg to about 60 mg daily, from about 0.1 mg to about 55 mg daily, from about 0.1 mg to about 50 mg daily, from about 0.1 mg to about 45 mg daily, from about 0.1 mg to about 40 mg daily, from about 0.1 mg to about 35 mg daily, from about 0.1 mg to about 30 mg daily, from about 0.1 mg to about 25 mg daily, from about 0.1 mg to about 20 mg daily, from about 0.1 mg to about 15 mg daily, from about 0.1 mg to about 10 mg daily, from about 0.1 mg to about 5 mg daily, from about 0.1 mg to about 3 mg daily, from about 0.1 mg to about 1 mg daily, or from about 5 mg to about 80 mg daily. In various embodiments, the CD8 binding agent is administered at a daily dose of about 0.01 mg, about 0.02 mg, about 0.03 mg, about 0.04 mg, about 0.05 mg, about 0.06 mg, about 0.07 mg, about 0.08 mg, about 0.09 mg, about 0.1 mg, about 0.2 mg, about 0.3 mg, about 0.4 mg, about 0.5 mg, about 0.6 mg, about 0.7 mg, about 0.8 mg, about 0.9 mg, about 1 mg, about 2 mg, about 3 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg about 10 mg, about 15 mg, about 20 mg, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, about 55 mg, about 60 mg, about 65 mg, about 70 mg, about 75 mg, about 80 mg, about 85 mg, about 90 mg, about 95 mg, about 100 mg, about 200 mg, about 500 mg, about 1 g, about 2.5 g, about 5 g, about 7.5 g, about 10 g, about 25 g, about 50 g, about 75 g, about 100 g, inclusive of all values and ranges therebetween.

In accordance with certain embodiments of the invention, the pharmaceutical composition comprising the CD8 binding agent may be administered, for example, more than once daily (*e.g.*, about two times, about three times, about four times, about five times, about six times, about seven times, about eight times, about nine times, or about ten times daily), about once per day, about every other day, about every third day, about once a week, about once every two weeks, about once every month, about once every two months, about once every three months, about once every six months, or about once every year.

Combination Therapy and Additional Therapeutic Agents

In various embodiments, the pharmaceutical composition of the present invention is co-administered in conjunction with additional therapeutic agent(s). Co-administration can be simultaneous or sequential.

In one embodiment, the additional therapeutic agent and the CD8 binding agent of the present invention are administered to a subject simultaneously. The term "simultaneously" as used herein, means that the additional therapeutic agent and the CD8 binding agent are administered with a time separation of no more than about 60 minutes, such as no more than about 30 minutes, no more than about 20 minutes, no more than about 10 minutes, no more than about 5 minutes, or no more than about 1 minute. Administration of the additional therapeutic agent and the CD8 binding agent can be by simultaneous administration of a single formulation (*e.g.*, a formulation comprising the additional therapeutic agent and the CD8 binding agent) or of separate formulations (*e.g.*, a first formulation including the additional therapeutic agent and a second formulation including the CD8 binding agent).

Co-administration does not require the therapeutic agents to be administered simultaneously, if the timing of their administration is such that the pharmacological activities of the additional therapeutic agent and the CD8 binding agent overlap in time, thereby exerting a combined therapeutic effect. For example, the additional therapeutic agent and the CD8 binding agent can be administered sequentially. The term "sequentially" as used herein means that the additional therapeutic agent and the CD8 binding agent are administered with a time separation of more than about 60 minutes. For example, the time between the sequential administration of the additional therapeutic agent and the CD8 binding agent can be more than about 60 minutes, more than about 2 hours, more than about 5 hours, more than about 10 hours, more than about 1 day, more than about 2 days, more than about 3 days, more than about 1 week apart, more than about 2 weeks apart, or more than about one month apart. The optimal administration times will depend on the rates of metabolism, excretion, and/or the pharmacodynamic activity of the additional therapeutic agent and the CD8 binding agent being administered. Either the additional therapeutic agent or the CD8 binding agent cell may be administered first.

Co-administration also does not require the therapeutic agents to be administered to the subject by the same route of administration. Rather, each therapeutic agent can be administered by any appropriate route, for example, parenterally or non-parenterally.

In some embodiments, the CD8 binding agent described herein acts synergistically when co-administered with another therapeutic agent. In such embodiments, the CD8 binding agent and the additional therapeutic agent may be administered at doses that are lower than the doses employed when the agents are used in the context of monotherapy.

In some embodiments, the present invention pertains to chemotherapeutic agents as additional therapeutic agents. For example, without limitation, such combination of the present CD8 binding agents and chemotherapeutic agent find use in the treatment of cancers, as described elsewhere herein. Examples of chemotherapeutic agents include, but are not limited to, alkylating agents such as thiotepa and CYTOXAN cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as

benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (*e.g.*, bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; cally statin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (*e.g.*, cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB 1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammall and calicheamicin omegall (*see*, *e.g.*, Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxy doxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as minoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (*e.g.*, T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*, TAXOL paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, 111.), and TAXOTERE doxetaxel (Rhone-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE. vinorelbine; novantrone; teniposide; edatrexate; daunomycin;

aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib (Tykerb); inhibitors of PKC-α, Raf, H-Ras, EGFR (*e.g.*, erlotinib (Tarceva)) and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above. In addition, the methods of treatment can further include the use of radiation. In addition, the methods of treatment can further include the use of photodynamic therapy.

In some embodiments, inclusive of, without limitation, infectious disease applications, the present invention pertains to anti-infectives as additional therapeutic agents. In some embodiments, the anti-infective is an anti-viral agent including, but not limited to, Abacavir, Acyclovir, Adefovir, Amprenavir, Atazanavir, Cidofovir, Darunavir, Delavirdine, Didanosine, Docosanol, Efavirenz, Elvitegravir, Emtricitabine, Enfuvirtide, Etravirine, Famciclovir, and Foscarnet. In some embodiments, the anti-infective is an anti-bacterial agent including, but not limited to, cephalosporin antibiotics (cephalexin, cefuroxime, cefadroxil, cefazolin, cephalothin, cefaclor, cefamandole, cefoxitin, cefprozil, and ceftobiprole); fluoroquinolone antibiotics (cipro, Levaquin, floxin, tequin, avelox, and norflox); tetracycline antibiotics (tetracycline, minocycline, oxytetracycline, and doxycycline); penicillin antibiotics (amoxicillin, ampicillin, penicillin V, dicloxacillin, carbenicillin, vancomycin, and methicillin); monobactam antibiotics (aztreonam); and carbapenem antibiotics (ertapenem, doripenem, imipenem/cilastatin, and meropenem). In some embodiments, the anti-infectives include anti-malarial agents (*e.g.*, chloroquine, quinine, mefloquine, primaquine, doxycycline, artemether/lumefantrine, atovaquone/proguanil and sulfadoxine/pyrimethamine), metronidazole, tinidazole, ivermectin, pyrantel pamoate, and albendazole.

In some embodiments, inclusive, without limitation, of autoimmmune applications, the additional therapeutic agent is an immunosuppressive agent. In some embodiments, the immunosuppressive agent is an anti-inflammatory agent such as a steroidal anti-inflammatory agent or a non-steroidal anti-inflammatory agent (NSAID). Steroids, particularly the adrenal corticosteroids and their synthetic analogues, are well known in the art. Examples of corticosteroids useful in the present invention include, without limitation, hydroxyltriamcinolone, alpha-methyl dexamethasone, beta-methyl betamethasone, beclomethasone dipropionate, betamethasone benzoate, betamethasone dipropionate, betamethasone valerate, clobetasol valerate, desonide, desoxymethasone, dexamethasone, diflorasone diacetate, diflucortolone valerate, fluadrenolone, fluclorolone acetonide, flumethasone pivalate, fluosinolone acetonide, fluocinonide, flucortine butylester, fluocortolone, fluprednidene (fluprednylidene) acetate, flurandrenolone, halcinonide, hydrocortisone acetate, hydrocortisone butyrate, methylprednisolone, triamcinolone acetonide, cortisone, cortodoxone, flucetonide, fludrocortisone, difluorosone diacetate, fluradrenolone acetonide, medrysone, amcinafel, amcinafide, betamethasone and the balance of its esters, chloroprednisone, clocortelone, clescinolone, dichlorisone, difluprednate, flucloronide, flunisolide, fluoromethalone, fluperolone, fluprednisolone, hydrocortisone, meprednisone, paramethasone, prednisolone, prednisone, beclomethasone dipropionate. (NSAIDS) that may be used in the present invention, include but are not limited to, salicylic acid, acetyl salicylic acid, methyl salicylate, glycol salicylate, salicylmides,

benzyl-2,5-diacetoxybenzoic acid, ibuprofen, fulindac, naproxen, ketoprofen, etofenamate, phenylbutazone, and indomethacin. In some embodiments, the immunosupressive agent may be cytostatics such as alkylating agents, antimetabolites (*e.g.*, azathioprine, methotrexate), cytotoxic antibiotics, antibodies (*e.g.*, basiliximab, daclizumab, and muromonab), anti-immunophilins (*e.g.*, cyclosporine, tacrolimus, sirolimus), inteferons, opioids, TNF binding proteins, mycophenolates, and small biological agents (*e.g.*, fingolimod, myriocin). Additional anti-inflammatory agents are described, for example, in U.S. Patent No. 4,537,776, the entire contents of which is incorporated by reference herein.

In some embodiments, the present invention relates to combination therapy with one or more immune-modulating agents, for example, without limitation, agents that modulate immune checkpoint. In various embodiments, the immune-modulating agent targets one or more of PD-1, PD-L1, and PD-L2. In various embodiments, the immune-modulating agent is PD-1 inhibitor. In various embodiments, the immune-modulating agent is an antibody specific for one or more of PD-1, PD-L1, and PD-L2. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, nivolumab, (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, MERCK), pidilizumab (CT-011, CURE TECH), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), MPDL328OA (ROCHE). In some embodiments, the immune-modulating agent targets one or more of CD137 or CD137L. In various embodiments, the immune-modulating agent is an antibody specific for one or more of CD137 or CD137L. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, urelumab (also known as BMS-663513 and anti-4-1BB antibody). In some embodiments, the present CD8 binding agent is combined with urelumab (optionally with one or more of nivolumab, lirilumab, and urelumab) for the treatment of solid tumors and/or B-cell non-Hodgkins lymphoma and/or head and neck cancer and/or multiple myeloma. In some embodiments, the immune-modulating agent is an agent that targets one or more of CTLA-4, AP2M1, CD80, CD86, SHP-2, and PPP2R5A. In various embodiments, the immune-modulating agent is an antibody specific for one or more of CTLA-4, AP2M1, CD80, CD86, SHP-2, and PPP2R5A. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, ipilimumab (MDX-010, MDX-101, Yervoy, BMS) and/or tremelimumab (Pfizer). In some embodiments, the present CD8 binding agent is combined with ipilimumab (optionally with bavituximab) for the treatment of one or more of melanoma, prostate cancer, and lung cancer. In various embodiments, the immune-modulating agent targets CD20. In various embodiments, the immune-modulating agent is an antibody specific CD20. For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, Ofatumumab (GENMAB), obinutuzumab (GAZYVA), AME-133v (APPLIED MOLECULAR EVOLUTION), Ocrelizumab (GENENTECH), TRU-015 (TRUBION/EMERGENT), veltuzumab (IMMU-106).

In some embodiments, the present invention relates to combination therapy with one or more chimeric agents described in WO 2013/10779, WO 2015/007536, WO 2015/007520, WO 2015/007542, and WO 2015/007903, the entire contents of which are hereby incorporated by reference in their entireties.

In some embodiments, the CD8 binding agent described herein, include derivatives that are modified, *i.e.*, by the covalent attachment of any type of molecule to the composition such that covalent attachment does not prevent the activity of the composition. For example, but not by way of limitation, derivatives include composition that have been modified by, *inter alia*, glycosylation, lipidation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc*. Any of numerous chemical modifications can be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, *etc.*

In still other embodiments, the CD8 binding agent described herein further comprise a cytotoxic agent, comprising, in illustrative embodiments, a toxin, a chemotherapeutic agent, a radioisotope, and an agent that causes apoptosis or cell death. Such agents may be conjugated to a composition described herein.

The CD8 binding agent described herein may thus be modified post-translationally to add effector moieties such as chemical linkers, detectable moieties such as for example fluorescent dyes, enzymes, substrates, bioluminescent materials, radioactive materials, and chemiluminescent moieties, or functional moieties such as for example streptavidin, avidin, biotin, a cytotoxin, a cytotoxic agent, and radioactive materials.

Illustrative cytotoxic agents include, but are not limited to, methotrexate, aminopterin, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine; alkylating agents such as mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU), mitomycin C, lomustine (CCNU), 1-methylnitrosourea, cyclothosphamide, mechlorethamine, busulfan, dibromomannitol, streptozotocin, mitomycin C, cis-dichlorodiamine platinum (II) (DDP) cisplatin and carboplatin (paraplatin); anthracyclines include daunorubicin (formerly daunomycin), doxorubicin (adriamycin), detorubicin, carminomycin, idarubicin, epirubicin, mitoxantrone and bisantrene; antibiotics include dactinomycin (actinomycin D), bleomycin, calicheamicin, mithramycin, and anthramycin (AMC); and antimytotic agents such as the vinca alkaloids, vincristine and vinblastine. Other cytotoxic agents include paclitaxel (taxol), ricin, pseudomonas exotoxin, gemcitabine, cytochalasin B, gramicidin D, ethidium bromide, emetine, etoposide, tenoposide, colchicin, dihydroxy anthracin dione, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, procarbazine, hydroxyurea, asparaginase, corticosteroids, mytotane (O,P'-(DDD)), interferons, and mixtures of these cytotoxic agents.

Further cytotoxic agents include, but are not limited to, chemotherapeutic agents such as carboplatin, cisplatin, paclitaxel, gemcitabine, calicheamicin, doxorubicin, 5-fluorouracil, mitomycin C, actinomycin D, cyclophosphamide, vincristine, bleomycin, VEGF antagonists, EGFR antagonists, platins, taxols, irinotecan, 5-fluorouracil, gemcytabine, leucovorine, steroids, cyclophosphamide, melphalan, vinca alkaloids (*e.g.*, vinblastine, vincristine, vindesine and vinorelbine), mustines, tyrosine kinase inhibitors, radiotherapy, sex hormone antagonists, selective androgen receptor modulators, selective estrogen receptor modulators, PDGF antagonists, TNF antagonists, IL-1 antagonists, interleukins (*e.g.* IL-12 or IL-2), IL-12R antagonists, Toxin conjugated monoclonal antibodies, tumor antigen specific monoclonal antibodies, Erbitux, Avastin, Pertuzumab, anti-CD20

antibodies, Rituxan, ocrelizumab, ofatumumab, DXL625, HERCEPTIN®, or any combination thereof. Toxic enzymes from plants and bacteria such as ricin, diphtheria toxin and Pseudomonas toxin may be conjugated to the therapeutic agents (*e.g.* antibodies) to generate cell-type-specific-killing reagents (Youle, *et al.*, Proc. Nat'l Acad. Sci. USA 77:5483 (1980); Gilliland, *et al.*, Proc. Nat'l Acad. Sci. USA 77:4539 (1980); Krolick, *et al.*, Proc. Nat'l Acad. Sci. USA 77:5419 (1980)).

Other cytotoxic agents include cytotoxic ribonucleases as described by Goldenberg in U.S. Pat. No. 6,653,104. Embodiments of the invention also relate to radioimmunoconjugates where a radionuclide that emits alpha or beta particles is stably coupled to the CD8 binding agent, with or without the use of a complex-forming agent. Such radionuclides include beta-emitters such as Phosphorus-32, Scandium-47, Copper-67, Gallium-67, Yttrium-88, Yttrium-90, Iodine-125, Iodine-131, Samarium-153, Lutetium-177, Rhenium-186 or Rhenium-188, and alpha-emitters such as Astatine-211, Lead-212, Bismuth-212, Bismuth-213 or Actinium-225.

Illustrative detectable moieties further include, but are not limited to, horseradish peroxidase, acetylcholinesterase, alkaline phosphatase, beta-galactosidase and luciferase. Further illustrative fluorescent materials include, but are not limited to, rhodamine, fluorescein, fluorescein isothiocyanate, umbelliferone, dichlorotriazinylamine, phycoerythrin and dansyl chloride. Further illustrative chemiluminescent moieties include, but are not limited to, luminol. Further illustrative bioluminescent materials include, but are not limited to, luciferin and aequorin. Further illustrative radioactive materials include, but are not limited to, Iodine-125, Carbon-14, Sulfur-35, Tritium and Phosphorus-32.

Methods of Treatment

Methods and compositions described herein have application to treating various diseases and disorders, including, but not limited to cancer, infections, immune disorders, inflammatory diseases or conditions, and autoimmune diseases.

Further, any of the present agents may be for use in the treating, or the manufacture of a medicament for treating, various diseases and disorders, including, but not limited to cancer, infections, immune disorders, inflammatory diseases or conditions, and autoimmune diseases.

In some embodiments, the present invention relates to the treatment of, or a patient having cancer. As used herein, cancer refers to any uncontrolled growth of cells that may interfere with the normal functioning of the bodily organs and systems, and includes both primary and metastatic tumors. Primary tumors or cancers that migrate from their original location and seed vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs. A metastasis is a cancer cell or group of cancer cells, distinct from the primary tumor location, resulting from the dissemination of cancer cells from the primary tumor to other parts of the body. Metastases may eventually result in death of a subject. For example, cancers can include benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases.

Illustrative cancers that may be treated include, but are not limited to, basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (*e.g.* that associated with brain tumors), and Meigs' syndrome.

Illustrative cancers that may be treated include, but are not limited to, carcinomas, *e.g.* various subtypes, including, for example, adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma), sarcomas (including, for example, bone and soft tissue), leukemias (including, for example, acute myeloid, acute lymphoblastic, chronic myeloid, chronic lymphocytic, and hairy cell), lymphomas and myelomas (including, for example, Hodgkin and non-Hodgkin lymphomas, light chain, non-secretory, MGUS, and plasmacytomas), and central nervous system cancers (including, for example, brain (*e.g.* gliomas (*e.g.* astrocytoma, oligodendroglioma, and ependymoma), meningioma, pituitary adenoma, and neuromas, and spinal cord tumors (*e.g.* meningiomas and neurofibroma).

In some embodiments, the present invention relates to the treatment of, or a patient having a microbial infection and/or chronic infection. Illustrative infections include, but are not limited to, HIV/AIDS, tuberculosis, osteomyelitis, hepatitis B, hepatitis C, Epstein-Barr virus or parvovirus, T cell leukemia virus, bacterial overgrowth syndrome, fungal or parasitic infections.

In various embodiments, the present compositions are used to treat or prevent one or more inflammatory diseases or conditions, such as inflammation, acute inflammation, chronic inflammation, respiratory disease, atherosclerosis, restenosis, asthma, allergic rhinitis, atopic dermatitis, septic shock, rheumatoid arthritis, inflammatory bowel disease, inflammatory pelvic disease, pain, ocular inflammatory disease, celiac disease, Leigh Syndrome, Glycerol Kinase Deficiency, Familial eosinophilia (FE), autosomal recessive spastic ataxia,

laryngeal inflammatory disease; Tuberculosis, Chronic cholecystitis, Bronchiectasis, Silicosis and other pneumoconioses.

In various embodiments, the present compositions are used to treat or prevent one or more autoimmune diseases or conditions, such as multiple sclerosis, diabetes mellitus, lupus, celiac disease, Crohn's disease, ulcerative colitis, Guillain-Barre syndrome, scleroderms, Goodpasture's syndrome, Wegener's granulomatosis, autoimmune epilepsy, Rasmussen's encephalitis, Primary biliary sclerosis, Sclerosing cholangitis, Autoimmune hepatitis, Addison's disease, Hashimoto's thyroiditis, Fibromyalgia, Menier's syndrome; transplantation rejection (*e.g.*, prevention of allograft rejection) pernicious anemia, rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, Sjogren's syndrome, lupus erythematosus, multiple sclerosis, myasthenia gravis, Reiter's syndrome, Grave's disease, and other autoimmune diseases.

Kits

The present invention also provides kits for the administration of any CD8 binding agent described herein (*e.g.* with or without additional therapeutic agents). The kit is an assemblage of materials or components, including at least one of the inventive pharmaceutical compositions described herein. Thus, in some embodiments, the kit contains at least one of the pharmaceutical compositions described herein.

The exact nature of the components configured in the kit depends on its intended purpose. In one embodiment, the kit is configured for the purpose of treating human subjects.

Instructions for use may be included in the kit. Instructions for use typically include a tangible expression describing the technique to be employed in using the components of the kit to effect a desired therapeutic outcome, such as to treat cancer. Optionally, the kit also contains other useful components, such as, diluents, buffers, pharmaceutically acceptable carriers, syringes, catheters, applicators, pipetting or measuring tools, bandaging materials or other useful paraphernalia as will be readily recognized by those of skill in the art.

The materials and components assembled in the kit can be provided to the practitioner stored in any convenience and suitable ways that preserve their operability and utility. For example, the components can be provided at room, refrigerated or frozen temperatures. The components are typically contained in suitable packaging materials. In various embodiments, the packaging material is constructed by well-known methods, preferably to provide a sterile, contaminant-free environment. The packaging material may have an external label which indicates the contents and/or purpose of the kit and/or its components.

Definitions

As used herein, “a,” “an,” or “the” can mean one or more than one.

Further, the term “about” when used in connection with a referenced numeric indication means the referenced numeric indication plus or minus up to 10% of that referenced numeric indication. For example, the language “about 50” covers the range of 45 to 55.

An "effective amount," when used in connection with medical uses is an amount that is effective for providing a measurable treatment, prevention, or reduction in the rate of pathogenesis of a disease of interest.

As used herein, something is "decreased" if a read-out of activity and/or effect is reduced by a significant amount, such as by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or more, up to and including at least about 100%, in the presence of an agent or stimulus relative to the absence of such modulation. As will be understood by one of ordinary skill in the art, in some embodiments, activity is decreased and some downstream read-outs will decrease but others can increase.

Conversely, activity is "increased" if a read-out of activity and/or effect is increased by a significant amount, for example by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, or more, up to and including at least about 100% or more, at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 50-fold, at least about 100-fold, in the presence of an agent or stimulus, relative to the absence of such agent or stimulus.

As referred to herein, all compositional percentages are by weight of the total composition, unless otherwise specified. As used herein, the word "include," and its variants, is intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that may also be useful in the compositions and methods of this technology. Similarly, the terms "can" and "may" and their variants are intended to be non-limiting, such that recitation that an embodiment can or may comprise certain elements or features does not exclude other embodiments of the present technology that do not contain those elements or features.

Although the open-ended term "comprising," as a synonym of terms such as including, containing, or having, is used herein to describe and claim the invention, the present invention, or embodiments thereof, may alternatively be described using alternative terms such as "consisting of" or "consisting essentially of."

As used herein, the words "preferred" and "preferably" refer to embodiments of the technology that afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the technology.

The amount of compositions described herein needed for achieving a therapeutic effect may be determined empirically in accordance with conventional procedures for the particular purpose. Generally, for administering therapeutic agents for therapeutic purposes, the therapeutic agents are given at a pharmacologically effective dose. A "pharmacologically effective amount," "pharmacologically effective dose," "therapeutically effective amount," or "effective amount" refers to an amount sufficient to produce the desired physiological effect or

amount capable of achieving the desired result, particularly for treating the disorder or disease. An effective amount as used herein would include an amount sufficient to, for example, delay the development of a symptom of the disorder or disease, alter the course of a symptom of the disorder or disease (*e.g.*, slow the progression of a symptom of the disease), reduce or eliminate one or more symptoms or manifestations of the disorder or disease, and reverse a symptom of a disorder or disease. Therapeutic benefit also includes halting or slowing the progression of the underlying disease or disorder, regardless of whether improvement is realized.

Effective amounts, toxicity, and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to about 50% of the population) and the ED50 (the dose therapeutically effective in about 50% of the population). The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. In some embodiments, compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from in vitro assays, including, for example, cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 as determined in cell culture, or in an appropriate animal model. Levels of the described compositions in plasma can be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment.

In certain embodiments, the effect will result in a quantifiable change of at least about 10%, at least about 20%, at least about 30%, at least about 50%, at least about 70%, or at least about 90%. In some embodiments, the effect will result in a quantifiable change of about 10%, about 20%, about 30%, about 50%, about 70%, or even about 90% or more. Therapeutic benefit also includes halting or slowing the progression of the underlying disease or disorder, regardless of whether improvement is realized.

As used herein, “methods of treatment” are equally applicable to use of a composition for treating the diseases or disorders described herein and/or compositions for use and/or uses in the manufacture of a medicaments for treating the diseases or disorders described herein.

EXAMPLES

The term “AcTaferon” is occasionally used herein to reference an interferon-based chimera.

In the following examples, unless noted, mutations to IFN are relative to human IFN-α2 - SEQ ID NO:25.

The Q124R mutant is representative of an attenuated human IFN alpha 2 mutant that can be assayed *in vivo* in a murine model. Specifically, Q124R is a human IFN mutation that is suitable for use in the mouse (*i.e.* it is a human mutant IFN that functions in mouse). *See Nat. Comm.* 2014;5:3016. doi: 10.1038/ncomms4016, the entire contents of which are hereby incorporated by reference.

The R33A/E120R mutant is representative of human IFN alpha 2 mutant that is non-functional (and is used as a control)

Anti-BclI10 VHH is used in these Examples as a control (targeting an irrelevant antigen, *i.e.* "untargeted").

Example 1. Construction and Evaluation of VHHs Specific for Mouse CD8

Isolation of antigen-specific VHHs

A VHH library was subject to 2 consecutive rounds of panning (in solution), performed on stably transfected CHO-K1 cells expressing mouse CD8. The enrichment for antigen-specific phages was assessed after each round of panning by comparing the number of phagemid particles eluted from transfected cells (output) with the number of phagemid particles used for panning (input). The phage output increased about 10^2-fold in the 2nd round, as compared to the output from the 1st round. The input phage was always about 5×10^{11} and the output from first round was about 10^9 phage particles. 190 randomly selected colonies from the 1st and 2nd panning rounds (95 from each round) were sequenced and then grouped based on CDR3 sequences. Using crude periplasmic extracts including VHHs, representative(s) of each group was (were) analyzed by flow cytometry for specificity to mouse CD8 using CHO-K1 cells stably transfected with mouse CD8. The parental non-transfected CHO-K1 cells served as negative control cell. An irrelevant VHH was used as negative VHH control. The flow cytometry experiments revealed that 34 different VHHs, belonging to 7 different groups, were specific for mouse CD8. Table 1 below provides a description of 34 clones representing the 34 different anti-mouse CD8 VHH genes. *E. coli* TG1 harboring recombinant phagemid pHEN4 containing anti-mouse CD8 VHH sequences was generated and stored at -80°C. The vector pHEN4 codes for ampicillin resistance.

Table 1.

***E. coli* strain + Vector**	**VHH (Nb)**	**NSF Reference No. (Glycerol stock)**
TG1, pHEN4	R1CDE 4	1946
TG1, pHEN4	R1CDE 6	1947
TG1, pHEN4	R1CDE 10	1948
TG1, pHEN4	R1CDE 16	1949
TG1, pHEN4	R1CDE 17	1950
TG1, pHEN4	R1CDE 20	1951
TG1, pHEN4	R1CDE 24	1952
TG1, pHEN4	R1CDE 26	1953
TG1, pHEN4	R1CDE 27	1954
TG1, pHEN4	R1CDE 28	1955
TG1, pHEN4	R1CDE 32	1956
TG1, pHEN4	R1CDE 35	1957
TG1, pHEN4	R1CDE 43	1958
TG1, pHEN4	R1CDE 46	1959
TG1, pHEN4	R1CDE 52	1960
TG1, pHEN4	R1CDE 60	1961
TG1, pHEN4	R1CDE 88	1962
TG1, pHEN4	R1CDE 95	1963
TG1, pHEN4	R2CDE 5	1964
TG1, pHEN4	R2CDE 21	1965

TG1, pHEN4	R2CDE 23	1966
TG1, pHEN4	R2CDE 33	1967
TG1, pHEN4	R2CDE 36	1968
TG1, pHEN4	R2CDE 37	1969
TG1, pHEN4	R2CDE 39	1970
TG1, pHEN4	R2CDE 51	1971
TG1, pHEN4	R2CDE 55	1972
TG1, pHEN4	R2CDE 69	1973
TG1, pHEN4	R2CDE 71	1974
TG1, pHEN4	R2CDE 75	1975
TG1, pHEN4	R2CDE 84	1976
TG1, pHEN4	R2CDE 86	1977
TG1, pHEN4	R2CDE 88	1978
TG1, pHEN4	R2CDE 93	1979

Expression and purification of VHHs

The VHH genes were recloned from pHEN4 to pHEN6c vectors. Specifically, the VHH gene was amplified by PCR using recombinant pHEN4 harboring the VHH gene as template and primers A6E and 38. Primers A6E and 38 were framework1 and framework4 primers, respectively. The primer sequences were as follows:

- Primer A6E (5' GAT GTG CAG CTG CAG GAG TCT GGR* GGA GG 3') (SEQ ID NO:228).
- Primer 38 (5' GGA CTA GTG CGG CCG CTG GAG ACG GTG ACC TGG GT 3') (SEQ ID NO:229).
- Universal reverse primer (5' TCA CAC AGG AAA CAG CTA TGA C 3') (SEQ ID NO:230).
- Universal forward primer (5' CGC CAG GGT TTT CCC AGT CAC GAC 3') (SEQ ID NO:231).

*R stands for A or G.

The amplification protocol included about 30 cycles of PCR, each cycle included 30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C, followed by 10 minutes extension at 72°C at the end of PCR. A fragment of about 400 bp was amplified.

The PCR product was purified (*e.g.* by Qiaquick PCR purification kit from Qiagen) and digested overnight with PstI. The purified PCR product was then digested with BstEII overnight (or with Eco91I from Fermentas). The temperature used for digestion varied. For example, digestion with BstEII was done at 50°C or 60°C depending on the supplier of the enzyme.

For ligation, the PCR product was purified. The pHEN6c vector was digested with PstI for 3 hours, purified as described above, and then digested with BstEII for 2 to 3 hours. Alternatively, digestion was carried out using Eco91I from Fermentas. The digested vector was ran on 1% agarose gel, with the vector band excised out of the gel and purified (*e.g.* by Qiaquick gel extraction kit from Qiagen). The PCR fragment was subsequently ligated to the vector.

Electrocompetent WK6 cells were transformed with the ligation reaction, and transformants were selected using LB/agar/ampicillin (100 μg/ml)/glucose (1%) plates. Positive clones were screened by PCR using universal reverse and universal forward primers. A fragment of about 550 bp was amplified, if the insert was present. To verify the identity of the clones, at least 2 clones per each VHH were sequenced using universal reverse primers. Antigen binding capacity was retested by ELISA or any other appropriate assay.

Following the above protocol, the VHH gene cloned in pHEN6c vector was generated which contained PelB signal sequence at the N-terminus and His_6-tail at the C-terminus. The PelB leader sequence directed the VHH to the periplasmic space of the *E.coli,* and the His-tag was used for the purification and detection of VHH (*e.g.* in ELISA, Western Blot, *etc.*).

Expression and purification of VHHs were carried out. Specifically, on day 1, 10-20 ml of LB + ampicillin (100 μg/ml) + glucose (1%) were innoculated with a freshly transformed WK6 colony. This pre-culture was incubated at 37°C overnight with shaking at 200-250 rpm. On day 2, a TB medium was used for expressing the VHHs. The TB medium included, per liter: 2.3 g KH_2PO_4, 16.4 g $K_2HPO_4.3H_2O$, 12 g Tryptone (Duchefa Biochemie), 24 g Yeast (Duchefa Biochemie), and 4 ml 100% glycerol (Duchefa Biochemie).

A baffled shaker flask of 1 liter was filled with 330 ml TB and autoclaved. KH_2PO_4 and $K_2HPO_4.3H_2O$ were not autoclaved. Instead, KH_2PO_4 and $K_2HPO_4.3H_2O$ were prepared, filter sterilized, and then added to the rest of the medium that was already autoclaved. About 1 ml of the pre-culture was added to 330 ml of TB supplemented with 100 μg/ml Ampicillin, 2 mM $MgCl_2$ and 0.1% glucose and subsequently grew at 37°C with shaking (200-250 rpm) until an OD_{600} of 0.6-0.9 was reached. IPTG (final concentration of 1 mM) was added to induce VHH expression. The culture was incubated at 28°C with shaking overnight (about 16-18 hours). The OD_{600} after overnight induction was usually between 25 and 30. At least 1 liter of culture (3 bottles) per clone was prepared with an average yield of between 1 and 15 mg/l.

Extraction of the VHHs from the periplasm of E. coli was carried out on day 3. The solutions used included: TES: 0.2 M Tris pH 8.0, 0.5 mM EDTA, 0.5 M sucrose, and TES/4: TES diluted 4 times in water.

The overnight induced cultures were centrifuged for 8 minutes at 8000 rpm. The cell pellets from 1 liter culture were resuspended in 12 ml TES by pipetting up and down and shaken for 1 hour on ice. Per each 12 ml TES used, about 18 ml TES/4 were added and incubated on ice for an additional hour with shaking followed by centrifugation for 30 minutes at 8000 rpm at 4°C. The supernatant which contained proteins extracted from the periplasmic spaced was transferred to fresh falcon tubes.

The VHHs were subsequently purified by IMAC which utilized the following solution: HIS-select (SIGMA), PBS, and 50 mM NaAcetate pH 4.6.

His-select was equilibrated with PBS. Specifically, per periplasmic extract derived from 1 liter culture, 1 ml of Resin (about 2 ml His-select solution) was added to a 50 ml falcon tube. PBS was also added to final volume of 50 ml and mixed. Centrifugation was carried out at 2000 rpm for 2 minutes, and the supernatant was discarded.

The resin was washed with PBS twice as described above. The periplasmic extract was added to the resin, incubated for 30 minutes to 1 hour at room temperature with gentle shaking. The samples were loaded on PD-10 columns with a filter at the bottom (GE healthcare, cat. No. 17-0435-01) and washed with 50 to 100 ml PBS (50-100 ml PBS per 1 ml resin used). Elution was carried out for 3 times, each time with 1 ml PBS/0.5 M imidazole per 1 ml resin used (for efficient elution, resuspend the beads and leave overnight at 4°C with the bottom of the column closed). Dialysis was performed overnight at 4°C against PBS (cutoff 3500 daltons) to remove imidazole. For efficient dialysis, the dialysis buffer (PBS) was changed 2-3 times. Alternatively, instead of elution with imidazole, the bound VHHs could be eluted with 10 ml 50 mM Na-acetate pH 4.6. If 50 mM Na-acetate pH 4.6 was used to elute VHHs, the eluted VHHs was immediately neutralized with 1 M Tris pH 8.0, and no dialysis was required.

The amount of protein was estimated by OD_{280} measurement of eluted sample. Extinction coefficient of each clone was determined by protParam tool under primary structure analysis at the Expasy proteomics server. Further purification of VHHs could be achieved by different methods. For example, the samples could be concentrated (Vivaspin 5000 MW cutoff, Vivascience) by centrifuging at 2000 rpm at 4°C until an appropriate volume for loading on a Superdex 75 16/60 was obtained (max. 4 ml). The concentrated sample was loaded on a Superdex 75 16/60 column equilibrated with PBS. Peak fractions were pooled, and OD_{280} measurements were performed for quantification. In general, VHHs eluted after 85-95 minutes when run at 1 ml/min. Aliquots of concentrated VHH samples were stored at -20°C at a concentration of about 1 mg/ml.

Functional analysis of VHHs

The VHHs were tested for binding to CD8. Specifically, mouse splenocytes were stained with anti-CD8 VHHs at 1 ug/ml and anti-His FITC mAb. Out of 31 positive VHHs, six were stained positive on splenocytes. **Figure 1**, panels A-B show binding of the six VHHs to CHO cells transfected with CD8α or to splenocytes. In all experiments, the six selected VHHs were labeled as follows:

Group	Nb Code	Tube label	single cells | Geometric Mean (FL1-H)
		20140908 CHO mCD8 negative control	4.05
1		20140908 CHO mCD8.11259	13.2
1		20140908 CHO mCD8.11260	12.4
1		20140908 CHO mCD8.11261	11.3
1		20140908 CHO mCD8.11262	12.5
5		20140908 CHO mCD8.11264	11.2
2	R1CDE24	20140908 CHO mCD8.11265	11.5
1		20140908 CHO mCD8.11266	4.14
3		20140908 CHO mCD8.11267	9.69
4	R1CDE28	20140908 CHO mCD8.11268	10.5
2	R1CDE32	20140908 CHO mCD8.11269	12.3
2		20140908 CHO mCD8.11270	9.14
2	R1CDE43	20140908 CHO mCD8.11271	9.05
3		20140908 CHO mCD8.11272	4.9

1		20140908 CHO mCD8.11273	5.56
1		20140908 CHO mCD8.11274	10.3
5		20140908 CHO mCD8.11275	4.26
1		20140908 CHO mCD8.11276	10.8
1		20140908 CHO mCD8.11277	11.3
2	R2CDE21	20140908 CHO mCD8.11278	11
5		20140908 CHO mCD8.11279	9.96
1		20140911 CHO_mCD8.11280	13.1
2		20140911 CHO_mCD8.11281	8.53
1		20140911 CHO_mCD8.11282	11.3
3		20140911 CHO_mCD8.11283	10.1
1		20140911 CHO_mCD8.11284	12.5
1		20140911 CHO_mCD8.11285	12.2
1		20140911 CHO_mCD8.11286	12.4
2	R2CDE71	20140911 CHO_mCD8.11287	12.1
1		20140911 CHO_mCD8.11288	11
6		20140911 CHO_mCD8.11289	12.3
1		20140911 CHO_mCD8.11290	12.8
3		20140911 CHO_mCD8.11291	10.3
1		20140911 CHO_mCD8.11292	12.7

The effects of the VHHs on T cell proliferation and activation were assessed. Specifically, OT-I Rag-/- T cells were cultured for 48 hours in a 50:1 ratio with unloaded or OVA peptide (SIINFEKL, SEQ ID NO:232)-loaded dendritic cells. Co-cultures were supplemented with 50 ng of the mCD8 VHHs, an irrelevant VHH or a monoclonal Ab known to block TCR activation (CT-CD8). OVA-induced T cell activation was determined by measuring CFSE dilution and CD25 expression. Co-cultures were performed in triplicate, cells were pooled for flow cytometric analysis. Results are shown in **Figure 2**. Clearly, none of the selected VHHs affected CTL activation, in stark contrast to CT-mAB, a monoclonal antibody known to interfere with the antigen presentation process.

Experiments were carried out to characterize the activity of the VHHs in treating tumors.

For **Figure 3**, female C57BL/6J mice were injected s.c. with 6 x 10^5 B16Bl6-mCD20 cells in 50 µl PBS. Seven days later, perilesional (= s.c. at the edge of the tumor) treatments were started; 5500 IU of chimeric proteins were injected while control mice received 100 µl PBS. Treatments were given at days 8, 9, 11, 12, 14, 16 and 17 after tumor cell inoculation. Tumor growth was monitored using a digital caliper. Plotted are means ± SEM (n=5).

For **Figure 4**, female C57BL/6J mice were injected s.c. with 6 x 10^5 B16Bl6-mCD20 cells in 50 µl PBS. Seven days later, perilesional (= s.c. at the edge of the tumor) treatments were started; 5500 IU of chimeric proteins were injected while control mice received 100 µl PBS. Treatments were given at days 8, 9, 11, 12, 14, 16 and 17 after tumor cell inoculation. One day after the last treatment (day 18), EDTA-blood was collected from the tail vein for analysis of hematological parameters using a Hemavet 950FS Analyzer (Drew Scientific). Plotted are means ± SEM (n=5)..

As shown in **Figure 3**, administration of VHHs against mouse CD8, fused to a modified human interferon (Q124R mutant), effectively reduced tumor size. **Figure 4** demonstrates that treatment with the VHHs against mouse CD8, fused to a modified human interferon (Q124R mutant), did not result in weight loss or hematological toxicity.

Example 2. Construction and Evaluation of VHHs Specific for Human CD8

Isolation of Antigen-specific VHHs

Three consecutive rounds of panning of a VHH library were performed on solid-phase coated His_6-tagged extracellular domain of human CD8A (200 μg/ml, 20 μg/well). The enrichment for antigen-specific phages was assessed after each round of panning by comparing the number of phagemid particles eluted from antigen-coated wells with the number of phagemid particles eluted from only-blocked wells (negative control wells). These experiments suggested that the phage population was enriched about 4-fold, 10^2-fold and 10^2-fold for antigen-specific phages after 1st, 2nd and 3rd rounds of panning, respectively. 285 colonies (95 & 190 from 2nd and 3rd rounds, respectively) were randomly selected and analyzed by ELISA for the presence of antigen-specific VHHs in their periplasmic extracts (ELISA using crude periplasmic extracts including soluble VHHs). The antigen used for the ELISA screening was the same as the one used for immunization and panning. Out of these 285 colonies, 64 colonies (3 and 61 from 2nd and 3rd rounds of panning, respectively) scored positive in this assay. Based on sequence data, the 64 positive colonies represented 16 different VHHs. The 16 different VHHs belong to 6 different CDR3 groups. Table 2 below provides a description of 3 clones representing the 3 different anti-human CD8A VHH genes. *E. coli* TG1 harboring recombinant phagemid pMECS containing anti-human CD8A VHH sequences was generated and stored at -80°C. The vector pMECS codes for ampicillin resistance.

Table 2.

***E. coli* strain + Vector**	**VHH (Nb)**	**NSF glycerol stock No.**
TG1, pMECS	R2HCD 26	2293
TG1, pMECS	R3HCD 27	2297
TG1, pMECS	R3HCD 129	2302

Transformation of non-suppressor strain (e.g. WK6) with recombinant pMECS

The VHH gene cloned in pMECS vector contained PelB signal sequence at the N-terminus and HA tag and His_6 tag at the C-terminus (PelB leader-VHH-HA-His_6). The PelB leader sequence directed the VHH to the periplasmic space of the *E.coli,* and the HA and His_6 tags was used for the purification and detection of VHH (*e.g.* in ELISA, Western Blot, *etc.*).

In pMECS vector, the His_6 tag was followed by an amber stop codon (TAG) and this amber stop codon was followed by gene III of M13 phage. In suppressor *E. coli* strains (*e.g.* TG1), the amber stop codon was read as glutamine and therefore the VHH was expressed as fusion protein with protein III of the phage which allowed the

display of the VHH on the phage coat for panning. In TG1 supressor strains, the efficiency of suppression is not 100% and therefore the expression of VHHs in suppressor strains led to two different types of VHH molecules, fused to protein III and without protein III). In non-suppressor *E. coli* strains (e. g., WK6), the amber stop codon was read as stop codon and therefore the resulting VHH was not fused to protein III.

In order to express and purify VHHs cloned in pMECS vector, pMECS was prepared containing the gene of the VHH of interest, and the plasmid was transformed into a non-suppressor strain (*e.g.*, WK6). The VHH of the resulting clone was sequenced using the MP057 primer (5'-TTATGCTTCCGGCTCGTATG-3') (SEQ ID NO:233) to verify the identity of the clone. Antigen binding capacity was retested by ELISA or any other appropriate assay. The non-suppressor strain (*e.g.*, WK6) containing the recombinant pMECS vector with the VHH gene was used for the expression and purification of the VHH.

In pMECS vector, the His_6 tag was cleaved off upon storage of the VHH. Accordingly, the VHH gene was recloned from pMECS into pHEN6c vector, if the His_6 tag was to be used for detection, *etc.* Specifically, the VHH gene was amplified by PCR using recombinant pMECS harboring the VHH gene as template and primers A6E and PMCF. Primers A6E and PMCF were framework1 and framework4 primers, respectively. The primer sequences were as follows:

- Primer A6E (5' GAT GTG CAG **CTG CAG** GAG TCT GGR* GGA GG 3') (SEQ ID NO:228).
- Primer PMCF (5' CTA GT*G CGG CCG C*TG AGG AGA CGG TGA CCT GGG T 3') (SEQ ID NO:234).
- Universal reverse primer (5' TCA CAC AGG AAA CAG CTA TGA C 3') (SEQ ID NO:230).
- Universal forward primer (5' CGC CAG GGT TTT CCC AGT CAC GAC 3') (SEQ ID NO:231).

*R stands for A or G. PstI, NotI and BstEII (Eco91I) recognition sequences are shown in bold, italic and underline, respectively.

The amplification protocol included about 30 cycles of PCR, each cycle included 30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C, followed by 10 minutes extension at 72°C at the end of PCR. A fragment of about 400 bp was amplified.

The PCR product was purified (*e.g.* by Qiaquick PCR purification kit from Qiagen) and digested overnight with PstI. The purified PCR product was digested with BstEII overnight (or with Eco91I from Fermentas). The temperature used for digestion varied. For example, digestion with BstEII was done at 50°C or 60°C depending on the supplier of the enzyme.

For ligation, the PCR product was purified. The pHEN6c vector was digested with PstI for 3 hours, purified as described above, and then digested with BstEII for 2 to 3 hours. Alternatively, digestion was carried out using Eco91I from Fermentas. The digested vector was ran on 1% agarose gel, with the vector band excised out of the gel and purified (*e.g.* by Qiaquick gel extraction kit from Qiagen). The PCR fragment was subsequently ligated to the vector.

Electrocompetent WK6 cells were transformed with the ligation reaction, and transformants were selected using LB/agar/ampicillin (100 µg/ml)/glucose (1-2%) plates. Positive clones were screened by PCR using universal reverse and universal forward primers. A fragment of about 550 bp was amplified, if the insert was present. To verify the identity of the clones, at least 2 clones per each VHH were sequenced using universal reverse primers. Antigen binding capacity was retested by ELISA or any other appropriate assay.

Following the above protocol, the VHH gene cloned in pHEN6c vector was generated which contained PelB signal sequence at the N-terminus and His_6-tail at the C-terminus. The PelB leader sequence directed the VHH to the periplasmic space of the *E.coli*, and the His-tag was used for the purification and detection of VHH (*e.g.* in ELISA, Western Blot, *etc.*).

Expression and purification of VHHs

Expression and purification of VHHs were carried out. Specifically, on day 1, 10-20 ml of LB + ampicillin (100 µg/ml) + glucose (1%) were innoculated with a freshly transformed WK6 colony. This pre-culture was incubated at 37°C overnight with shaking at 200-250 rpm. On day 2, a TB medium was used for expressing the VHHs. The TB medium included, per liter: 2.3 g KH_2PO_4, 16.4 g $K_2HPO_4.3H_2O$, 12 g Tryptone (Duchefa Biochemie), 24 g Yeast (Duchefa Biochemie), and 4 ml 100% glycerol (Duchefa Biochemie)

A baffled shaker flask of 1 liter was filled with 330 ml TB and autoclaved. KH_2PO_4 and $K_2HPO_4.3H_2O$ were not autoclaved. Instead, KH_2PO_4 and $K_2HPO_4.3H_2O$ were prepared, filter sterilized, and then added to the rest of the medium that was already autoclaved. About 1 ml of the pre-culture was added to 330 ml of TB supplemented with 100 µg/ml Ampicillin, 2 mM $MgCl_2$ and 0.1% glucose and subsequently grew at 37°C with shaking (200-250 rpm) until an OD_{600} of 0.6-0.9 was reached. IPTG (final concentration of 1 mM) was added to induce VHH expression. The culture was incubated at 28°C with shaking overnight (about 16-18 hours). The OD_{600} after overnight induction was usually between 25 and 30. At least 1 liter of culture (3 bottles) per clone was prepared with an average yield of between 1 and 15 mg/l.

Extraction of the VHHs from the periplasm of E. coli was carried out on day 3. The solutions used included: TES: 0.2 M Tris pH 8.0, 0.5 mM EDTA, 0.5 M sucrose, TES/4: TES diluted 4 times in water.

The overnight induced cultures were centrifuged for 8 minutes at 8000 rpm. The cell pellets from 1 liter culture were resuspended in 12 ml TES by pipetting up and down and shaken for 1 hour on ice. Per each 12 ml TES used, about 18 ml TES/4 were added and incubated on ice for an additional hour with shaking followed by centrifuge for 30 minutes at 8000 rpm at 4°C. The supernatant which contained proteins extracted from the periplasmic spaced was transferred to fresh falcon tubes.

The VHHs were subsequently purified by IMAC which utilized the following solution: HIS-select (SIGMA), PBS, and 50 mM NaAcetate pH 4.6.

His-select was equilibrated with PBS. Specifically, per periplasmic extract derived from 1 liter culture, 1 ml of Resin (about 2 ml His-select solution) was added to a 50 ml falcon tube. PBS was also added to final volume of

50 ml and mixed. Centrifugation was carried out at 2000 rpm for 2 minutes, and the supernatant was discarded. The resin was washed with PBS twice as described above. The periplasmic extract was added to the resin, incubated for 30 minutes to 1 hour at room temperature with gentle shaking. The samples were loaded on PD-10 columns with a filter at the bottom (GE healthcare, cat. No. 17-0435-01) and washed with 50 to 100 ml PBS (50-100 ml PBS per 1 ml resin used). Elution was carried out for 3 times, each time with 1 ml PBS/0.5 M imidazole per 1 ml resin used (for efficient elution, resuspend the beads and leave overnight at 4°C with the bottom of the column closed). Dialysis was performed overnight at 4°C against PBS (cutoff 3500 daltons) to remove imidazole. For efficient dialysis, the dialysis buffer (PBS) was changed 2-3 times. Alternatively, instead of elution with imidazole, the bound VHHs could be eluted with 10 ml 50 mM Na-acetate pH 4.6. If 50 mM Na-acetate pH 4.6 was used to elute VHHs, the eluted VHHs was immediately neutralized with 1M Tris pH 8.0, and no dialysis was required.

The amount of protein was estimated by OD_{280} measurement of eluted sample. Extinction coefficient of each clone was determined by protParam tool under primary structure analysis at the Expasy proteomics server. Further purification of VHHs could be achieved by different methods. For example, the samples could be concentrated (Vivaspin 5000 MW cutoff, Vivascience) by centrifuging at 2000 rpm at 4°C until an appropriate volume for loading on a Superdex 75 16/60 was obtained (max. 4 ml). The concentrated sample was loaded on a Superdex 75 16/60 column equilibrated with PBS. Peak fractions were pooled, and OD_{280} measurements were performed for quantification. In general, VHHs eluted after 85-95 minutes when run at 1 ml/min. Aliquots of concentrated VHH samples were stored at -20°C at a concentration of about 1 mg/ml.

Example 3. Functional Characterization of Human CD8 Binding VHHs

Three VHHs comprising antigen recognition domains against human CD8 were constructed. The VHHs were named R3HCD27, R3HCD129, and R2HCD26. The amino acid sequences of the VHHs are as follows:

R3HCD27 (SEQ ID NO:19)

QVQLQESGGGSVQPGGSLRLSCAASGFTFDDYAMSWVRQVPGKGLEWV
STINWNGGSAEYAEPVKGRFTISRDNAKNTVYLQMNSLKLEDTAVYYCAK
DADLVWYNLSTGQGTQVTVSSAAAYPYDVPDYGS

R3HCD129 (SEQ ID NO:20)

QVQLQESGGGLVQPGGSLRLSCAASGFTFDDYAMSWVRQVPGKGLEWV
STINWNGGSAEYAEPVKGRFTISRDNAKNTVYLQMNSLKLEDTAVYYCAK
DADLVWYNLRTGQGTQVTVSSAAAYPYDVPDYGS

R2HCD26 (SEQ ID NO:21)

QVQLQESGGGLVQAGGSLRLSCAASGFTFDDYAIGWFRQAPGKEREGVS
CIRVSDGSTYYADPVKGRFTISSDNAKNTVYLQMNSLKPEDAAVYYCAAGS
LYTCVQSIVVVPARPYYDMDYWGKGTQVTVSSAAAYPYDVPDYGS

The binding characteristics of the three VHHs were tested by flow cytometry. HEK293-T cells were transfected with a human CD8α expression plasmid and stained with the three His-tagged VHHs (*i.e.*, R2HCD26, R3HCD27, and R3HCD129) at 2 µg/ml, followed by staining with an anti-His Fitc conjugated antibody. Cellular fluorescence was detected via flow cytometry. Results as shown in **Figure 5** show that the VHHs bound to CD8.

In addition, human peripheral blood mononuclear cells (PBMCs) were stained with the three His-tagged CD8 VHHs (*i.e.*, R2HCD26, R3HCD27, and R3HCD129) at 1 µg/ml, followed by staining with an anti-His FITC conjugated antibody. Staining was also performed using a control VHH that did not recognize CD8. As depicted in panel A of **Figure 6,** all three His-tagged CD8 VHHs showed specific binding to CD8+ cells present among the human PBMCs. Median fluorescence intensity (MFI) was also calculated for five VHH dilutions and compared to binding obtained with the control VHH (**Figure 6**, panel B). Results indicate that at the 10 nM and 100 nM concentrations, all three VHHs had higher MFI than the control antibody (suggesting specific binding to CD8) with the R2HCD26 antibody showing the highest MFI. Panel C of **Figure 6** shows that a significant portion of CD3-antigen positive cells in the PBMC also stained positive for CD8.

Example 4. Anti-tumor Effects of Murine CD8-based Chimeras

Figure 7 shows the anti-tumor activities of a murine bispecific chimera ("CD8-Q124R-PD-L1") analyzed using the B16 melanoma model. As shown, the bi-specific (anti-murine CD8 and anti-murine PD-L1) fusion to modified human IFN alpha (Q124R) provided better anti-tumor activity as compared to a fusion of anti-CD8 to modified human IFN alpha (Q124R).

Figure 8 shows a 4T1 mammary tumor model study in which a bi-specific (anti-murine CD8 and anti-murine PD-L1) fusion to modified human IFN alpha (Q124R) provided better anti-tumor activity as compared to the co-administration of a fusion of anti-CD8 to modified human IFN alpha (Q124R) and a fusion of anti-PD-L1 to modified human IFN alpha (Q124R) or the co-administration of a fusion of anti-CD8 to modified human IFN alpha (Q124R) and an anti-PD-L1 VHH.

Figure 9 shows in the 4T1 mammary tumor model, the use of the bi-specific (anti-murine CD8 and anti-murine PD-L1) fusion to modified human IFN alpha (Q124R) with doxorubicin resulted in a curative effect in 3 out of 6 mice (i.e., the mice were completely tumor free).

Example 5. Efficiency of Human CD8 Targeting of Mono-Specific Human Chimeras

The efficiency of human CD8 targeting of mono-specific human chimeras was examined by quantification of STAT1 phosphorylation in CD8-positive and CD8-negative peripheral blood mononuclear cells (PBMCs) by FACS.

A chimera of anti-human CD8 VHH/human IFN R149A (i.e., pmTW-SIgK-hCD8_R2HCD26 (SEQ ID NO:21)-$(GGS)_{20}$-hIFNa2_R149A-GGS-$(His)_9$ construct) and anti-human CD8 VHH/human IFN R33A/E120A (i.e., pmTW-SIgK-hCD8_R2HCD26 (SEQ ID NO:21)-$(GGS)_{20}$-hIFNa2_R33A/E120A-GGS-$(His)_9$ construct) were produced in Hek293F cells. Cells were grown to a density of $0.6x10^6$ cells per ml in Freestyle medium and transfected with

25K PEI (polyethylenimine) according to standard protocols. Three days after transfection, fresh medium was added to the cultures and cells were grown for two or three additional days. Medium was harvested, cells removed by centrifugation and filter-sterilized. Recombinant proteins were purified using Ni Excel resin (GE Healthcare) according to the manufacturer's instructions and imidazole removed from the samples with PD10 columns (GE Healthcare).

PBMCs from buffy coats of healthy donors were isolated using density gradient centrifugation with Ficoll-Paque (GE Healthcare). Cells were washed twice with FACS buffer (2% FBS, 1 mM EDTA in PBS) and stained with anti-human CD8 APC (clone RPE-T8; BD Pharmingen) for 20 minutes at 4°C. After two washes, cells were stimulated with a serial dilution of CD8-targeting chimeras for 15 minutes at 37°C. After fixation (10 minutes, 37°C, Fix Buffer I; BD Biosciences) and permeabilisation (30 minutes, on ice, Perm III Buffer I; BD Biosciences) and washing, cells were stained with anti-STAT1 pY701 Ab (BD Biosciences). Samples were acquired with a FACSCalibur (BD Biosciences), with the CellQuest Pro Version 4.0.2 software (BD Biosciences).

Isolated PBMCs were stimulated with a serial dilution of CD8-targeting chimeras (anti-human CD8 VHH/human IFN R149A, anti-human CD8 VHH/human IFN R33A/E120A, or anti-BcII10 VHH/human IFN R149A) and stained for CD8 (APC) and pSTAT1 (PE). Data clearly showed that the biological activity of anti-human CD8 VHH/human IFN R149A and anti-BcII10 VHH- human IFN R149A were comparable in CD8-negative cells (**Figure 10,** panel C), but CD8 targeting resulted in a clear and pronounced increase (at least 500 fold) in STAT1 phosphorylation by anti-human CD8 VHH/human IFN R149A (**Figure 10,** panels A and B). The combined IFN mutations in the anti-human CD8 VHH/human IFN R33A/E120A chimera completely blocked STAT1 phosphorylation, and CD8 targeting did not rescue biological activity. This was in contrast to the IFN R149A mutation which maintained STAT1 phosphorylation.

EQUIVALENTS

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

INCORPORATION BY REFERENCE

All patents and publications referenced herein are hereby incorporated by reference in their entireties.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

As used herein, all headings are simply for organization and are not intended to limit the disclosure in any manner. The content of any individual section may be equally applicable to all sections.

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CLAIMS

What is claimed is:

1. A CD8 binding agent comprising at least one targeting moiety comprising three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:

(a) CDR1 comprises an amino acid sequence selected from GFTFDDYAMS (SEQ ID NO:12) and GFTFDDYAIG (SEQ ID NO:13);

(b) CDR2 comprises an amino acid sequence selected from TINWNGGSAEYAEPVKG (SEQ ID NO:14) and CIRVSDGSTYYADPVKG (SEQ ID NO:15); and

(c) CDR3 comprises an amino acid sequence selected from KDADLVWYNLS (SEQ ID NO:16), KDADLVWYNLR (SEQ ID NO:17), and AGSLYTCVQSIVVVPARPYYDMDY (SEQ ID NO:18).

2. The CD8 binding agent of claim 1, wherein the targeting moiety is a full-length antibody, a single-domain antibody, a recombinant heavy-chain-only antibody (VHH), a single-chain antibody (scFv), a shark heavy-chain-only antibody (VNAR), a microprotein, a darpin, an anticalin, an adnectin, an aptamer, a Fv, a Fab, a Fab', a $F(ab')_2$, a peptide mimetic molecule, a natural ligand for a receptor, or a synthetic molecule.

3. The CD8 binding agent of claim 1 or 2, wherein the targeting moiety is a single-domain antibody.

4. The CD8 binding agent of claim 3, wherein the targeting moiety comprises a V_HH, a humanized V_HH, or a camelized V_HH.

5. The CD8 binding agent of any one of the above claims, wherein the targeting moiety comprises a CDR1 comprising the amino acid sequence of GFTFDDYAMS (SEQ ID NO:12), a CDR2 comprising the amino acid sequence of TINWNGGSAEYAEPVKG (SEQ ID NO:14), and a CDR3 comprising the amino acid sequence of KDADLVWYNLS (SEQ ID NO:16).

6. The CD8 binding agent of claim 5, comprising an amino acid sequence having at least 90% sequence similarity with SEQ ID NO:19.

7. The CD8 binding agent of any one of claims 1-4, wherein the targeting moiety comprises a CDR1 comprising the amino acid sequence of GFTFDDYAMS (SEQ ID NO:12), a CDR2 comprising the amino acid sequence of TINWNGGSAEYAEPVKG (SEQ ID NO:14), and a CDR3 comprising the amino acid sequence of KDADLVWYNLR (SEQ ID NO:17).

8. The CD8 binding agent of claim 7, comprising an amino acid sequence having at least 90% sequence similarity with SEQ ID NO:20.

9. The CD8 binding agent of any one of claims 1-4, wherein the targeting moiety comprises a CDR1 comprising the amino acid sequence of GFTFDDYAIG (SEQ ID NO:13), a CDR2 comprising the amino acid sequence of CIRVSDGSTYYADPVKG (SEQ ID NO:15), and a CDR3 comprising the amino acid sequence of AGSLYTCVQSIVVVPARPYYDMDY (SEQ ID NO:18).

10. The CD8 binding agent of claim 9, comprising an amino acid sequence having at least 90% sequence similarity with SEQ ID NO:21.

11. The CD8 binding agent of any one of the above claims, wherein the CD8 binding agent comprises one or more signaling agents.

12. The CD8 binding agent of claim 11, wherein the signaling agent is selected from one or more of an interferon, an interleukin, and a tumor necrosis factor, any of which are optimally modified.

13. The CD8 binding agent of any one of the above claims, wherein the CD8 binding agent comprises one or more additional targeting moieties.

14 The CD8 binding agent of claim 13, wherein the one or more additional targeting moieties recognize and optionally functionally modulate a tumor antigen.

15. The CD8 binding agent of claim 14, wherein the one or more additional targeting moieties recognize and optionally functionally modulate an antigen on an immune cell.

16. The CD8 binding agent of claim 15, wherein the immune cell is selected from a T cell, a B cell, a dendritic cell, a macrophage, neutrophil, and a NK cell.

17. The CD8 binding agent of any of the above claims, wherein the CD8 binding agent recruits cytotoxic T cells to tumor cells or to the tumor environment.

18. The CD8 binding agent of any of the above claims, wherein the CD8 binding agent recognizes and binds CD8 without substantially functionally modulating its activity.

19. A recombinant nucleic acid composition encoding the CD8 binding agents of any one of the above claims.

20. A host cell comprising a nucleic acid of claim 19.

21. The CD8 binding agent of any one of the above claims, wherein the CD8 binding agent is suitable for use in a patient having one or more of: cancer, infections, immune disorders, and/or autoimmune diseases.

22. A method for treating or preventing cancer, comprising administering to a patient in need thereof an effective amount of a chimera comprising a targeting moiety comprising an antigen or receptor recognition domain targeting CD8 and a signaling agent, selected from one or more of an interferon, an interleukin, and a tumor necrosis factor.

23. The method of claim 22, wherein the signaling agent is modified.

24. The method of claim 22 or 23, wherein the CD8 binding agent is a CD8 binding agent of any of the above claims.

25. The method of any one of claims 22-24, wherein the CD8 binding agent comprises at least one targeting moiety comprising three complementarity determining regions (CDR1, CDR2, and CDR3), wherein:

(a) CDR1 comprises an amino acid sequence selected from GFTFDDYAMS (SEQ ID NO:12) and GFTFDDYAIG (SEQ ID NO:13);

(b) CDR2 comprises an amino acid sequence selected from TINWNGGSAEYAEPVKG (SEQ ID NO:14) and CIRVSDGSTYYADPVKG (SEQ ID NO:15); and

(c) CDR3 comprises an amino acid sequence selected from KDADLVWYNLS (SEQ ID NO:16), KDADLVWYNLR (SEQ ID NO:17), and AGSLYTCVQSIVVVPARPYYDMDY (SEQ ID NO:18).

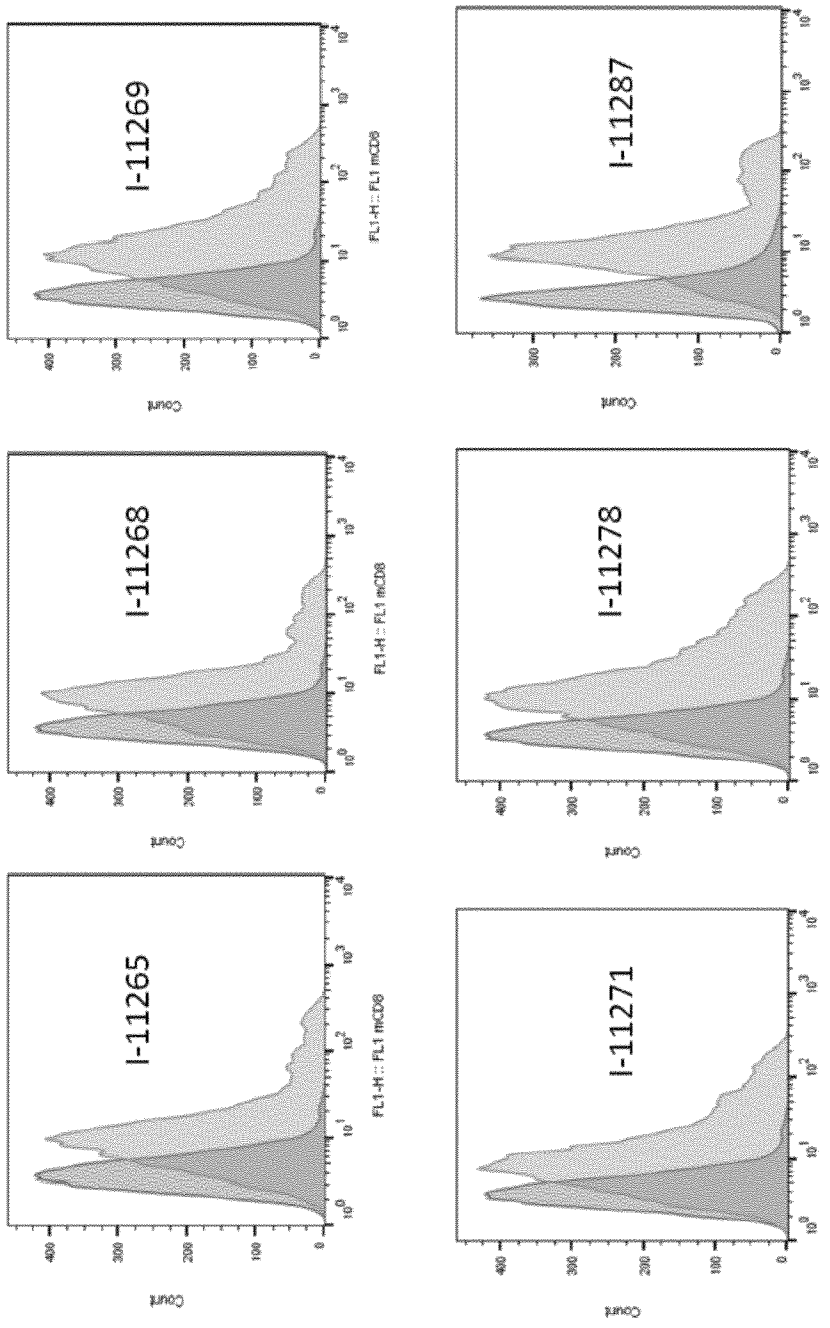
26. The method of any of any of claims 22-25, wherein the cancer is selected form one or more of basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma; hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); melanoma; myeloma; neuroblastoma; oral cavity cancer (lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; lymphoma including Hodgkin's and non-Hodgkin's lymphoma, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia; chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; as well as other carcinomas and sarcomas; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (*e.g.* that associated with brain tumors), and Meigs' syndrome.

27. A CD8 binding agent of any one of the above claims for use in the treatment of one or more of: cancer, infections, immune disorders, and/or autoimmune diseases, as described herein.

28. Use of a CD8 binding agent of any one of the above claims for the manufacture of a medicament for treating one or more of: cancer, infections, immune disorders, and/or autoimmune diseases, as described herein.

FIGURE 1

A.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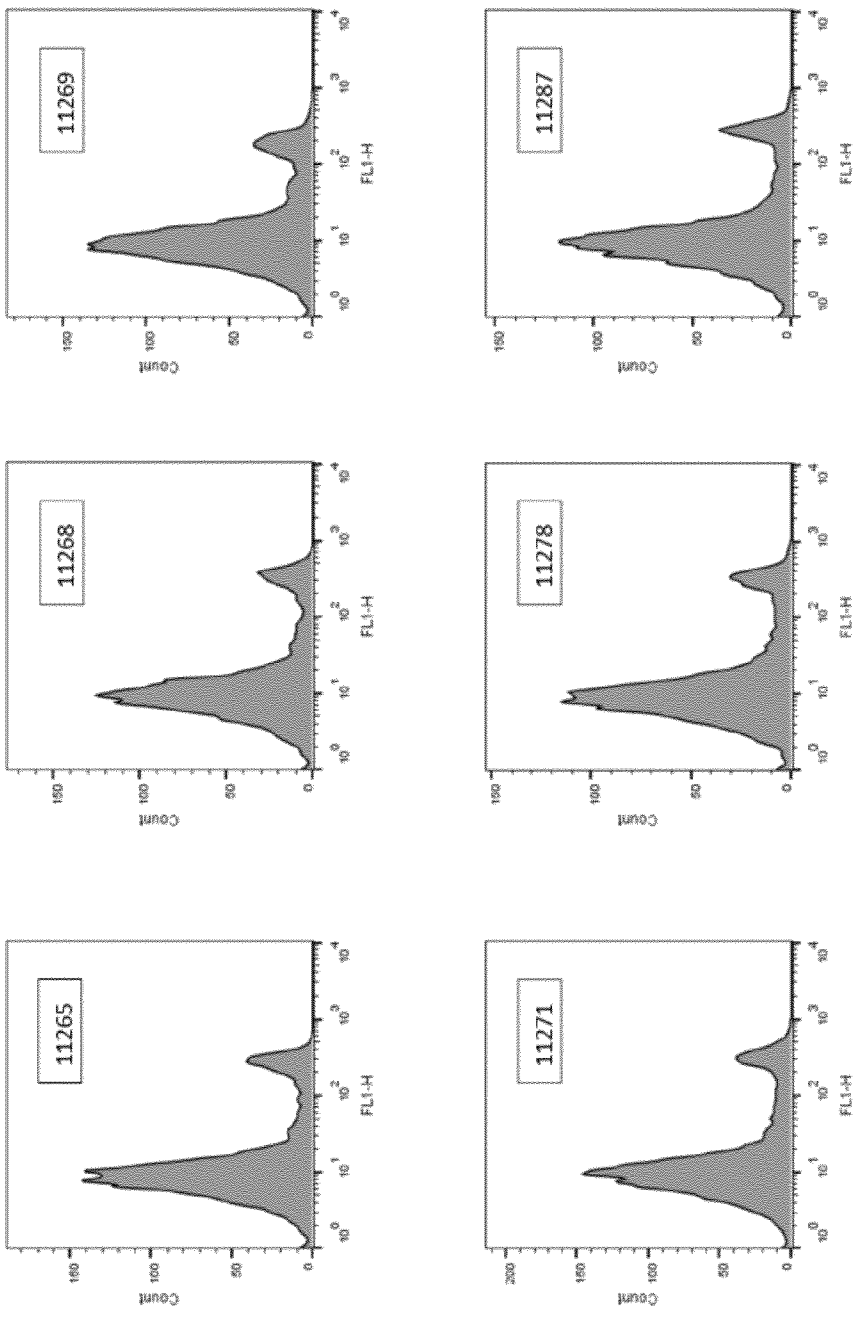
I-11268
I-11269
I-11271
I-11278
I-11287
Count
FL1-H :: FL1 mCD8

FIGURE 1

B.



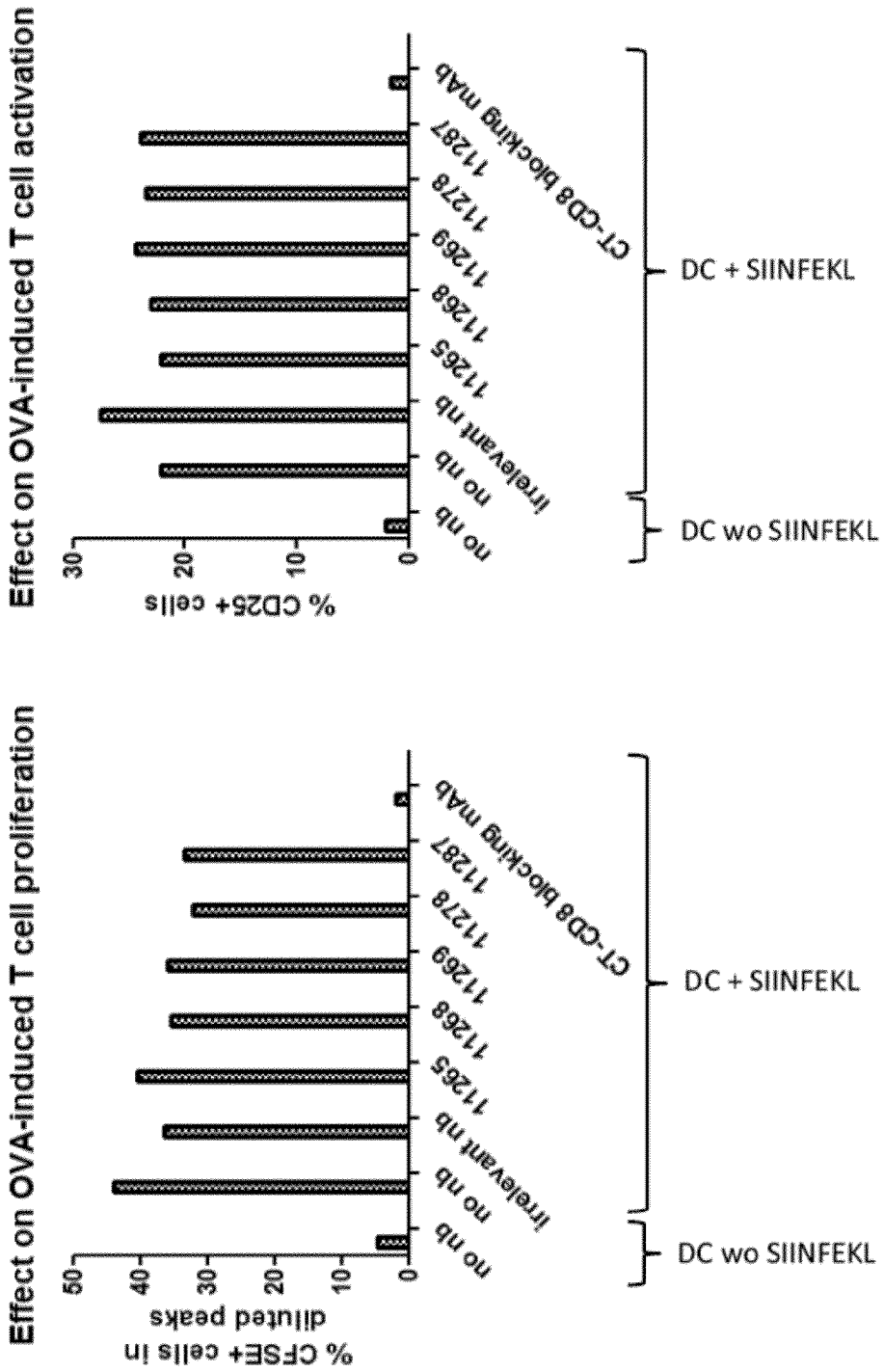

Effect on OVA-induced T cell activation
% CD25+ cells
0
10
20
30
no nb
no nb
irrelevant nb
1265
1268
1269
1278
1287
CT-CD8 blocking mAb
DC + SIINFEKL
DC wo SIINFEKL
Effect on OVA-induced T cell proliferation
% CFSE+ cells in diluted peaks
0
10
20
30
40
50
no nb
no nb
irrelevant nb
1265
1268
1269
1278
1287
CT-CD8 blocking mAb
DC + SIINFEKL
DC wo SIINFEKL

FIGURE 2

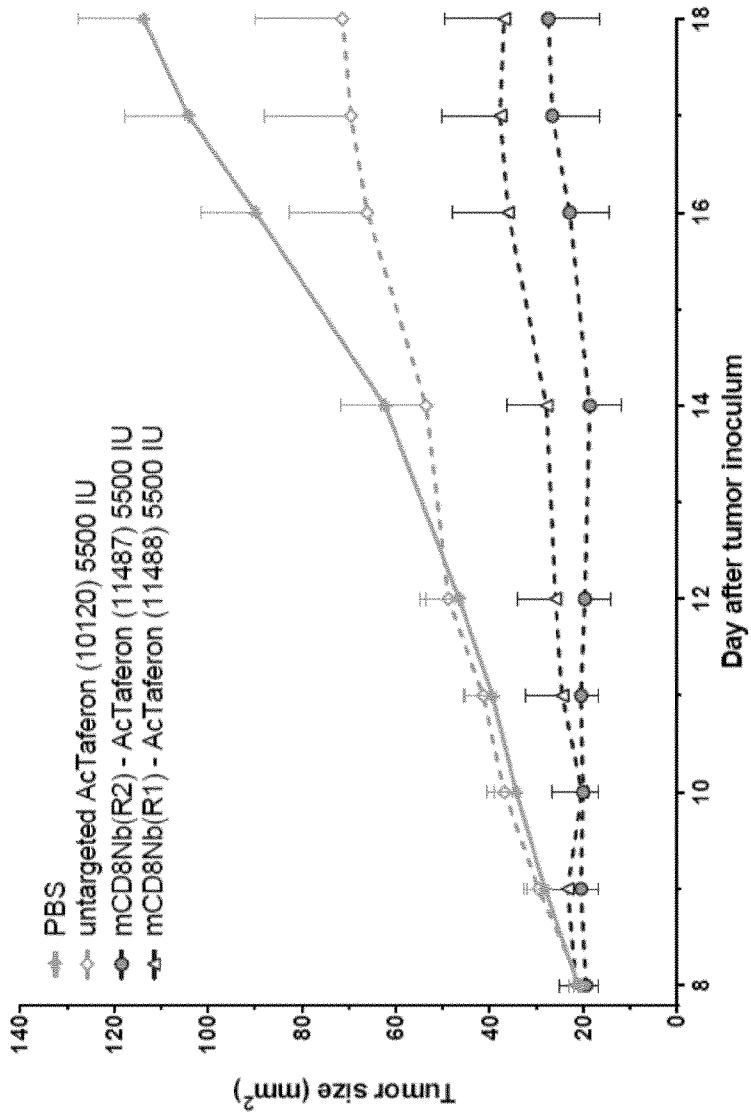
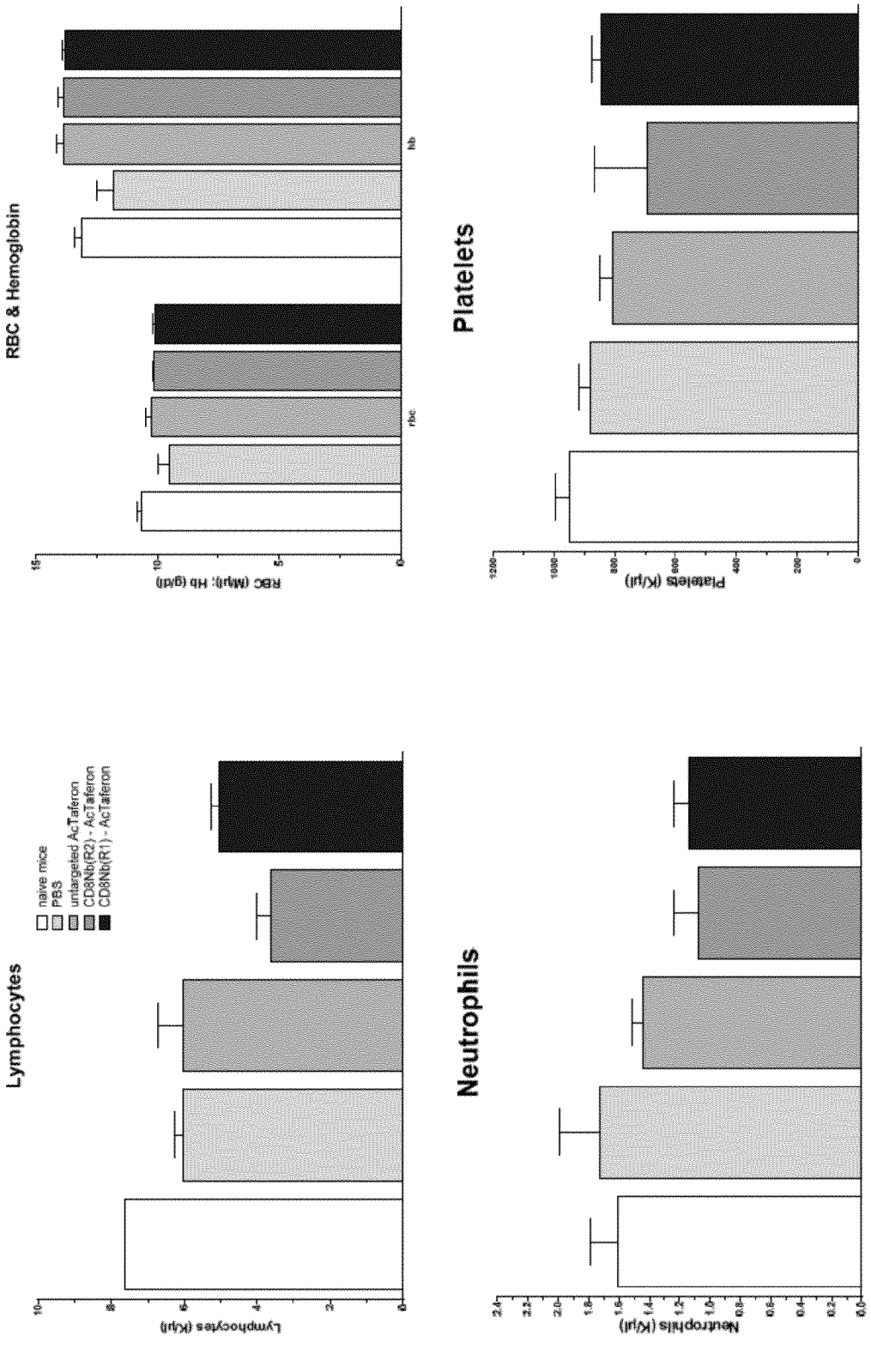

IFN012 tumor sizes
(treat d8-9/11-12/14/16-17)
PBS
untargeted AcTaferon (10120) 5500 IU
mCD8Nb(R2) - AcTaferon (11487) 5500 IU
mCD8Nb(R1) - AcTaferon (11488) 5500 IU
Tumor size (mm²)
Day after tumor inoculum
0
20
40
60
80
100
120
140
8
10
12
14
16
18

FIGURE 3

FIGURE 4


RBC & Hemoglobin
RBC (Mpl); Hb (g/dl)
rbc
hb
Platelets
Platelets (K/µl)
Lymphocytes
Lymphocytes (K/µl)
naive mice
PBS
untargeted AcTaferon
CD8Nb(R2) - AcTaferon
CD8Nb(R1) - AcTaferon
Neutrophils
Neutrophils (K/µl)

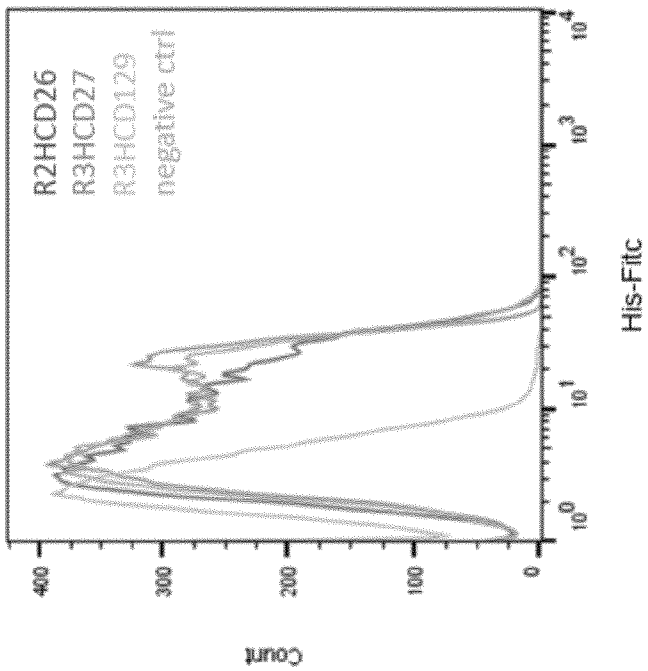

R2HCD26
R3HCD27
R3HCD129
negative ctrl
Count
His-Fitc
0
100
200
300
400
10^0
10^1
10^2
10^3
10^4

FIGURE 5

FIGURE 6

A.

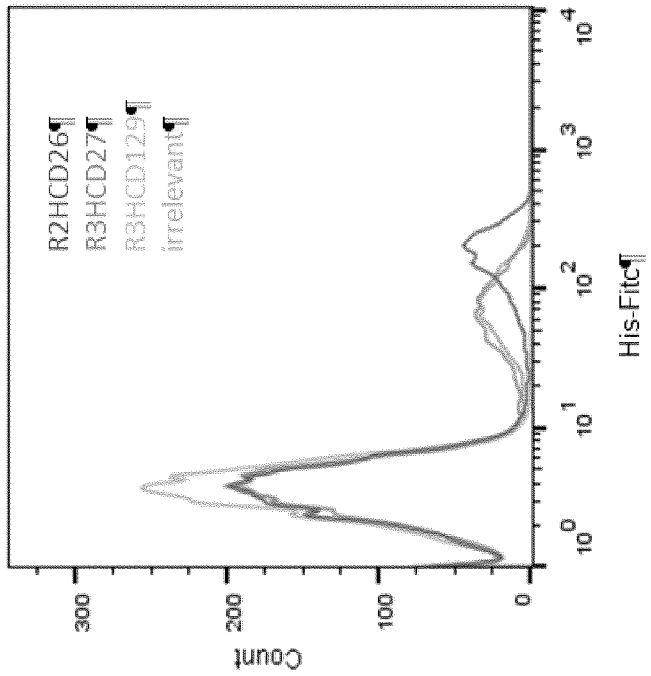

R2HCD26
R3HCD27
R3HCD129
irrelevant
Count
0
100
200
300
10^0
10^1
10^2
10^3
10^4
His-Fitc

FIGURE 6 (CONT.)

B.

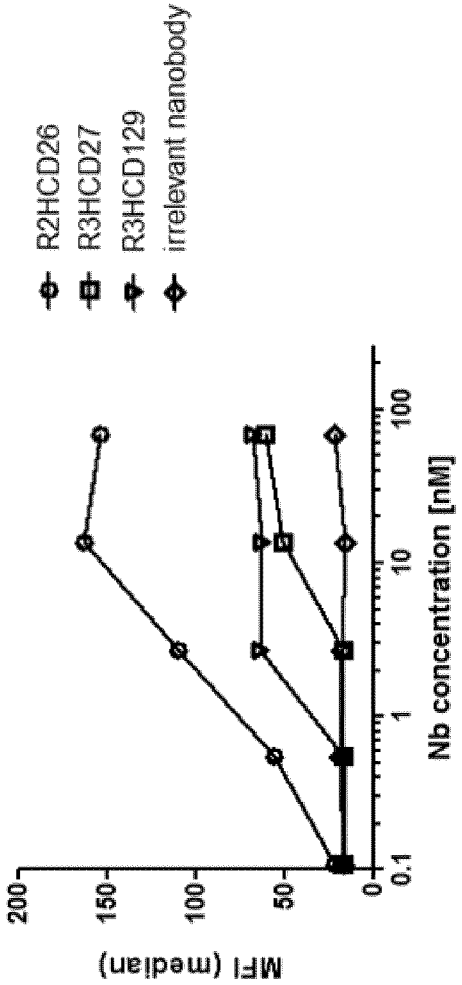
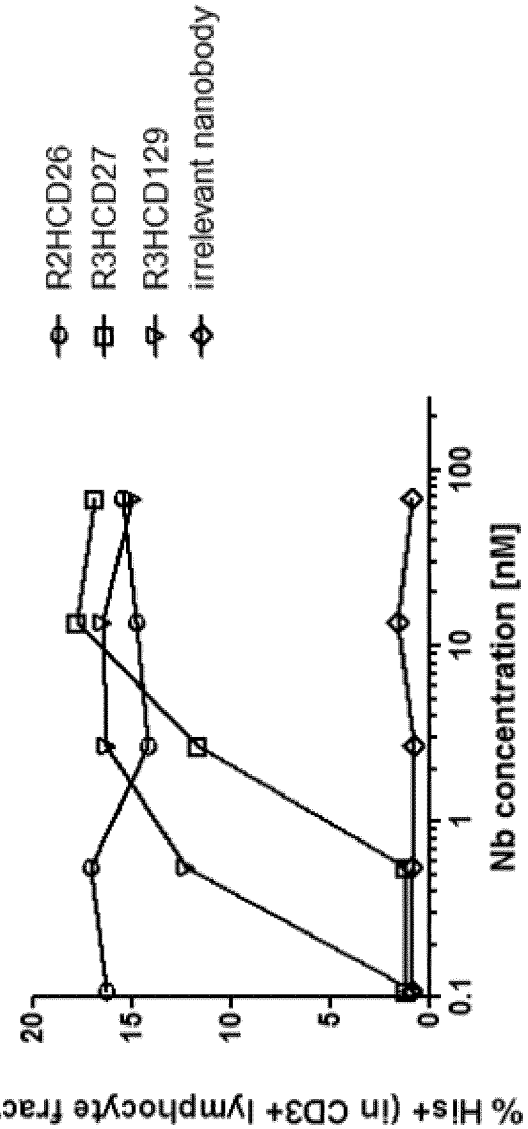


FIGURE 6 (CONT.)

C.



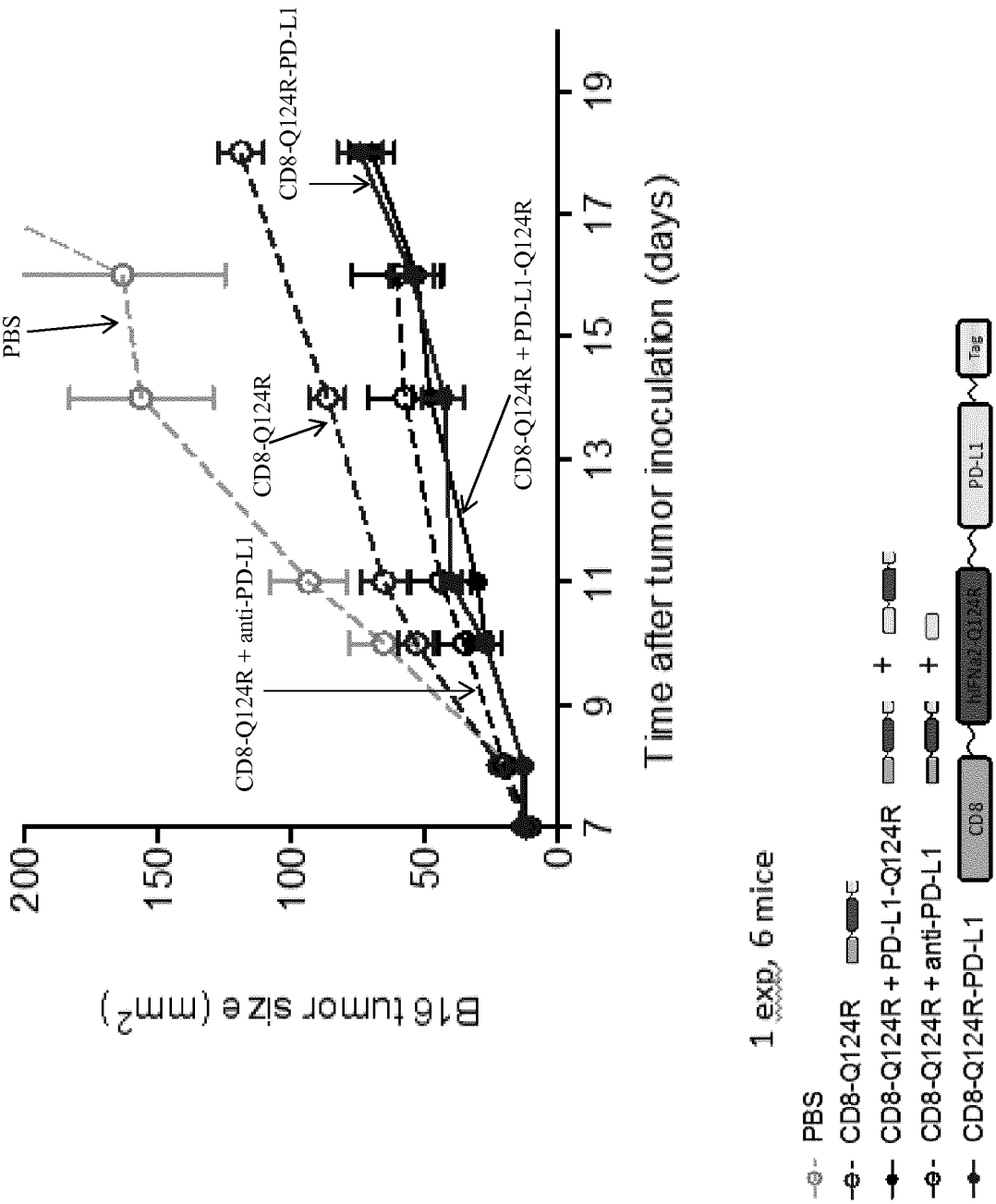

B16 tumor size (mm²)
200
150
100
50
0
7
9
11
13
15
17
19
Time after tumor inoculation (days)
PBS
CD8-Q124R
CD8-Q124R + anti-PD-L1
CD8-Q124R-PD-L1
CD8-Q124R + PD-L1-Q124R
1 exp, 6 mice
PBS
CD8-Q124R
CD8-Q124R + PD-L1-Q124R
CD8-Q124R + anti-PD-L1
CD8-Q124R-PD-L1
CD8
PD-L1
Tag

FIGURE 7

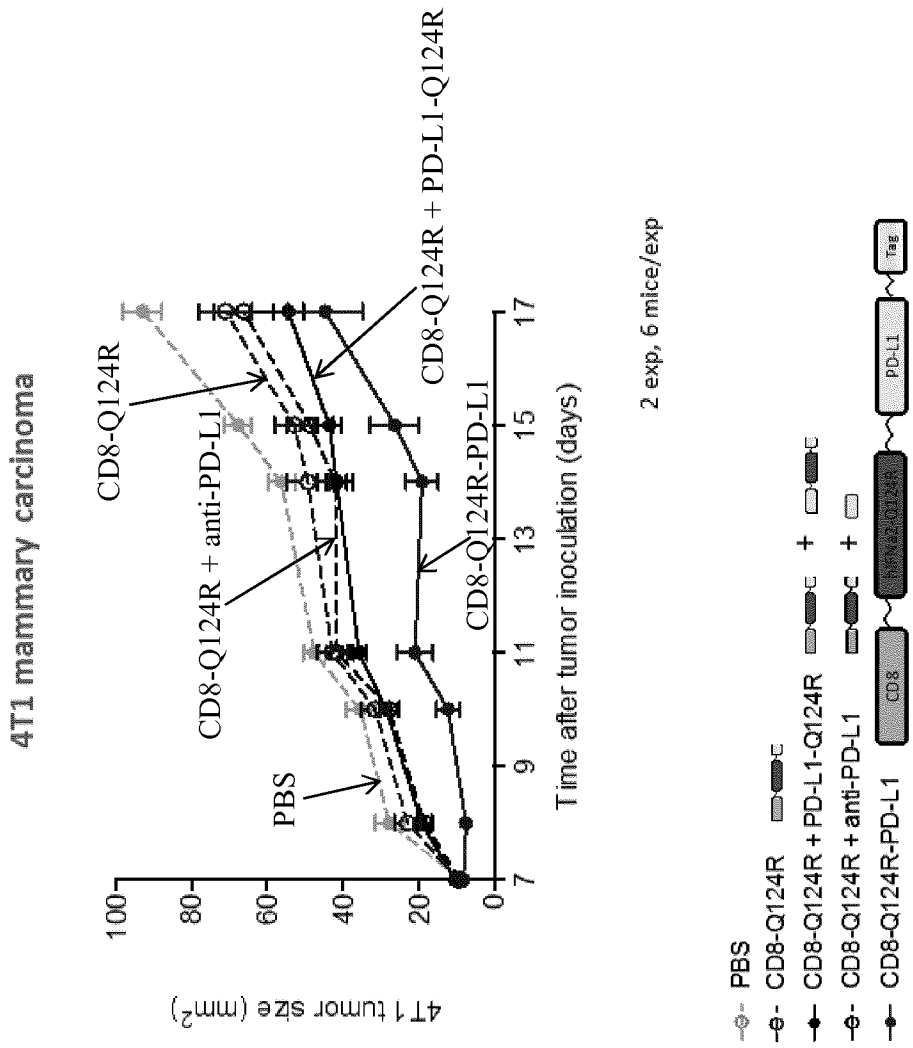

4T1 mammary carcinoma
4T1 tumor size (mm²)
Time after tumor inoculation (days)
CD8-Q124R
CD8-Q124R + anti-PD-L1
PBS
CD8-Q124R + PD-L1-Q124R
CD8-Q124R-PD-L1
2 exp, 6 mice/exp
PBS
CD8-Q124R
CD8-Q124R + PD-L1-Q124R
CD8-Q124R + anti-PD-L1
CD8-Q124R-PD-L1
CD8
PD-L1
Tag

FIGURE 8

FIGURE 9

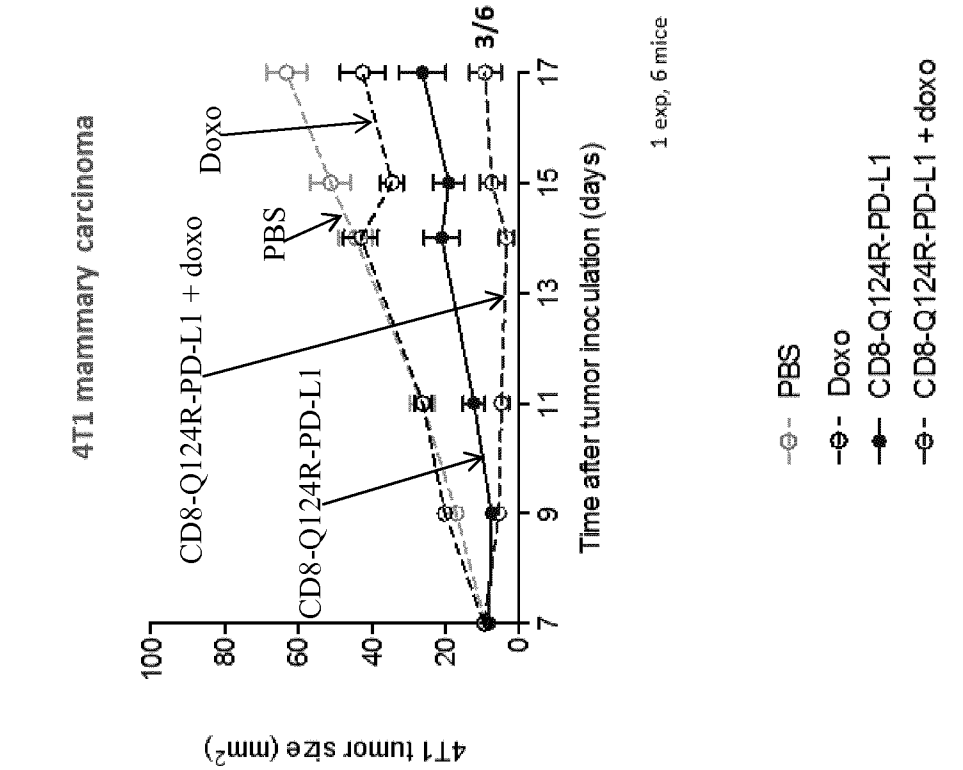
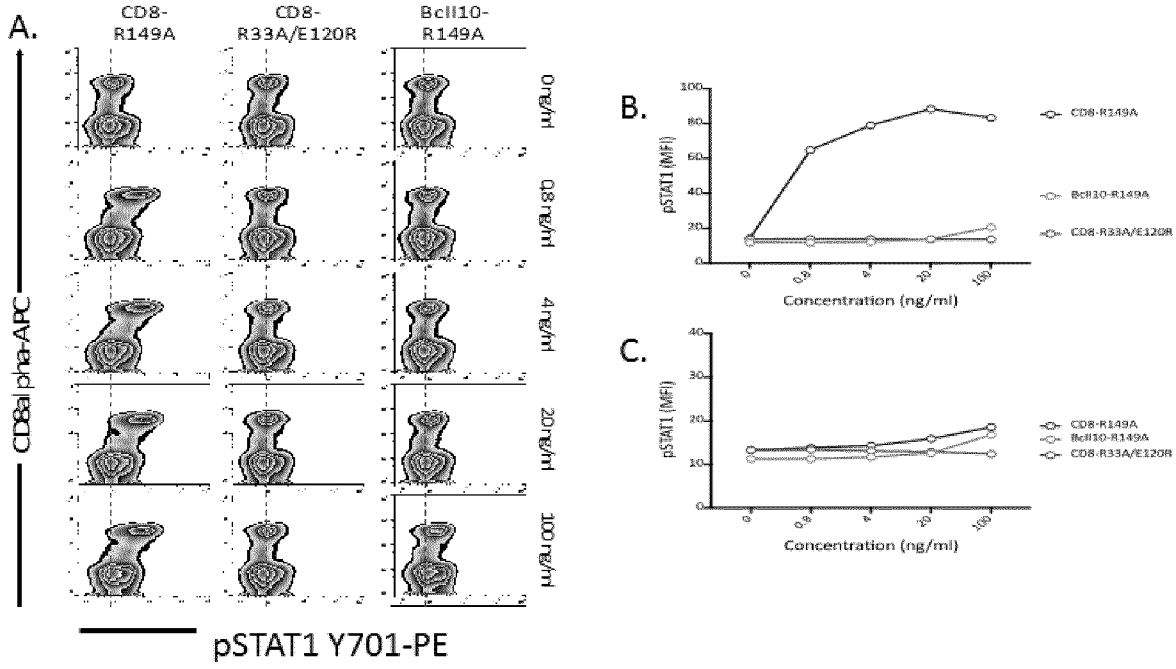

4T1 mammary carcinoma
4T1 tumor size (mm²)
Time after tumor inoculation (days)
CD8-Q124R-PD-L1 + doxo
Doxo
PBS
CD8-Q124R-PD-L1
3/6
1 exp, 6 mice
PBS
Doxo
CD8-Q124R-PD-L1
CD8-Q124R-PD-L1 + doxo

FIGURE 10


A.
CD8-R149A
CD8-R33A/E120R
BclI10-R149A
0ng/ml
0.8ng/ml
4ng/ml
20ng/ml
100ng/ml
CD8alpha-APC
pSTAT1 Y701-PE
B.
pSTAT1 (MFI)
Concentration (ng/ml)
CD8-R149A
BclI10-R149A
CD8-R33A/E120R
C.
pSTAT1 (MFI)
Concentration (ng/ml)
CD8-R149A
BclI10-R149A
CD8-R33A/E120R

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/052553

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/715 C07K16/28
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

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, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2013/230517 A1 (GREWAL IQBAL [US] ET AL) 5 September 2013 (2013-09-05) paragraph [0010]; table 1	1-8, 11-21, 24-28
Y	US 2014/271462 A1 (HO DAVID T [US] ET AL) 18 September 2014 (2014-09-18) abstract	1-8, 11-21, 24-28
Y	RU 2011 127226 A (EPSHTEJN OLEG ILICH [RU]) 10 January 2013 (2013-01-10) the whole document	1-8, 11-21, 24-28

☐ Further documents are listed in the continuation of Box C.

☒ 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
3 April 2017	12/07/2017

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	Scheffzyk, Irmgard

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2017/052553

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

5-8(completely); 1-4, 11-21, 24-28(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5-8(completely); 1-4, 11-21, 24-28(partially)

   CD8 binding agent as defined in claims 5 and 7

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2. claims: 9, 10(completely); 1-4, 11-21, 24-28(partially)

   CD8 binding agent as defined in claim 9

   ---

3. claims: 22, 23

   Method of treating or preventing cancer characterized by the administration of a chimera containing a recognition domain targeting CD8 and a signaling agent

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/052553

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2013230517 A1	05-09-2013	AU 2013230484 A1	12-02-2015
		CA 2866126 A1	12-09-2013
		CN 104470536 A	25-03-2015
		EP 2822575 A1	14-01-2015
		JP 2015515453 A	28-05-2015
		US 2013230517 A1	05-09-2013
		US 2015139951 A1	21-05-2015
		US 2017073388 A1	16-03-2017
		WO 2013134138 A1	12-09-2013
US 2014271462 A1	18-09-2014	AU 2014249243 A1	08-10-2015
		CA 2904969 A1	09-10-2014
		CN 105163765 A	16-12-2015
		EP 2968622 A1	20-01-2016
		JP 2016520514 A	14-07-2016
		KR 20150131208 A	24-11-2015
		US 2014271462 A1	18-09-2014
		US 2016024209 A1	28-01-2016
		US 2017029507 A1	02-02-2017
		WO 2014164553 A1	09-10-2014
RU 2011127226 A	10-01-2013	NONE	