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(54) Title: CD33 TARGETED IMMUNOTHERAPIES

(57) Abstract: The present disclosure provides improved CD33 targeting polypeptides and compositions for adoptive T cell therapies for treating, preventing, or ameliorating at least one symptom of a cancer, infectious disease, autoimmune disease, inflammatory disease, and immunodeficiency, or condition associated therewith.

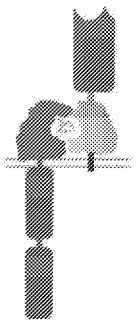

FIG. 1A

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CD33 TARGETED IMMUNOTHERAPIES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/898,392, filed September 10, 2019, and U.S. Provisional Application No. 62/845,304, filed May 8, 2019, each of which is incorporated by reference herein in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is BLBD_119_02WO_ST25.txt. The text file is 302 KB, was created on May 5, 2020, and is being submitted electronically via EFS-Web, concurrent with the filing of the specification.

BACKGROUND

Technical Field

The present disclosure relates to improved adoptive cell therapies directed against CD33. More particularly, the disclosure relates to anti-CD33 VHH-containing chemically regulated signaling molecules, anti-CD33 VHH-containing chimeric antigen receptors, cells, and related methods of treatment using the same.

Description of the Related Art

The global burden of cancer doubled between 1975 and 2000. Cancer is the second leading cause of morbidity and mortality worldwide, with approximately 14.1 million new cases and 8.2 million cancer related deaths in 2012. The most common cancers are breast cancer, lung and bronchus cancer, prostate cancer, colon and rectum cancer, bladder cancer, melanoma of the skin, non-Hodgkin lymphoma, thyroid cancer, kidney and renal

pelvis cancer, endometrial cancer, leukemia, and pancreatic cancer. The number of new cancer cases is projected to rise to 22 million within the next two decades.

Adoptive cellular therapy is emerging as a powerful paradigm for delivering complex biological signals to treat cancer. In contrast to small molecule and biologic drug compositions, adoptive cell therapies have the potential to execute unique therapeutic tasks owing to their myriad sensory and response programs and increasingly defined mechanisms of genetic control. Existing methods have focused primarily on scFv-based chimeric antigen receptors (CARs). CAR T cell therapy has met with limited success due to poor CAR expression, *in vivo* expansion of CAR T cells, rapid disappearance of the cells after infusion, disappointing clinical activity, and antigen escape.

There is a need to retrofit immune effector cells with improved CAR architectures (CARchitectures) and/or improved machinery for sensing and integrating chemical and/or biological information associated with local physiological environments.

BRIEF SUMMARY

The present disclosure generally relates, in part, to VHH-based dimerizing agent regulated immunoreceptor complexes (DARICs) and VHH-based chimeric antigen receptors (CARs) directed against CD33, polynucleotides encoding the same, compositions thereof, and methods of making and using the same to treat cancer.

In particular embodiments, a VHH DARIC or VHH CAR binds full-length CD33. In particular embodiments, a VHH DARIC or VHH CAR binds a CD33 splice variant. In certain embodiments, the CD33 splice variant lacks the124 amino acids encoded by exon 2 of the human CD33 gene (CD33 C2 variant). In certain embodiments, the CD33 splice variant lacks 54 carboxy-terminal amino acids due to an early translation stop signal residing in exon 7a. In certain embodiments, the CD33 splice variant lacks the 124 amino acids encoded by exon 2 and 54 carboxy-terminal amino acids due to an early translation stop signal residing in exon 7a.

In particular embodiments, a VHH DARIC or VHH CAR binds both full-length CD33 and a CD33 splice variant.

In various embodiments, a non-natural cell comprises: a first polypeptide comprising: an FRB multimerization domain polypeptide or variant thereof; a CD8α transmembrane domain or a CD4 transmembrane domain; a CD137 co-stimulatory domain; and/or a CD3ζ primary signaling domain; and a second polypeptide comprising: an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21; an FKBP multimerization domain polypeptide or variant thereof; and a CD4 transmembrane domain or a CD8α transmembrane domain; wherein a bridging factor promotes the formation of a polypeptide complex on the non-natural cell surface with the bridging factor associated with and disposed between the multimerization domains of the first and second polypeptides.

In particular embodiments, the anti-CD33 VHH antibody has an amino acid sequence set forth in SEQ ID NO: 10.

In particular embodiments, the anti-CD33 VHH antibody has an amino acid sequence set forth in SEQ ID NO: 20.

In particular embodiments, the FKBP multimerization domain is FKBP12.

In some embodiments, the FRB polypeptide is FRB T2098L.

In certain embodiments, the bridging factor is selected from the group consisting of: AP21967, sirolimus, everolimus, novolimus, pimecrolimus, ridaforolimus, tacrolimus, temsirolimus, umirolimus, and zotarolimus.

In various embodiments, the first polypeptide comprises a signal peptide, a CD8α transmembrane domain; a CD137 co-stimulatory domain; and a CD3ζ primary signaling domain.

In particular embodiments, the second polypeptide comprises a signal peptide and a CD4 transmembrane domain.

In further embodiments, the second polypeptide comprises a costimulatory domain.

In some embodiments, the costimulatory domain of the second polypeptide is selected from a costimulatory molecule selected from the group consisting of: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28,

CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNFRS14, TNFRS18, TNRFS25, and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

In additional embodiments, the costimulatory domain of the second polypeptide is a costimulatory domain isolated from OX40 or TNFR2.

In further embodiments, the second polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 22-31.

In particular embodiments, the second polypeptide comprises the sequence set forth in SEQ ID NO: 30.

In preferred embodiments, a first polypeptide comprises an amino acid sequence set forth in SEQ ID NO 82.

In various embodiments, a non-natural cell comprises a polypeptide complex that comprises: a first polypeptide comprising: an FRB multimerization domain polypeptide or variant thereof; a CD8α transmembrane domain or a CD4 transmembrane domain; a CD137 co-stimulatory domain; and/or a CD3ζ primary signaling domain; a second polypeptide comprising: an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21; an FKBP multimerization domain polypeptide or variant thereof; and a CD4 transmembrane domain or a CD8α transmembrane domain; and a bridging factor associated with and disposed between the multimerization domains of the first and second polypeptides.

In particular embodiments, the anti-CD33 VHH antibody has an amino acid sequence set forth in SEQ ID NO: 10.

In particular embodiments, the anti-CD33 VHH antibody has an amino acid sequence set forth in SEQ ID NO: 20.

In particular embodiments, the FKBP multimerization domain is FKBP12.

In certain embodiments, the FRB polypeptide is FRB T2098L.

In some embodiments, the bridging factor is selected from the group consisting of: AP21967, sirolimus, everolimus, novolimus, pimecrolimus, ridaforolimus, tacrolimus, temsirolimus, umirolimus, and zotarolimus.

In additional embodiments, the first polypeptide comprises a signal peptide, a CD8α transmembrane domain; a CD137 co-stimulatory domain; and a CD3ζ primary signaling domain.

In particular embodiments, the second polypeptide comprises a signal peptide and a CD4 transmembrane domain.

In some embodiments, the second polypeptide comprises a costimulatory domain.

In various embodiments, the costimulatory domain of the second polypeptide is selected from a costimulatory molecule selected from the group consisting of: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNFRS14, TNFRS18, TNRFS25, and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

In additional embodiments, the costimulatory domain of the second polypeptide is a costimulatory domain isolated from OX40 or TNFR2.

In further embodiments, the second polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 22-31.

In particular embodiments, the second polypeptide comprises the sequence set forth in SEQ ID NO: 30.

In preferred embodiments, a first polypeptide comprises an amino acid sequence set forth in SEQ ID NO 82.

In certain embodiments, the cell is a hematopoietic cell.

In particular embodiments, the cell is a T cell, an αβ T cell, or a γδ T cell.

In further embodiments, the cell is a CD3+, CD4+, and/or CD8+ cell.

In various embodiments, the cell is an immune effector cell.

In some embodiments, the cell is a cytotoxic T lymphocytes (CTLs), a tumor infiltrating lymphocytes (TILs), or a helper T cell.

In additional embodiments, the cell is a natural killer (NK) cell or natural killer T (NKT) cell.

In various embodiments, the source of the cell is peripheral blood mononuclear cells, bone marrow, lymph nodes tissue, cord blood, thymus issue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, or tumors.

In particular embodiments, the FRB multimerization domain and the FKBP multimerization domain localize extracellularly when of the first polypeptide and the second polypeptide are expressed.

In some embodiments, a fusion polypeptide comprises: a first polypeptide comprising: an FRB multimerization domain polypeptide or variant thereof; a CD8α transmembrane domain or a CD4 transmembrane domain; a CD137 co-stimulatory domain; and/or a CD3ζ primary signaling domain; a polypeptide cleavage signal; and a second polypeptide comprising: an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21; an FKBP multimerization domain polypeptide or variant thereof; and a CD4 transmembrane domain or a CD8α transmembrane domain.

In particular embodiments, the anti-CD33 VHH antibody has an amino acid sequence set forth in SEQ ID NO: 10.

In particular embodiments, the FKBP multimerization domain is FKBP12.

In certain embodiments, the FRB polypeptide is FRB T2098L.

In some embodiments, the bridging factor is selected from the group consisting of: AP21967, sirolimus, everolimus, novolimus, pimecrolimus, ridaforolimus, tacrolimus, temsirolimus, umirolimus, and zotarolimus.

In additional embodiments, the first polypeptide comprises a signal peptide, a CD8α transmembrane domain; a CD137 co-stimulatory domain; and a CD3ζ primary signaling domain.

In particular embodiments, the second polypeptide comprises a signal peptide and a CD4 transmembrane domain.

In certain embodiments, the fusion polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 32-41.

In certain embodiments, the fusion polypeptide comprises the sequence set forth in SEQ ID NO: 40.

In further embodiments, the second polypeptide comprises a costimulatory domain.

In various embodiments, the costimulatory domain of the second polypeptide is selected from a costimulatory molecule selected from the group consisting of: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNFRS14, TNFRS18, TNRFS25, and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

In additional embodiments, the costimulatory domain of the second polypeptide is a costimulatory domain isolated from OX40 or TNFR2.

In certain embodiments, the polypeptide cleavage signal is a viral self-cleaving polypeptide.

In particular embodiments, the polypeptide cleavage signal is a viral self-cleaving 2A polypeptide.

In various embodiments, the polypeptide cleavage signal is a viral self-cleaving polypeptide selected from the group consisting of: a foot-and-mouth disease virus (FMDV) (F2A) peptide, an equine rhinitis A virus (ERAV) (E2A) peptide, a Thosea asigna virus (TaV) (T2A) peptide, a porcine teschovirus-1 (PTV-1) (P2A) peptide, a Theilovirus 2A peptide, and an encephalomyocarditis virus 2A peptide.

In some embodiments, the fusion polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 42-61.

In some embodiments, the fusion polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 50 or 60.

In further embodiments, the FRB multimerization domain and the FKBP multimerization domain localize extracellularly when of the first polypeptide and the second polypeptide are expressed.

In various embodiments, polypeptide complex comprises: a first polypeptide comprising: an FRB multimerization domain polypeptide or variant thereof; a CD8α transmembrane domain or a CD4 transmembrane domain; a CD137 co-stimulatory domain; and/or a CD3ζ primary signaling domain; a second polypeptide comprising: an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21; an FKBP multimerization domain polypeptide or variant thereof; and a CD4 transmembrane domain or a CD8α transmembrane domain; and a bridging factor associated with and disposed between the multimerization domains of the first and second polypeptides.

In particular embodiments, the anti-CD33 VHH antibody has an amino acid sequence set forth in SEQ ID NO: 10.

In particular embodiments, the FKBP multimerization domain is FKBP12.

In additional embodiments, the FRB polypeptide is FRB T2098L.

In particular embodiments, the bridging factor is selected from the group consisting of: AP21967, sirolimus, everolimus, novolimus, pimecrolimus, ridaforolimus, tacrolimus, temsirolimus, umirolimus, and zotarolimus.

In certain embodiments, the first polypeptide comprises a CD8α transmembrane domain; a CD137 co-stimulatory domain; and a CD3ζ primary signaling domain.

In various embodiments, the second polypeptide comprises a CD4 transmembrane domain.

In further embodiments, the second polypeptide comprises a costimulatory domain.

In some embodiments, the costimulatory domain of the second polypeptide is selected from a costimulatory molecule selected from the group consisting of: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10,

caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNFRS14, TNFRS18, TNRFS25, and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

In particular embodiments, the costimulatory domain of the second polypeptide is a costimulatory domain isolated from OX40 or TNFR2.

In certain embodiments, the cell is a hematopoietic cell.

In various embodiments, the cell is a T cell, an αβ T cell, or a γδ T cell.

In various embodiments, the cell is a CD3+, CD4+, and/or CD8+ cell.

In additional embodiments, the cell is an immune effector cell.

In some embodiments, the cell is a cytotoxic T lymphocytes (CTLs), a tumor infiltrating lymphocytes (TILs), or a helper T cell.

In particular embodiments, the cell is a natural killer (NK) cell or natural killer T (NKT) cell.

In additional embodiments, the source of the cell is peripheral blood mononuclear cells, bone marrow, lymph nodes tissue, cord blood, thymus issue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, or tumors.

In further embodiments, the FRB multimerization domain and the FKBP multimerization domain localize extracellularly when of the first polypeptide and the second polypeptide are expressed.

In preferred embodiments, a first polypeptide comprises an amino acid sequence set forth in SEQ ID NO 82.

In particular embodiments, a chimeric antigen receptor (CAR) comprises: an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21; a hinge domain; a transmembrane domain; one or more intracellular costimulatory signaling domains; and/or a primary signaling domain.

In particular embodiments, the anti-CD33 VHH antibody has an amino acid sequence set forth in SEQ ID NO: 10.

In particular embodiments, the anti-CD33 VHH antibody has an amino acid sequence set forth in SEQ ID NO: 20.

In various embodiments, the CAR comprises from 5′ to 3′: an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21; a hinge domain; a transmembrane domain; one or more intracellular costimulatory signaling domains; and/or a primary signaling domain.

In particular embodiments, the anti-CD33 VHH antibody has an amino acid sequence set forth in SEQ ID NO: 10.

In particular embodiments, the anti-CD33 VHH antibody has an amino acid sequence set forth in SEQ ID NO: 20.

In certain embodiments, the hinge domain and transmembrane domain are isolated from CD8α, CD27, CD28, CD33, CD37, CD45, CD64, CD71, CD80, CD86, CD 134, CD137, CD152, CD154, AMN, and PD1.

In additional embodiments, the one or more costimulatory signaling domains are isolated from a costimulatory molecule selected from the group consisting of: CD28, CD134, CD137, and CD278.

In particular embodiments, the CAR comprises a CD8α signal peptide, a CD8α hinge and transmembrane domain, a CD134 costimulatory domain, and a CD3ζ primary signaling domain.

In further embodiments, a CAR comprises the amino acid sequence set forth in any one of SEQ ID NOs: 62-81.

In further embodiments, a CAR comprises the amino acid sequence set forth in any one of SEQ ID NOs: 70 or 80.

In some embodiments, polynucleotide encoding a first or second polypeptide, a fusion polypeptide, or a CAR contemplated herein is provided.

In various embodiments, a cDNA encoding a first or second polypeptide, a fusion polypeptide, or a CAR contemplated herein is provided.

In particular embodiments, an RNA encoding a first or second polypeptide, a fusion polypeptide, or a CAR contemplated herein is provided.

In additional embodiments, a vector comprising a polynucleotide contemplated herein is provided.

In certain embodiments, the vector is an expression vector.

In certain embodiments, the vector is a transposon.

In further embodiments, the vector is a piggyBAC transposon or a Sleeping Beauty transposon.

In particular embodiments, the vector is a viral vector.

In particular embodiments, the vector is an adenoviral vector, an adeno-associated viral (AAV) vector, a herpes virus vector, a vaccinia virus vector, or a retroviral vector.

In additional embodiments, the retroviral vector is a lentiviral vector.

In various embodiments, the lentiviral vector is selected from the group consisting of: human immunodeficiency virus 1 (HIV-1); human immunodeficiency virus 2 (HIV-2), visna-maedi virus (VMV) virus; caprine arthritis-encephalitis virus (CAEV); equine infectious anemia virus (EIAV); feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV).

In further embodiments, a cell comprising a first and second polypeptide, a fusion polypeptide, or a CAR contemplated herein is provided.

In particular embodiments, the cell is a hematopoietic cell.

In certain embodiments, the cell is an immune effector cell.

In various embodiments, the cell is a T cell, an αβ T cell, or a γδ T cell.

In some embodiments, the cell expresses CD3+, CD4+, CD8+, or a combination thereof.

In particular embodiments, the cell is a cytotoxic T lymphocyte (CTL), a tumor infiltrating lymphocyte (TIL), or a helper T cell.

In further embodiments, the cell is a natural killer (NK) cell or natural killer T (NKT) cell.

In certain embodiments, a composition comprises a cell contemplated herein.

In particular embodiments, a composition comprises a physiologically acceptable carrier and a cell contemplated herein.

In additional embodiments, method of treating a subject in need thereof comprising administering the subject an effective amount of a composition contemplated herein.

In particular embodiments, a method of treating, preventing, or ameliorating at least one symptom of a cancer, infectious disease, autoimmune disease, inflammatory disease, and immunodeficiency, or condition associated therewith, comprises administering to the subject an effective amount of a composition contemplated herein.

In some embodiments, a method of treating a solid cancer comprises administering to the subject an effective amount of a composition contemplated herein.

In various embodiments, the solid cancer is selected from the group consisting of: lung cancer, liver cancer, gastric cancer, colorectal cancer, head and neck cancer, urothelial cancer, prostate cancer, testicular cancer, endometrial cancer, pancreatic cancer, breast cancer, cervical cancer, ovarian cancer, skin cancer, and melanoma.

In certain embodiments, a method of treating a hematological malignancy comprises administering to the subject an effective amount of a composition contemplated herein.

In various embodiments, the hematological malignancy is a leukemia, lymphoma, or multiple myeloma.

In particular embodiments, the hematological malignancy is acute myelogenous leukemia (AML).

BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

Figure 1A shows a cartoon of a VHH-DARIC polypeptide complex.

Figure 1B shows a cartoon of a CD33 VHH DARIC architecture.

Figure 2A shows the expression of CD33 VHH1-5 DARICs in transduced T cells, as detected by anti-VHH staining (top row) and by CD33-Fc binding (bottom row).

Figure 2B shows the expression of CD33 VHH9-10 DARICs in transduced T cells, as detected by CD33-Fc binding.

Figure 3A shows the phenotype of T cells transduced with CD33 VHH1-5 DARICs or controls.

Figure 3B shows the phenotype of T cells transduced with CD33 VHH9-10 DARICs or controls.

Figure 4A shows IFNγ secretion from CD33 VHH1-5 DARICs or control cells cultured with $CD33^+$ THP-1 cells at an E:T ratio of 1:1 in the presence or absence of AP21967 for 24 hours.

Figure 4B shows IFNγ secretion from CD33 VHH9-10 DARICs or control cells cultured with $CD33^+$ THP-1 cells at an E:T ratio of 1:1 in the presence or absence of AP21967 for 24 hours.

Figure 4C shows IFNγ secretion from CD33 VHH9-10 DARICs or control cells cultured with modified 293T cells that express full-length CD33 (CD33M) or a CD33 splice variant (CD33m, C2) at an E:T ratio of 1:1 in the presence or absence of AP21967 for 24 hours.

Figure 5A shows CD33 expression on MV4-11 cells, MV4-11 cells engineered to knock out the CD33 gene (CD33-KO cells), and in an unstained control.

Figure 5B shows IFNγ secretion from anti-CD33 VHH9 DARIC T cells or UTD T cells co-cultured with MV4-11 cells or CD33-KO cells at an E:T ratio of 1:1 in the presence or absence of AP21967 for 24 hours.

Figure 5C shows IFNγ secretion from UTD T cells, anti-CD33 CAR T cells, or anti-CD33 VHH DARIC T cells co-cultured with MV4-11 cells (left panel) or CD33-KO cells engineered to express a CD33m splice variant (CD33-KO-C2 cells; right panel) at an E:T ratio of 1:1 in the presence or absence of AP21967 for 24 hours.

Figure 6 shows IFNγ secretion from anti-CD33 VHH DARIC T cells co-cultured with $CD33^+$ THP-1 cells at an E:T ratio of 1:1 in the presence or absence of soluble CD33 (CD33-Fc) and AP21967 for 24 hours.

Figure 7 shows IFNγ secretion from anti-CD33 VHH DARIC T cells co-cultured with $CD33^{neg}$ 293T cells transfected with different amounts of mRNA encoding CD33 at an E:T ratio of 1:1 in the presence or absence of AP21967 for 24 hours.

Figure 8A shows tumor growth measured as a function of luminescence in immunodeficient NSG mice inoculated with HL60 AML tumor cells expressing a luciferase reporter and treated, 10 days post-inoculation (Day 0), with UTD T cells or anti-CD33 VHH DARIC T cells in the absence of rapamycin.

Figure 8B shows tumor growth measured as a function of luminescence in immunodeficient NSG mice inoculated with HL60 AML tumor cells expressing a luciferase reporter and treated, 10 days post-inoculation (Day 0), with UTD T cells or anti-CD33 VHH DARIC T cells and .1 mg/kg rapamycin.

BRIEF DESCRIPTION OF THE SEQUENCE IDENTIFIERS

SEQ ID NO: 1 sets forth the amino acid sequence for full-length human CD33.

SEQ ID NOs: 2-21 set forth the amino acid sequences for an anti-CD33 VHH domains.

SEQ ID NOs: 22-31 set forth the amino acid sequences for anti-CD33 VHH DARIC binding components.

SEQ ID NOs: 32-41 set forth the amino acid sequences for anti-CD33 VHH DARIC fusion proteins.

SEQ ID NOs: 42-51 set forth the amino acid sequences for anti-CD33 VHH DARIC.OX40 fusion proteins.

SEQ ID NOs: 52-61 set forth the amino acid sequences for anti-CD33 VHH DARIC.TNFR2 fusion proteins.

SEQ ID NOs: 62-81 set forth the amino acid sequences for anti-CD33 VHH CARs.

SEQ ID NO: 82 sets forth the amino acid sequence for an anti-CD33 VHH DARIC signaling component.

SEQ ID NO: 83 sets forth the polynucleotide sequence for a Kozak sequence.

SEQ ID NOs: 84-94 set forth the amino acid sequences of various linkers.

SEQ ID NOs: 95-119 set forth the amino acid sequences of protease cleavage sites and self-cleaving polypeptide cleavage sites.

In the foregoing sequences, Xaa, if present, may refer to any amino acid or the absence of an amino acid. In preferred embodiments, XaaXaa refers to the amino acid sequence SS or KP.

DETAILED DESCRIPTION

A. OVERVIEW

Cancer is among the leading causes of death worldwide. About 10% of cancers are hematological malignancies, which includes leukemia, lymphomas, and myelomas. Acute myeloid leukemia (AML) is the most common and fatal hematological malignancy in adults. Despite major scientific discoveries and novel therapies over the past four decades, the treatment outcomes of AML, especially in the adult patient population remain dismal. Standard chemotherapies can induce complete remission in selected patients; however, a majority of patients eventually relapse and succumb to the disease. In 2012, the worldwide incidence of AML was about 351,965 and about 265,461 people died from AML.

CD33 is expressed on the majority of acute myeloid leukemia (AML) leukemic blasts and, possibly, leukemic stem cells. CD33 is a challenging target because of its low expression and slow internalization; these characteristics limit antibody-dependent cell-mediated cytotoxicity and intracellular drug accumulation and, consequently, the activity of unlabeled and toxin-carrying antibodies.

The disclosure generally relates to improved compositions and methods for regulating the spatial and temporal control of adoptive cell therapies using dimerizing agent regulated immunoreceptor complexes (DARICs) that bind CD33. Without wishing to be bound by any particular theory, DARIC compositions and methods contemplated herein provide numerous advantages over CAR T cell therapies existing in the art, including but not limited to, both spatial and temporal control over immune effector cell signal transduction binding and signaling activities. DARIC temporal control primes the DARIC machinery for signaling through bridging factor mediated association of a DARIC binding component to a DARIC signaling component. DARIC spatial control engages the

signaling machinery through recognition of CD33 by the DARIC binding domain of the DARIC binding component. In this manner, DARIC immune effector cells become activated when both a target cell expressing CD33 and a bridging factor are present.

The disclosure also relates to improved anti-CD33 CAR architectures that overcome potential limitations of existing CAR T therapies including but not limited to tonic signaling or antigen independent signaling, poor expression and/or subtherapeutic activity.

In various embodiments, the disclosure contemplates anti-CD33 VHH DARICs or anti-CD33 VHH CARs that generate an anti-cancer response against cancers, *e.g.,* AML that express CD33, *e.g.,* full-length CD33 and/or a CD33 splice variant.

In particular embodiments, a DARIC includes a polypeptide (DARIC signaling component) that comprises a multimerization domain polypeptide or variant thereof, a transmembrane domain, a costimulatory domain; and/or a primary signaling domain; and a polypeptide (DARIC binding component) that comprises an anti-CD33 VHH, a multimerization domain polypeptide or variant thereof, a transmembrane domain; and optionally a costimulatory domain. In the presence of a bridging factor, the DARIC binding and signaling components associate with one another through the bridging factor to form a functionally active DARIC that targets cells expressing CD33.

In particular embodiments, the multimerization domains of the DARIC binding and DARIC signaling components are positioned extracellularly. Extracellular position of the multimerization domains provides numerous advantages over intracellular positioning including, but not limited to, more efficient positioning of the anti-CD33 VHH domain, higher temporal sensitivity to bridging factor regulation, and less toxicity due to ability to use non-immunosuppressive doses of particular bridging factors.

Polynucleotides encoding DARICs, DARIC binding components, and DARIC signaling components; DARIC binding components, DARIC signaling components, DARIC protein complexes, DARIC fusion proteins; cells comprising polynucleotides encoding DARICs, DARIC binding components, and DARIC signaling components and/or

expressing the same; and methods of using the same to treat an immune disorder are contemplated herein.

Techniques for recombinant (*i.e.*, engineered) DNA, peptide and oligonucleotide synthesis, immunoassays, tissue culture, transformation (*e.g.*, electroporation, lipofection), enzymatic reactions, purification and related techniques and procedures may be generally performed as described in various general and more specific references in microbiology, molecular biology, biochemistry, molecular genetics, cell biology, virology and immunology as cited and discussed throughout the present specification. *See*, *e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 3d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; *Current Protocols in Molecular Biology* (John Wiley and Sons, updated July 2008); *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience; Glover, *DNA Cloning: A Practical Approach*, vol. I & II (IRL Press, Oxford Univ. Press USA, 1985); *Current Protocols in Immunology* (Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober 2001 John Wiley & Sons, NY, NY); *Real-Time PCR: Current Technology and Applications*, Edited by Julie Logan, Kirstin Edwards and Nick Saunders, 2009, Caister Academic Press, Norfolk, UK; Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); *Oligonucleotide Synthesis* (N. Gait, Ed., 1984); *Nucleic Acid* The *Hybridization* (B. Hames & S. Higgins, Eds., 1985); *Transcription and Translation* (B. Hames & S. Higgins, Eds., 1984); *Animal Cell Culture* (R. Freshney, Ed., 1986); Perbal, *A Practical Guide to Molecular Cloning* (1984); *Next-Generation Genome Sequencing* (Janitz, 2008 Wiley-VCH); *PCR Protocols (Methods in Molecular Biology)* (Park, Ed., 3rd Edition, 2010 Humana Press); *Immobilized Cells And Enzymes* (IRL Press, 1986); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Harlow and Lane, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998); *Immunochemical Methods In Cell And*

Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir andCC Blackwell, eds., 1986); Roitt, *Essential Immunology,* 6th Edition, (Blackwell Scientific Publications, Oxford, 1988); *Current Protocols in Immunology* (Q. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach and W. Strober, eds., 1991); *Annual Review of Immunology*; as well as monographs in journals such as *Advances in Immunology*.

B. DEFINITIONS

Prior to setting forth this disclosure in more detail, it may be helpful to an understanding thereof to provide definitions of certain terms to be used herein.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of particular embodiments, preferred embodiments of compositions, methods and materials are described herein. For the purposes of the present disclosure, the following terms are defined below.

The articles "a," "an," and "the" are used herein to refer to one or to more than one (*i.e.,* to at least one, or to one or more) of the grammatical object of the article. By way of example, "an element" means one element or one or more elements.

The use of the alternative (*e.g.,* "or") should be understood to mean either one, both, or any combination thereof of the alternatives.

The term "and/or" should be understood to mean either one, or both of the alternatives.

As used herein, the term "about" or "approximately" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In one embodiment, the term "about" or "approximately" refers a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length ± 15%, ± 10%, ± 9%, ± 8%, ± 7%, ± 6%, ± 5%, ± 4%, ± 3%, ± 2%, or ± 1% about a

reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

In one embodiment, a range, *e.g.,* 1 to 5, about 1 to 5, or about 1 to about 5, refers to each numerical value encompassed by the range. For example, in one non-limiting and merely illustrative embodiment, the range "1 to 5" is equivalent to the expression 1, 2, 3, 4, 5; or 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0; or 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, or 5.0.

As used herein, the term "substantially" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that is 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher compared to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In one embodiment, "substantially the same" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that produces an effect, e.g., a physiological effect, that is approximately the same as a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises," and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that no other elements are present that materially affect the activity or action of the listed elements.

Reference throughout this specification to "one embodiment," "an embodiment," "a particular embodiment," "a related embodiment," "a certain embodiment," "an additional embodiment," or "a further embodiment" or combinations thereof means that a particular

feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments. It is also understood that the positive recitation of a feature in one embodiment, serves as a basis for excluding the feature in a particular embodiment.

An "antigen (Ag)" refers to a compound, composition, or substance that can stimulate the production of antibodies or a T cell response in an animal, including compositions (such as one that includes a cancer-specific protein) that are injected or absorbed into an animal. Exemplary antigens include but are not limited to lipids, carbohydrates, polysaccharides, glycoproteins, peptides, or nucleic acids. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous antigens, such as the disclosed antigens.

A "target antigen" or "target antigen of interest" refers to a portion of CD33, that a binding domain contemplated herein, is designed to bind. In particular embodiments, the target antigen is an epitope of the amino acid sequence set forth in SEQ ID NO: 1.

"CD33" refers to a cell surface receptor also known as sialic-acid-binding immunoglobulin-like lectin 3 (SIGLEC-3) or GP67. The CD33 gene is located on chromosome 19 and produces a glycosylated protein of about 67 kD. CD33 has two Ig-like domains, one V-set domain and one C2-set domain. CD33 plays a role in mediating cell-cell interactions and in maintaining immune cells in a resting state. CD33 recognizes and binds alpha-2,3- and more avidly alpha-2,6-linked sialic acid-bearing glycans. Upon engagement of ligands such as C1q or sialylated glycoproteins, two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) located in CD33 cytoplasmic tail are phosphorylated by Src-like kinases such as LCK. These phosphorylations provide docking sites for the recruitment and activation of protein-tyrosine phosphatases PTPN6/SHP-1 and PTPN11/SHP-2. CD33 also has at least three identified splice variants. $CD33^{\Delta E2}$ splice variant lacks the amino acid sequence encoded by exon 2 of the human CD33 gene (amino acids 13-139 of full-length CD33; *e.g.,* NP_001076087.1, C2). $CD33^{7a}$ splice variant lacks

54 carboxy-terminal amino acids due to an early translation stop signal residing in exon 7a (*e.g.*, NP_001171079.1). $CD33^{\Delta E2/7a}$ lacks the amino acids encoded by exon 2 and 54 carboxy-terminal amino acids. CD33 is normally expressed on subsets of normal B cells and activated T cells and natural killer cells but is not expressed on hematopoietic stem cells or outside the hematopoietic system. Both full-length CD33 and/or CD33 splice variants are also expressed in acute myeloid leukemia (AML) blast cells in a majority of AML patients.

An "antibody" refers to a binding agent that is a polypeptide comprising at least a light chain or heavy chain immunoglobulin variable region which specifically recognizes and binds an epitope of an antigen, such as a lipid, carbohydrate, polysaccharide, glycoprotein, peptide, or nucleic acid containing an antigenic determinant, such as those recognized by an immune cell.

References to "V_H" or "VH" refer to the variable region of an immunoglobulin heavy chain or antigen binding fragments thereof.

A "heavy chain antibody" refers to an antibody that contains two V_H domains and no light chains (Riechmann L. *et al*, *J. Immunol. Methods* 231:25–38 (1999); WO94/04678; WO94/25591; U.S. Patent No. 6,005,079). A "camelid antibody" refers to an antibody isolated from a Camel, Alpaca, or Llama that contains two V_H domains and no light chains. A "humanized VHH" or "humanized camelid antibody" refers to a non-human VHH or camelid antibody that has undergone humanization to reduce potential immunogenicity of the antibody in human recipients.

A "V_HH," "V_HH antibody," or "V_HH domain" as used herein refers an antibody fragment that contains the smallest known antigen-binding unit of the variable region of a heavy chain antibody (Koch-Nolte, *et al*, *FASEB J.*, 21: 3490-3498 (2007)).

A "linker" refers to a plurality of amino acid residues between the various polypeptide domains added for appropriate spacing and conformation of the molecule. In particular embodiments, a linker separates one or more VHH domains, hinge domains, multimerization domains, transmembrane domains, co-stimulatory domains, and/or primary signaling domains.

Illustrated examples of linkers suitable for use in particular embodiments contemplated herein include, but are not limited to the following amino acid sequences: GGG; DGGGS (SEQ ID NO: 84); TGEKP (SEQ ID NO: 85) (see, *e.g.,* Liu *et al.*, PNAS 5525-5530 (1997)); GGRR (SEQ ID NO: 86) (Pomerantz *et al.* 1995, supra); $(GGGGS)_n$ wherein n = 1, 2, 3, 4 or 5 (SEQ ID NO: 87) (Kim *et al.*, PNAS 93, 1156-1160 (1996.); EGKSSGSGSESKVD (SEQ ID NO: 88) (Chaudhary *et al.*, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:1066-1070); KESGSVSSEQLAQFRSLD (SEQ ID NO: 89) (Bird *et al.*, 1988, Science 242:423-426), GGRRGGGS (SEQ ID NO: 90); LRQRDGERP (SEQ ID NO: 91); LRQKDGGGSERP (SEQ ID NO: 92); $LRQKD(GGGS)_2$ ERP (SEQ ID NO: 93). Alternatively, flexible linkers can be rationally designed using a computer program capable of modeling both DNA-binding sites and the peptides themselves (Desjarlais & Berg, *PNAS* 90:2256-2260 (1993), *PNAS* 91:11099-11103 (1994) or by phage display methods. In one embodiment, the linker comprises the following amino acid sequence: GSTSGSGKPGSGEGSTKG (SEQ ID NO: 94) (Cooper *et al., Blood*, 101(4): 1637-1644 (2003)).

A "spacer domain," refers to a polypeptide that separates two domains. In one embodiment, a spacer domain moves a VHH domain away from the effector cell surface to enable proper cell/cell contact, antigen binding and activation (Patel *et al.*, *Gene Therapy*, 1999; 6: 412-419). In particular embodiments, a spacer domain separates one or more VHH domains, multimerization domains, transmembrane domains, co-stimulatory domains, and/or primary signaling domains. The spacer domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. In certain embodiments, a spacer domain is a portion of an immunoglobulin, including, but not limited to, one or more heavy chain constant regions, *e.g.,* CH2 and CH3. The spacer domain can include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region.

A "hinge domain," refers to a polypeptide that plays a role in positioning the antigen binding domain away from the effector cell surface to enable proper cell/cell contact, antigen binding and activation. In particular embodiments, polypeptides may comprise one or more hinge domains between the binding domain and the multimerization

domain, between the binding domain and the transmembrane domain (TM), or between the multimerization domain and the transmembrane domain. The hinge domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. The hinge domain can include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region.

A "multimerization domain," as used herein, refers to a polypeptide that preferentially interacts or associates with another different polypeptide directly or via a bridging molecule, *e.g.,* a chemically inducible dimerizer, wherein the interaction of different multimerization domains substantially contributes to or efficiently promotes multimerization (*i.e.*, the formation of a dimer, trimer, or multipartite complex, which may be a homodimer, heterodimer, homotrimer, heterotrimer, homomultimer, heteromultimer). A multimerization domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source.

Illustrative examples of multimerization domains suitable for use in particular embodiments contemplated herein include an FK506 binding protein (FKBP) polypeptide or variants thereof, an FKBP-rapamycin binding (FRB) polypeptide or variants thereof, a calcineurin polypeptide or variants thereof, a cyclophilin polypeptide or variants thereof, a bacterial dihydrofolate reductase (DHFR) polypeptide or variants thereof, a PYR1-like 1 (PYL1) polypeptide or variants thereof, an abscisic acid insensitive 1 (ABI1) polypeptide or variants thereof, a GIB1 polypeptide or variants thereof, or a GAI polypeptide or variants thereof.

As used herein, the term "FKBP-rapamycin binding polypeptide" refers to an FRB polypeptide. In particular embodiments, the FRB polypeptide is an FKBP12-rapamycin binding polypeptide. FRB polypeptides suitable for use in particular embodiments contemplated herein generally contain at least about 85 to about 100 amino acid residues. In certain embodiments, the FRB polypeptide comprises a 93 amino acid sequence Ile-2021 through Lys -2113 and a mutation of T2098L, with reference to GenBank Accession No. L34075.1. An FRB polypeptide contemplated herein binds to an FKBP polypeptide through a bridging factor, thereby forming a ternary complex.

As used herein, the term "FK506 binding protein" refers to an FKBP polypeptide. In particular embodiments, the FKBP polypeptide is an FKBP12 polypeptide or an FKBP12

polypeptide comprising an F36V mutation. In certain embodiments, an FKBP domain may also be referred to as a "rapamycin binding domain". Information concerning the nucleotide sequences, cloning, and other aspects of various FKBP species is known in the art (*see*, *e.g.*, Staendart *et al.*, *Nature 346*:671, 1990 (human FKBP12); Kay, *Biochem. J. 314*:361, 1996). An FKBP polypeptide contemplated herein binds to an FRB polypeptide through a bridging factor, thereby forming a ternary complex.

A "bridging factor" refers to a molecule that associates with and that is disposed between two or more multimerization domains. In particular embodiments, multimerization domains substantially contribute to or efficiently promote formation of a polypeptide complex only in the presence of a bridging factor. In particular embodiments, multimerization domains do not contribute to or do not efficiently promote formation of a polypeptide complex in the absence of a bridging factor. Illustrative examples of bridging factors suitable for use in particular embodiments contemplated herein include, but are not limited to AP21967, rapamycin (sirolimus) or a rapalog thereof, coumermycin or a derivative thereof, gibberellin or a derivative thereof, abscisic acid (ABA) or a derivative thereof, methotrexate or a derivative thereof, cyclosporin A or a derivative thereof, FKCsA or a derivative thereof, trimethoprim (Tmp)-synthetic ligand for FKBP (SLF) or a derivative thereof, or any combination thereof.

Rapamycin analogs (rapalogs) include, but are not limited to, those disclosed in U.S. Pat. No. 6,649,595, which rapalog structures are incorporated herein by reference in their entirety. In certain embodiments, a bridging factor is a rapalog with substantially reduced immunosuppressive effect as compared to rapamycin. In a preferred embodiment, the rapalog is AP21967 (also known as C-16-(S)-7-methylindolerapamycin, IC_{50} = 10nM, a chemically modified non-immunosuppressive rapamycin analogue). Other illustrative rapalogs suitable for use in particular embodiments contemplated herein include, but are not limited to, everolimus, novolimus, pimecrolimus, ridaforolimus, tacrolimus, temsirolimus, umirolimus, and zotarolimus.

A "substantially reduced immunosuppressive effect" refers to at least less than 0.1 to 0.005 times the immunosuppressive effect observed or expected for the same dose measured either clinically or in an appropriate *in vitro* (*e.g.*, inhibition of T cell proliferation) or *in vivo* surrogate of human immunosuppressive activity.

A "transmembrane domain" or "TM domain" is a domain that anchors a polypeptide to the plasma membrane of a cell. The TM domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source.

The term "effector function" or "effector cell function" refers to a specialized function of an immune effector cell. Effector function includes, but is not limited to, activation, cytokine production, proliferation and cytotoxic activity, including the release of cytotoxic factors, or other cellular responses elicited with antigen binding to the receptor expressed on the immune effector cell.

An "intracellular signaling domain" or "endodomain" refers to the portion of a protein which transduces the effector function signal and that directs the cell to perform a specialized function. While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire domain. To the extent that a truncated portion of an intracellular signaling domain is used, such truncated portion may be used in place of the entire domain as long as it transduces an effector function signal. The term intracellular signaling domain is meant to include any truncated portion of an intracellular signaling domain necessary or sufficient to transduce an effector function signal.

It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary or co-stimulatory signal is also required. Thus, T cell activation can be said to be mediated by two distinct classes of intracellular signaling domains: primary signaling domains that initiate antigen-dependent primary activation through the TCR (*e.g.*, a TCR/CD3 complex) and co-stimulatory signaling domains that act in an antigen-independent manner to provide a secondary or co-stimulatory signal.

A "primary signaling domain" refers to an intracellular signaling domain that regulates the primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary signaling domains that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs. Illustrative examples of ITAM containing primary signaling domains that are suitable for use in particular embodiments include, but are not limited to those derived from FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d.

As used herein, the term, "co-stimulatory signaling domain," or "co-stimulatory domain" refers to an intracellular signaling domain of a co-stimulatory molecule. Co-stimulatory molecules are cell surface molecules other than antigen receptors or Fc receptors that provide a second signal required for efficient activation and function of T lymphocytes upon binding to antigen. Illustrative examples of such co-stimulatory molecules from which co-stimulatory domains may be isolated include, but are not limited to: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNF receptor superfamily member 14 (TNFRS14; HVEM), TNF receptor superfamily member 18 (TNFRS18; GITR), TNF receptor superfamily member 25 (TNFRS25; DR3), and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

An "immune disorder" refers to a disease that evokes a response from the immune system. In particular embodiments, the term "immune disorder" refers to a cancer, an autoimmune disease, or an immunodeficiency.

As used herein, the term "cancer" relates generally to a class of diseases or conditions in which abnormal cells divide without control and can invade nearby tissues.

As used herein, the term "malignant" refers to a cancer in which a group of tumor cells display one or more of uncontrolled growth (*i.e.,* division beyond normal limits), invasion (*i.e.,* intrusion on and destruction of adjacent tissues), and metastasis (*i.e.,* spread to other locations in the body via lymph or blood). As used herein, the term "metastasize" refers to the spread of cancer from one part of the body to another. A tumor formed by cells that have spread is called a "metastatic tumor" or a "metastasis." The metastatic tumor contains cells that are like those in the original (primary) tumor.

As used herein, the term "benign" or "non-malignant" refers to tumors that may grow larger but do not spread to other parts of the body. Benign tumors are self-limited and typically do not invade or metastasize.

A "cancer cell" refers to an individual cell of a cancerous growth or tissue. Cancer cells include both solid cancers and liquid cancers. A "tumor" or "tumor cell" refers generally to a swelling or lesion formed by an abnormal growth of cells, which may be benign, pre-malignant, or malignant. Most cancers form tumors, but liquid cancers, *e.g.*, leukemia, do not necessarily form tumors. For those cancers that form tumors, the terms cancer (cell) and tumor (cell) are used interchangeably. The amount of a tumor in an individual is the "tumor burden" which can be measured as the number, volume, or weight of the tumor.

The term "relapse" refers to the diagnosis of return, or signs and symptoms of return, of a cancer after a period of improvement or remission.

"Remission," is also referred to as "clinical remission," and includes both partial and complete remission. In partial remission, some, but not all, signs and symptoms of cancer have disappeared. In complete remission, all signs and symptoms of cancer have disappeared, although cancer still may be in the body.

"Refractory" refers to a cancer that is resistant to, or non-responsive to, therapy with a particular therapeutic agent. A cancer can be refractory from the onset of treatment (*i.e.*, non-responsive to initial exposure to the therapeutic agent), or as a result of developing resistance to the therapeutic agent, either over the course of a first treatment period or during a subsequent treatment period.

As used herein, the terms "individual" and "subject" are often used interchangeably and refer to any animal that exhibits a symptom of cancer or other immune disorder that can be treated with the compositions and methods contemplated elsewhere herein. Suitable subjects (*e.g.*, patients) include laboratory animals (such as mouse, rat, rabbit, or guinea pig), farm animals, and domestic animals or pets (such as a cat or dog). Non-human primates and, preferably, human patients, are included. Typical subjects include human patients that have, have been diagnosed with, or are at risk or having, cancer or another immune disorder.

As used herein, the term "patient" refers to a subject that has been diagnosed with cancer or another immune disorder that can be treated with the compositions and methods disclosed elsewhere herein.

As used herein "treatment" or "treating," includes any beneficial or desirable effect on the symptoms or pathology of a disease or pathological condition, and may include even minimal reductions in one or more measurable markers of the disease or condition being treated. Treatment can involve optionally either the reduction of the disease or condition, or the delaying of the progression of the disease or condition, *e.g.,* delaying tumor outgrowth. "Treatment" does not necessarily indicate complete eradication or cure of the disease or condition, or associated symptoms thereof.

As used herein, "prevent," and similar words such as "prevented," "preventing" *etc.*, indicate an approach for preventing, inhibiting, or reducing the likelihood of the occurrence or recurrence of, a disease or condition. It also refers to delaying the onset or recurrence of a disease or condition or delaying the occurrence or recurrence of the symptoms of a disease or condition. As used herein, "prevention" and similar words also includes reducing the intensity, effect, symptoms and/or burden of a disease or condition prior to onset or recurrence of the disease or condition.

As used herein, the phrase "ameliorating at least one symptom of" refers to decreasing one or more symptoms of the disease or condition for which the subject is being treated. In particular embodiments, the disease or condition being treated is a cancer, wherein the one or more symptoms ameliorated include, but are not limited to, weakness, fatigue, shortness of breath, easy bruising and bleeding, frequent infections, enlarged lymph nodes, distended or painful abdomen (due to enlarged abdominal organs), bone or joint pain, fractures, unplanned weight loss, poor appetite, night sweats, persistent mild fever, and decreased urination (due to impaired kidney function).

By "enhance" or "promote," or "increase" or "expand" refers generally to the ability of a composition contemplated herein to produce, elicit, or cause a greater physiological response (*i.e.*, downstream effects) compared to the response caused by either vehicle or a control molecule/composition. A measurable physiological response may include an increase in T cell expansion, activation, persistence, cytokine secretion, and/or an increase in cancer cell killing ability, among others apparent from the understanding in the art and the description herein. An "increased" or "enhanced" amount is typically a "statistically significant" amount, and may include an increase that is 1.1, 1.2,

1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 or more times (*e.g.,* 500, 1000 times) (including all integers and decimal points in between and above 1, *e.g.,* 1.5, 1.6, 1.7. 1.8, *etc.*) the response produced by vehicle or a control composition.

By "decrease" or "lower," or "lessen," or "reduce," or "abate" refers generally to the ability of composition contemplated herein to produce, elicit, or cause a lesser physiological response (*i.e.*, downstream effects) compared to the response caused by either vehicle or a control molecule/composition. A "decrease" or "reduced" amount is typically a "statistically significant" amount, and may include a decrease that is 1.1, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 or more times (*e.g.,* 500, 1000 times) (including all integers and decimal points in between and above 1, *e.g.,* 1.5, 1.6, 1.7. 1.8, *etc.*) the response (reference response) produced by vehicle, a control composition, or the response in a particular cell lineage.

By "maintain," or "preserve," or "maintenance," or "no change," or "no substantial change," or "no substantial decrease" refers generally to the ability of a composition contemplated herein to produce, elicit, or cause a substantially similar or comparable physiological response (*i.e.*, downstream effects) in a cell, as compared to the response caused by either vehicle, a control molecule/composition, or the response in a particular cell lineage. A comparable response is one that is not significantly different or measurable different from the reference response.

Additional definitions are set forth throughout this disclosure.

C. CD33 VHH DARICs

In particular embodiments, a DARIC receptor comprising an anti-CD33 VHH domain that redirects cytotoxicity of immune effector cells toward cancer cells expressing CD33 is contemplated. As used herein, the terms "CD33 VHH DARIC receptor," "anti-CD33 VHH DARIC receptor" "CD33 VHH DARIC," or "anti-CD33 VHH DARIC" are used interchangeably and refer to one or more non-naturally occurring polypeptides that transduces an immunostimulatory signal in an immune effector cell upon exposure to a target cell expressing full-length CD33 or a CD33 splice variant and a multimerizing agent

or bridging factor, *e.g.*, stimulating immune effector cell activity and function, increasing production and/or secretion of proinflammatory cytokines. In preferred embodiments, a CD33 VHH DARIC is a multi-chain chimeric receptor comprising a DARIC signaling component and a DARIC binding component comprising a VHH domain that recognizes full-length CD33 and/or a CD33 splice variant.

In one embodiment, a DARIC signaling component and a DARIC binding component are expressed from the same cell. In another embodiment, a DARIC signaling component and a DARIC binding component are expressed from different cells. In a particular embodiment, a DARIC signaling component is expressed from a cell and a DARIC binding component is supplied exogenously, as a polypeptide. In one embodiment, a DARIC binding component pre-loaded with a bridging factor is supplied exogenously to a cell expressing a DARIC signaling component.

1. CD33 DARIC SIGNALING COMPONENT

The terms "DARIC signaling component," CD33 DARIC signaling component," "DARIC signaling polypeptide," or "DARIC signaling polypeptide" are used interchangeably and refer to a polypeptide comprising one or more multimerization domains, a transmembrane domain, and one or more intracellular signaling domains. In particular embodiments, a DARIC signaling component comprises a multimerization domain, a transmembrane domain, a costimulatory domain and/or a primary signaling domain. In particular embodiments, a DARIC signaling component comprises a first multimerization domain, a first transmembrane domain, a first costimulatory domain and/or a primary signaling domain.

In particular embodiments, a DARIC signaling component comprises one or more multimerization domains.

Illustrative examples of multimerization domains suitable for use in particular CD33 DARIC signaling components contemplated herein include, but are not limited to, an FK506 binding protein (FKBP) polypeptide or variants thereof, an FKBP-rapamycin binding (FRB) polypeptide or variants thereof, a calcineurin polypeptide or variants

thereof, a cyclophilin polypeptide or variants thereof, a bacterial dihydrofolate reductase (DHFR) polypeptide or variants thereof, a PYR1-like 1 (PYL1) polypeptide or variants thereof and an abscisic acid insensitive 1 (ABI1) polypeptide or variants thereof.

In particular embodiments, a CD33 DARIC signaling component comprises an FRB polypeptide.

In particular preferred embodiments, a CD33 DARIC signaling component comprises an FRB polypeptide comprising a T2098L mutation, or variant thereof. In certain preferred embodiments, a CD33 DARIC signaling component comprises an FKBP12 polypeptide or variant thereof.

In some embodiments, a CD33 VHH DARIC signaling component comprises a hinge domain.

Illustrative hinge domains suitable for use in a CD33 VHH DARIC signaling component described herein include the hinge region derived from the extracellular regions of type 1 membrane proteins such as CD28, CD8α, and CD4, which may be wild-type hinge regions from these molecules or may be altered.

In particular embodiments, a DARIC signaling component comprises a transmembrane domain.

In particular embodiments, a DARIC signaling component comprises a hinge domain and a transmembrane domain.

Illustrative examples of transmembrane domains suitable for use in particular CD33 DARIC signaling components contemplated herein include, but are not limited to, the transmembrane region(s) of the alpha, beta, gamma, or delta chain of a T-cell receptor, CD3ε, CD3ζ, CD4, CD5, CD8α, CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD71, CD80, CD86, CD 134, CD137, CD152, CD 154, amnionless (AMN), and programmed cell death 1 (PDCD1). In a preferred embodiment, a CD33 DARIC signaling component comprises a CD4 transmembrane domain. In a preferred embodiment, a CD33 DARIC signaling component comprises a CD8α transmembrane domain.

In particular embodiments, a DARIC signaling component comprises a linker that links the C-terminus of the transmembrane domain to the N-terminus of an intracellular

signaling domain. In various preferred embodiments, a short oligo- or poly-peptide linker, preferably between 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in length links the transmembrane domain and an intracellular signaling domain. A glycine-serine based linker provides a particularly suitable linker.

DARIC signaling components contemplated in particular embodiments herein comprise one or more intracellular signaling domains. In one embodiment, a CD33 DARIC signaling component comprises one or more costimulatory signaling domains and/or a primary signaling domain. In one embodiment, the intracellular signaling domain comprises an immunoreceptor tyrosine activation motif (ITAM).

Illustrative examples of ITAM containing primary signaling domains that are suitable for use in particular CD33 DARIC signaling components contemplated herein include, but are not limited to those derived from FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d. In preferred embodiments, a CD33 DARIC signaling component comprises a CD3ζ primary signaling domain and one or more costimulatory signaling domains. The primary signaling and costimulatory signaling domains may be linked in any order in tandem to the carboxyl terminus of the transmembrane domain.

Illustrative examples of costimulatory domains suitable for use in particular CD33 DARIC signaling components contemplated herein include, but are not limited to those domains isolated from the following costimulatory molecules: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNF receptor superfamily member 14 (TNFRS14; HVEM), TNF receptor superfamily member 18 (TNFRS18; GITR), TNF receptor superfamily member 25 (TNFRS25; DR3), and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

In particular embodiments, a CD33 DARIC signaling component contemplated herein comprises a signal peptide. Illustrative examples of signal peptides suitable for use in particular CD33 DARIC signaling components include but are not limited to an IgG1 heavy chain signal polypeptide, an Igκ light chain signal polypeptide, a CD8α signal polypeptide, or a human GM-CSF receptor alpha signal polypeptide. In various preferred embodiments, a CD33 DARIC signaling component comprises a CD8α signal polypeptide.

In particular embodiments, a CD33 DARIC signaling component comprises one or more costimulatory signaling domains selected from the group consisting of CD28, CD137, and CD134. In particular embodiments, a CD33 DARIC signaling component comprises one or more costimulatory signaling domains selected from the group consisting of CD28, CD137, and CD134, and a CD3ζ primary signaling domain. In a particular embodiment, a CD33 DARIC signaling component comprises a CD137 costimulatory domain and a CD3ζ primary signaling domain.

In a preferred embodiment, a CD33 DARIC signaling component comprises an FRB T2098L multimerization domain, a CD8α transmembrane domain, a CD137 costimulatory domain and a CD3ζ primary signaling domain.

In preferred embodiments, a CD33 VHH DARIC signaling component comprises an amino acid sequence set forth in SEQ ID NO 82.

2. CD33 DARIC BINDING COMPONENT

A "DARIC binding component," "DARIC binding polypeptide," "CD33 VHH DARIC binding component," or "CD33 VHH DARIC binding polypeptide" are used interchangeably and refer to a polypeptide comprising an anti-CD33 VHH domain, and one or more multimerization domains. In particular embodiments, the CD33 VHH DARIC binding component comprises an anti-CD33 VHH domain, a multimerization domain and a transmembrane domain. In particular embodiments, the CD33 VHH DARIC binding component comprises an anti-CD33 VHH domain, a second multimerization domain, and a second transmembrane domain. In other particular embodiments, the CD33 VHH DARIC

binding component comprises an anti-CD33 VHH domain, a multimerization domain, a transmembrane domain and one or more intracellular signaling domains. In particular embodiments, the CD33 VHH DARIC binding component comprises an anti-CD33 VHH domain, a second multimerization domain, a second transmembrane domain, and a second costimulatory domain.

In particular embodiments, the CD33 VHH DARIC binding component comprises one or more anti-CD33 VHH domains.

In particular preferred embodiments, the anti-CD33 VHH domain is a humanized camelid VHH. In particular embodiments, the anti-CD33 VHH domain is a humanized camelid VHH that binds one or more epitopes of full-length CD33 (*e.g.,* SEQ ID NO: 1) or one or more epitopes of a CD33 splice variant. In particular embodiments, the anti-CD33 VHH domain is a humanized camelid VHH that binds the same one or more epitopes displayed on both full-length CD33 and a CD33 splice variant.

In particular preferred embodiments, the anti-CD33 VHH domain is a humanized camelid VHH comprising amino acid sequence set forth in any one of SEQ ID NOs: 3-6, 10-11, 13-16, and 20-21. In certain preferred embodiments, the anti-CD33 VHH domain is a humanized camelid VHH comprising amino acid sequence set forth in SEQ ID NO: 10. In certain preferred embodiments, the anti-CD33 VHH domain is a humanized camelid VHH comprising amino acid sequence set forth in SEQ ID NO: 20.

In particular embodiments, a DARIC binding component comprises one or more multimerization domains.

Illustrative examples of multimerization domains suitable for use in particular CD33 VHH DARIC binding components contemplated herein include, but are not limited to, an FKBP polypeptide or variants thereof, an FRB polypeptide or variants thereof, a calcineurin polypeptide or variants thereof, a cyclophilin polypeptide or variants thereof, a DHFR polypeptide or variants thereof, a PYL1 polypeptide or variants thereof and an ABI1 polypeptide or variants thereof.

In particular embodiments, a CD33 VHH DARIC binding component comprises an FRB polypeptide or variant thereof and a DARIC signaling component comprises an FKBP

polypeptide or variant thereof. In a preferred embodiment, a CD33 VHH DARIC binding component comprises an FRB polypeptide comprising a T2098L mutation, or variant thereof and a DARIC signaling component comprises an FKBP12 polypeptide or variant thereof.

In particular embodiments, a CD33 VHH DARIC binding component comprises an FKBP polypeptide or variant thereof and a DARIC signaling component comprises an FRB polypeptide, or variant thereof. In a preferred embodiment, a CD33 VHH DARIC binding component comprises an FKBP12 polypeptide, or variant thereof and a DARIC signaling component comprises an FRB polypeptide comprising a T2098L mutation, or variant thereof.

In some embodiments, a CD33 VHH DARIC binding component comprises a hinge domain.

Illustrative hinge domains suitable for use in a CD33 VHH DARIC binding component described herein include the hinge region derived from the extracellular regions of type 1 membrane proteins such as CD28, CD8α, and CD4, which may be wild-type hinge regions from these molecules or may be altered.

In particular embodiments, a DARIC binding component comprises a transmembrane domain. In particular embodiments, a DARIC binding component comprises a hinge domain and a transmembrane domain. In one embodiment, the transmembrane domain may be the same as the transmembrane domain used in the DARIC signaling component. In one embodiment, the transmembrane domain may be different from the transmembrane domain used in the DARIC signaling component.

Illustrative examples of transmembrane domains suitable for use in particular CD33 VHH DARIC binding components contemplated herein include, but are not limited to, the transmembrane region(s) of the alpha, beta, gamma, or delta chain of a T-cell receptor, CD3ε, CD3ζ, CD4, CD5, CD8α, CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD71, CD80, CD86, CD 134, CD137, CD152, CD 154, amnionless (AMN), and programmed cell death 1 (PDCD1). In a preferred embodiment, a CD33 DARIC binding

component comprises a CD8α transmembrane domain. In a preferred embodiment, a CD33 VHH DARIC binding component comprises a CD4 transmembrane domain.

In various preferred embodiments, a short oligo- or poly-peptide linker, preferably between 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in length links the transmembrane domain and the intracellular signaling domain. A glycine-serine based linker provides a particularly suitable linker.

DARIC binding components contemplated in particular embodiments herein do not comprise one or more intracellular signaling domains.

In other particular embodiments, CD33 VHH DARIC binding components contemplated herein comprise one or more intracellular signaling domains. In preferred embodiments, wherein the CD33 VHH DARIC binding component comprises one or more intracellular signaling domains, those domains are different that the intracellular signaling domains present in the cognate CD33 DARIC signaling component. In one embodiment, a CD33 VHH DARIC binding component comprises a costimulatory signaling domain.

Illustrative examples of costimulatory domains suitable for use in particular CD33 VHH DARIC binding components contemplated herein include, but are not limited to those domains isolated from the following costimulatory molecules: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNF receptor superfamily member 14 (TNFRS14; HVEM), TNF receptor superfamily member 18 (TNFRS18; GITR), TNF receptor superfamily member 25 (TNFRS25; DR3), and zeta chain of T cell receptor associated protein kinase 70 (ZAP70). In preferred embodiments, the costimulatory domain is derived, obtained, or isolated from TNFR2 or OX40.

In particular embodiments, a DARIC binding component contemplated herein comprises a signal peptide. Illustrative examples of signal peptides suitable for use in

particular CD33 VHH DARIC binding components include but are not limited to an IgG1 heavy chain signal polypeptide, an Igκ light chain signal polypeptide, a CD8α signal polypeptide, or a human GM-CSF receptor alpha signal polypeptide. In various preferred embodiments, a CD33 VHH DARIC binding component comprises a CD8α signal polypeptide.

In particular embodiments, a CD33 VHH DARIC binding component comprises a VHH domain that binds to CD33, an FKBP12 multimerization domain, and a CD4 transmembrane domain and optionally, a costimulatory domain.

In certain embodiments, a CD33 VHH DARIC binding component comprises a VHH that binds to CD33, and an FKBP12 multimerization domain.

In some embodiments, a CD33 VHH DARIC binding component comprises a VHH domain comprising the amino acid sequence set forth in any one of SEQ ID NOs: 2-21, an FKBP12 multimerization domain, and a CD4 transmembrane domain and optionally, a costimulatory domain.

In some embodiments, a CD33 VHH DARIC binding component comprises a VHH domain comprising the amino acid sequence set forth in SEQ ID NO: 10 or SEQ ID NO: 20, an FKBP12 multimerization domain, and a CD4 transmembrane domain and optionally, a costimulatory domain.

In some embodiments, a CD33 VHH DARIC binding component comprises a VHH domain comprising the amino acid sequence set forth in any one of SEQ ID NOs: 2-21, and an FKBP12 multimerization domain.

In some embodiments, a CD33 VHH DARIC binding component comprises a VHH domain comprising the amino acid sequence set forth in SEQ ID NO: 10 or SEQ ID NO: 20, and an FKBP12 multimerization domain.

In some embodiments, a CD33 VHH DARIC binding component comprises the amino acid sequence set forth in any one of SEQ ID NOs: 22-31.

In some embodiments, a CD33 VHH DARIC binding component comprises the amino acid sequence set forth in SEQ ID NO: 30.

3. *BRIDGING FACTOR*

Bridging factors contemplated in particular embodiments herein, mediate or promote the association of a CD33 DARIC signaling component with a CD33 VHH DARIC binding component through multimerization domains in the respective components. A bridging factor associates with and is disposed between the multimerization domains to promote association of a CD33 DARIC signaling component and a CD33 VHH DARIC binding component. In the presence of a bridging factor, the CD33 VHH DARIC binding component and the CD33 DARIC signaling component associate and initiate immune effector cell activity against a target cell when the CD33 VHH DARIC binding component binds CD33 expressed on the target cell. In the absence of a bridging factor, the CD33 VHH DARIC binding component does not associate with the CD33 DARIC signaling component and the CD33 VHH DARIC is inactive.

In particular embodiments, a CD33 DARIC signaling component and a CD33 VHH DARIC binding component comprise a cognate pair of multimerization domains selected from the group consisting of: FKBP and FKBP12-rapamycin binding (FRB), FKBP and calcineurin, FKBP and cyclophilin, FKBP and bacterial dihydrofolate reductase (DHFR), calcineurin and cyclophilin, and PYR1-like 1 (PYL1) and abscisic acid insensitive 1 (ABI1).

In certain embodiments, the multimerization domains of CD33 VHH DARIC signaling and binding components associate with a bridging factor selected from the group consisting of: rapamycin or a rapalog thereof, coumermycin or a derivative thereof, gibberellin or a derivative thereof, abscisic acid (ABA) or a derivative thereof, methotrexate or a derivative thereof, cyclosporin A or a derivative thereof, FK506/cyclosporin A (FKCsA) or a derivative thereof, and trimethoprim (Tmp)-synthetic ligand for FK506 binding protein (FKBP) (SLF) or a derivative thereof.

In particular embodiments, a CD33 DARIC signaling component and a CD33 VHH DARIC binding component comprise one or more FRB and/or FKBP multimerization domains or variants thereof. In certain embodiments, a CD33 DARIC signaling component comprises an FRB multimerization domain or variant thereof and a CD33 VHH DARIC

binding component comprises an FKBP multimerization domain or variant thereof. In particular preferred embodiments, a CD33 DARIC signaling component comprises an FRB T2098L multimerization domain or variant thereof and a CD33 VHH DARIC binding component comprises an FKBP12 or FKBP12 F36V multimerization domains or variant thereof.

Illustrative examples of bridging factors suitable for use in particular embodiments contemplated herein include, but are not limited to, AP1903, AP20187, AP21967 (also known as C-16-(S)-7-methylindolerapamycin), everolimus, novolimus, pimecrolimus, ridaforolimus, tacrolimus, temsirolimus, umirolimus, and zotarolimus. In particular preferred embodiments, the bridging factor is AP21967. In certain preferred embodiments, the bridging factor is a non-immunosuppressive dose of sirolimus (rapamycin).

D. ANTI-CD33 CHIMERIC ANTIGEN RECEPTORS

In particular embodiments, immune effector cells contemplated herein comprise an anti-CD33 VHH CAR. Chimeric antigen receptors (CARs) are molecules that combine antibody-based specificity for a target antigen (*e.g.*, tumor antigen) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-tumor cellular immune activity. As used herein, the term, "chimeric," describes being composed of parts of different proteins or DNAs from different origins.

In particular embodiments, T cells are engineered by introducing a polynucleotide encoding an anti-CD33 VHH CAR.

In particular embodiments, T cells are engineered by introducing a vector comprising a polynucleotide encoding an anti-CD33 VHH CAR.

In various embodiments, an anti-CD33 CAR comprises a VHH domain that binds CD33, a transmembrane domain and one or more intracellular signaling domains. The main characteristic of CARs is their ability to redirect immune effector cell specificity, thereby triggering proliferation, cytokine production, phagocytosis or production of molecules that can mediate cell death of the target antigen expressing cell in a major

histocompatibility (MHC) independent manner, exploiting the cell specific targeting abilities of monoclonal antibodies, soluble ligands or cell specific coreceptors.

In some embodiments, an anti-CD33 VHH CAR comprises a spacer domain. In particular embodiments, the spacer domain comprises the CH2 and CH3 of IgG1, IgG4, or IgD.

Illustrative hinge domains suitable for use in the anti-CD33 VHH CARs described herein include the hinge region derived from the extracellular regions of type 1 membrane proteins such as CD28, CD8α, and CD4, which may be wild-type hinge regions from these molecules or may be altered. In another embodiment, the hinge domain comprises a CD8α hinge region.

The transmembrane (TM) domain of the CAR fuses the extracellular binding portion and intracellular signaling domain and anchors the CAR to the plasma membrane of the immune effector cell. The TM domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source.

Illustrative TM domains may be derived from (i.e., comprise at least the transmembrane region(s) of the alpha, beta, gamma, or delta chain of a T-cell receptor, CD3ε, CD3ζ, CD4, CD5, CD8α, CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD71, CD80, CD86, CD 134, CD137, CD152, CD 154, AMN, and PDCD1.

In one embodiment, an anti-CD33 VHH CAR comprises a TM domain derived from CD8α. In another embodiment, a CAR contemplated herein comprises a TM domain derived from CD8α and a short oligo- or polypeptide linker, preferably between 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in length that links the TM domain and the intracellular signaling domain of the CAR. A glycine-serine linker provides a particularly suitable linker.

In preferred embodiments, an anti-CD33 VHH CAR comprises an intracellular signaling domain that comprises one or more costimulatory signaling domains and a primary signaling domain.

Primary signaling domains that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs.

Illustrative examples of ITAM containing primary signaling domains suitable for use in anti-CD33 VHH CARs contemplated in particular embodiments include those derived from FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d. In particular preferred embodiments, a CAR comprises a CD3ζ primary signaling domain and one or more costimulatory signaling domains. The intracellular primary signaling and costimulatory signaling domains may be linked in any order in tandem to the carboxyl terminus of the transmembrane domain.

In particular embodiments, an anti-CD33 VHH CAR comprises one or more costimulatory signaling domains to enhance the efficacy and expansion of T cells expressing CAR receptors.

Illustrative examples of such costimulatory molecules suitable for use in anti-CD33 VHH CARs contemplated in particular embodiments include, but are not limited to, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DAP10, LAT, SLP76, TRAT1, TNFR2, and ZAP70. In one embodiment, a CAR comprises one or more costimulatory signaling domains selected from the group consisting of CD28, CD137, and CD134, and a CD3ζ primary signaling domain.

In various embodiments, the anti-CD33 VHH CAR comprises: a VHH that binds CD33; a transmembrane domain isolated from a polypeptide selected from the group consisting of: CD4, CD8α, CD154, and PD-1; one or more intracellular costimulatory signaling domains isolated from a polypeptide selected from the group consisting of: CD28, CD134, and CD137; and a signaling domain isolated from a polypeptide selected from the group consisting of: FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d.

In various embodiments, the anti-CD33 VHH CAR comprises: a VHH that binds CD33; a transmembrane domain isolated from a polypeptide selected from the group consisting of: CD4, CD8α, CD154, and PD-1; one or more intracellular costimulatory signaling domains isolated from a polypeptide selected from the group consisting of: CD28, CD134, and CD137; and a signaling domain isolated from a polypeptide selected

from the group consisting of: FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d.

In preferred embodiments, an anti-CD33 VHH CAR comprises a VHH that comprises an amino acid sequence set forth in any one of SEQ ID NOs: 2-21, a CD8α hinge domain, a CD8α transmembrane domain, a 4-1BB costimulatory domain, and a CD3ζ primary signaling domain.

In preferred embodiments, an anti-CD33 VHH CAR comprises a VHH that comprises an amino acid sequence set forth in SEQ ID NO: 10, a CD8α hinge domain, a CD8α transmembrane domain, a 4-1BB costimulatory domain, and a CD3ζ primary signaling domain.

In particular embodiments, an anti-CD33 VHH CAR comprises a sequence set forth in any one of SEQ ID NOs: 62-81.

In particular embodiments, an anti-CD33 VHH CAR comprises a sequence set forth in SEQ ID NO: 70 or SEQ ID NO: 80.

E. POLYPEPTIDES

Various polypeptides are contemplated herein, including, but not limited to, CD33 VHH DARICs, CD33 VHH DARIC binding components, CD33 DARIC signaling components, anti-CD33 VHH CARs, and fragments thereof. In preferred embodiments, a polypeptide comprises an amino acid sequence set forth in any one of SEQ ID NOs: 2-82. "Polypeptide," "peptide" and "protein" are used interchangeably, unless specified to the contrary, and according to conventional meaning, *i.e.*, as a sequence of amino acids. In one embodiment, a "polypeptide" includes fusion polypeptides and other variants. Polypeptides can be prepared using any of a variety of well-known recombinant and/or synthetic techniques. Polypeptides are not limited to a specific length, *e.g.*, they may comprise a full-length protein sequence, a fragment of a full-length protein, or a fusion protein, and may include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. In particular

preferred embodiments, fusion polypeptides, polypeptides, fragments and other variants thereof are prepared, obtained, or isolated from one or more human polypeptides.

An "isolated peptide" or an "isolated polypeptide" and the like, as used herein, refer to *in vitro* isolation and/or purification of a peptide or polypeptide molecule from a cellular environment, and from association with other components of the cell, *i.e.*, it is not significantly associated with *in vivo* substances. In particular embodiments, an isolated polypeptide is a synthetic polypeptide, a semi-synthetic polypeptide, or a polypeptide obtained or derived from a recombinant source.

Polypeptides include "polypeptide variants." Polypeptide variants may differ from a naturally occurring polypeptide in one or more substitutions, deletions, additions and/or insertions. Such variants may be naturally occurring, *e.g.,* a splice variant, or may be synthetically generated, for example, by modifying one or more of the above polypeptide sequences. For example, in particular embodiments, it may be desirable to improve the binding affinity and/or other biological properties of a polypeptide by introducing one or more substitutions, deletions, additions and/or insertions the polypeptide. In particular embodiments, polypeptides include polypeptides having at least about 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 86%, 97%, 98%, or 99% amino acid identity to any of the reference sequences contemplated herein, typically where the variant maintains at least one biological activity of the reference sequence. In particular embodiments, the biological activity is binding affinity. In particular embodiments, the biological activity is cytolytic activity.

Polypeptide variants include biologically active "polypeptide fragments." Illustrative examples of biologically active polypeptide fragments include anti-CD33 VHH domains, intracellular signaling domains, and the like. As used herein, the term "biologically active fragment" or "minimal biologically active fragment" refers to a polypeptide fragment that retains at least 100%, at least 90%, at least 80%, at least 70%, at least 60%, at least 50%, at least 40%, at least 30%, at least 20%, at least 10%, or at least 5% of the naturally occurring polypeptide activity. In certain embodiments, a polypeptide fragment can comprise an amino acid chain at least 5 to about 1700 amino acids long. It will be appreciated that in certain

embodiments, fragments are at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700 or more amino acids long.

In particular embodiments, the polypeptides set forth herein may comprise one or more amino acids denoted as "X" or "Xaa," which are used interchangeably. "X" if present in an amino acid SEQ ID NO, refers to any one or more amino acids. In particular embodiments, SEQ ID NOs denoting a fusion protein comprise a sequence of continuous X residues that cumulatively represent any amino acid sequence. In particular embodiments, "XX" represent any two amino acid combination. In certain embodiments, "XX" represents two serines, SS. In certain embodiments, "XX" represents any two amino acid combination that reduces immunogenicity.

In preferred embodiments, "XX" represents the amino acids KP.

As noted above, polypeptides may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of a reference polypeptide can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. *See*, for example, Kunkel (1985, *Proc. Natl. Acad. Sci. USA*. 82: 488-492), Kunkel *et al.*, (1987, *Methods in Enzymol*, 154: 367-382), U.S. Pat. No. 4,873,192, Watson, J. D. *et al.*, (*Molecular Biology of the Gene*, Fourth Edition, Benjamin/Cummings, Menlo Park, Calif., 1987) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.*, (1978) *Atlas of Protein Sequence and Structure* (*Natl. Biomed. Res. Found.*, Washington, D.C.).

In certain embodiments, a polypeptide variant comprises one or more conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Modifications may be made in the structure of the

polynucleotides and polypeptides contemplated in particular embodiments and still obtain a functional molecule that encodes a variant or derivative polypeptide with desirable characteristics. When it is desired to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, variant polypeptide, one skilled in the art, for example, can change one or more of the codons of the encoding DNA sequence, *e.g.*, according to Table 1.

TABLE 1- Amino Acid Codons

Amino Acids	**One letter code**	**Three letter code**	**Codons**					
Alanine	A	Ala	GCA	GCC	GCG	GCU		
Cysteine	C	Cys	UGC	UGU				
Aspartic acid	D	Asp	GAC	GAU				
Glutamic acid	E	Glu	GAA	GAG				
Phenylalanine	F	Phe	UUC	UUU				
Glycine	G	Gly	GGA	GGC	GGG	GGU		
Histidine	H	His	CAC	CAU				
Isoleucine	I	Iso	AUA	AUC	AUU			
Lysine	K	Lys	AAA	AAG				
Leucine	L	Leu	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	M	Met	AUG					
Asparagine	N	Asn	AAC	AAU				
Proline	P	Pro	CCA	CCC	CCG	CCU		
Glutamine	Q	Gln	CAA	CAG				
Arginine	R	Arg	AGA	AGG	CGA	CGC	CGG	CGU
Serine	S	Ser	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	T	Thr	ACA	ACC	ACG	ACU		
Valine	V	Val	GUA	GUC	GUG	GUU		
Tryptophan	W	Trp	UGG					
Tyrosine	Y	Tyr	UAC	UAU				

Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological activity can be found using computer programs well

known in the art, such as DNASTAR, DNA Strider, Geneious, Mac Vector, or Vector NTI software. Preferably, amino acid changes in the protein variants disclosed herein are conservative amino acid changes, *i.e.,* substitutions of similarly charged or uncharged amino acids. A conservative amino acid change involves substitution of one of a family of amino acids which are related in their side chains. Naturally occurring amino acids are generally divided into four families: acidic (aspartate, glutamate), basic (lysine, arginine, histidine), non-polar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), and uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine) amino acids. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in this art and generally can be made without altering a biological activity of a resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.,* Watson *et al. Molecular Biology of the Gene*, 4th Edition, 1987, The Benjamin/Cummings Pub. Co., p.224).

In one embodiment, where expression of two or more polypeptides is desired, the polynucleotide sequences encoding them can be separated by an IRES sequence or a polynucleotide sequence encoding a ribosomal skip sequence as disclosed elsewhere herein.

Polypeptides contemplated in particular embodiments include fusion polypeptides. In particular embodiments, fusion polypeptides and polynucleotides encoding fusion polypeptides are provided. Fusion polypeptides and fusion proteins refer to a polypeptide having at least two, three, four, five, six, seven, eight, nine, or ten polypeptide segments. In preferred embodiments, a fusion polypeptide comprises one or more CD33 VHH DARIC components. In other preferred embodiments, the fusion polypeptide comprises one or more CD33 VHH DARICs.

In another embodiment, two or more CD33 VHH DARIC components and/or other polypeptides can be expressed as a fusion protein that comprises one or more self-cleaving peptide sequences between the polypeptides as disclosed elsewhere herein.

In particular embodiments, a fusion polypeptide comprises a CD33 DARIC signaling component, a self-cleaving polypeptide sequence or ribosomal skip sequence, and a CD33 VHH DARIC binding component.

In particular embodiments, a fusion polypeptide comprises a CD33 DARIC signaling component, a self-cleaving polypeptide sequence or ribosomal skip sequence, a CD33 VHH DARIC binding component, another self-cleaving polypeptide sequence or ribosomal skip sequence, and another DARIC binding component that is directed against another target antigen.

Fusion polypeptides can comprise one or more polypeptide domains or segments including, but are not limited to signal peptides, cell permeable peptide domains (CPP), binding domains, signaling domains, *etc.*, epitope tags (*e.g.,* maltose binding protein ("MBP"), glutathione S transferase (GST), HIS6, MYC, FLAG, V5, VSV-G, and HA), polypeptide linkers, and polypeptide cleavage signals. Fusion polypeptides are typically linked C-terminus to N-terminus, although they can also be linked C-terminus to C-terminus, N-terminus to N-terminus, or N-terminus to C-terminus. In particular embodiments, the polypeptides of the fusion protein can be in any order. Fusion polypeptides or fusion proteins can also include conservatively modified variants, polymorphic variants, alleles, mutants, subsequences, and interspecies homologs, so long as the desired activity of the fusion polypeptide is preserved. Fusion polypeptides may be produced by chemical synthetic methods or by chemical linkage between the two moieties or may generally be prepared using other standard techniques. Ligated DNA sequences comprising the fusion polypeptide are operably linked to suitable transcriptional or translational control elements as disclosed elsewhere herein.

Fusion polypeptides may optionally comprise one or more linkers that can be used to link the one or more polypeptides or domains within a polypeptide. A peptide linker sequence may be employed to separate any two or more polypeptide components by a distance sufficient to ensure that each polypeptide folds into its appropriate secondary and tertiary structures so as to allow the polypeptide domains to exert their desired functions. Such a peptide linker sequence is incorporated into the fusion polypeptide using standard techniques in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary

structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. In particular embodiments, preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea *et al.*, *Gene* 40:39-46, 1985; Murphy *et al.*, *Proc. Natl. Acad. Sci. USA* 83:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. Linker sequences are not required when a particular fusion polypeptide segment contains non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference. In particular embodiments, preferred linkers are typically flexible amino acid subsequences which are synthesized as part of a recombinant fusion protein. Linker polypeptides can be between 1 and 200 amino acids in length, between 1 and 100 amino acids in length, or between 1 and 50 amino acids in length, including all integer values in between.

Exemplary polypeptide cleavage signals include polypeptide cleavage recognition sites such as protease cleavage sites, nuclease cleavage sites (*e.g.,* rare restriction enzyme recognition sites, self-cleaving ribozyme recognition sites), and self-cleaving viral oligopeptides (*see* deFelipe and Ryan, 2004. *Traffic*, 5(8); 616-26).

Suitable protease cleavages sites and self-cleaving peptides are known to the skilled person (*see, e.g.,* in Ryan *et al.*, 1997. *J. Gener. Virol.* 78, 699-722; Scymczak *et al.* (2004) Nature Biotech. 5, 589-594). Exemplary protease cleavage sites include, but are not limited to the cleavage sites of potyvirus NIa proteases (*e.g.,* tobacco etch virus protease), potyvirus HC proteases, potyvirus P1 (P35) proteases, byovirus NIa proteases, byovirus RNA-2-encoded proteases, aphthovirus L proteases, enterovirus 2A proteases, rhinovirus 2A proteases, picorna 3C proteases, comovirus 24K proteases, nepovirus 24K proteases, RTSV (rice tungro spherical virus) 3C-like protease, PYVF (parsnip yellow fleck virus) 3C-like protease, heparin, thrombin, factor Xa and enterokinase. Due to its high cleavage stringency, TEV (tobacco etch virus) protease cleavage sites are preferred in one embodiment, *e.g.,* EXXYXQ(G/S) (SEQ ID NO: 95), for example, ENLYFQG (SEQ ID NO: 96) and ENLYFQS (SEQ ID NO: 97), wherein X represents any amino acid (cleavage by TEV occurs between Q and G or Q and S).

In particular embodiments, the polypeptide cleavage signal is a viral self-cleaving peptide or ribosomal skipping sequence.

Illustrative examples of ribosomal skipping sequences include, but are not limited to: a 2A or 2A-like site, sequence or domain (Donnelly *et al.*, 2001. *J. Gen. Virol.* 82:1027-1041). In a particular embodiment, the viral 2A peptide is an aphthovirus 2A peptide, a potyvirus 2A peptide, or a cardiovirus 2A peptide.

In one embodiment, the viral 2A peptide is selected from the group consisting of: a foot-and-mouth disease virus (FMDV) 2A peptide, an equine rhinitis A virus (ERAV) 2A peptide, a Thosea asigna virus (TaV) 2A peptide, a porcine teschovirus-1 (PTV-1) 2A peptide, a Theilovirus 2A peptide, and an encephalomyocarditis virus 2A peptide.

Illustrative examples of 2A sites are provided in Table 2.

TABLE 2:

SEQ ID NO: 98	GSGATNFSLLKQAGDVEENPGP
SEQ ID NO: 99	ATNFSLLKQAGDVEENPGP
SEQ ID NO: 100	LLKQAGDVEENPGP
SEQ ID NO: 101	GSGEGRGSLLTCGDVEENPGP
SEQ ID NO: 102	EGRGSLLTCGDVEENPGP
SEQ ID NO: 103	LLTCGDVEENPGP
SEQ ID NO: 104	GSGQCTNYALLKLAGDVESNPGP
SEQ ID NO: 105	QCTNYALLKLAGDVESNPGP
SEQ ID NO: 106	LLKLAGDVESNPGP
SEQ ID NO: 107	GSGVKQTLNFDLLKLAGDVESNPGP
SEQ ID NO: 108	VKQTLNFDLLKLAGDVESNPGP
SEQ ID NO: 109	LLKLAGDVESNPGP
SEQ ID NO: 110	LLNFDLLKLAGDVESNPGP
SEQ ID NO: 111	TLNFDLLKLAGDVESNPGP
SEQ ID NO: 112	LLKLAGDVESNPGP
SEQ ID NO: 113	NFDLLKLAGDVESNPGP
SEQ ID NO: 114	QLLNFDLLKLAGDVESNPGP

SEQ ID NO: 115	APVKQTLNFDLLKLAGDVESNPGP
SEQ ID NO: 116	VTELLYRMKRAETYCPRPLLAIHPTEARHKQKIVAPVKQT
SEQ ID NO: 117	LNFDLLKLAGDVESNPGP
SEQ ID NO: 118	LLAIHPTEARHKQKIVAPVKQTLNFDLLKLAGDVESNPGP
SEQ ID NO: 119	EARHKQKIVAPVKQTLNFDLLKLAGDVESNPGP

In preferred embodiments, a polypeptide or fusion polypeptide comprises one or more CD33 VHH DARIC components, CD33 VHH DARICs, or anti-CD33 VHH CAR.

In preferred embodiments, a fusion polypeptide comprises a CD33 DARIC signaling component and a CD33 VHH DARIC binding component separated by a self-cleaving polypeptide sequence.

In particular embodiments, a fusion polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 32-61. In particular embodiments, a fusion polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 40, 50, or 60.

In particular embodiments, a fusion polypeptide comprises a CD33 DARIC signaling component comprising an FRB T2098L multimerization domain, a CD8α transmembrane domain, a CD137 costimulatory domain and a CD3ζ primary signaling domain; a viral self-cleaving 2A polypeptide; and a CD33 VHH DARIC binding component comprising an anti-CD33 VHH, an FKBP12 multimerization domain polypeptide, and a CD4 transmembrane domain.

In particular embodiments, a fusion polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 32-41. In particular embodiments, a fusion polypeptide comprises the sequence set forth in SEQ ID NO: 40.

In particular embodiments, a fusion polypeptide comprises a CD33 DARIC signaling component comprising an FRB T2098L multimerization domain, a CD8α transmembrane domain, a CD137 costimulatory domain and a CD3ζ primary signaling domain; a viral self-cleaving 2A polypeptide; and an anti-CD33 VHH, a CD4 transmembrane domain, and optionally a CD27, CD28, TNFRS14, TNFRS18, TNFRS25, OX40 or TNFR2 costimulatory domain.

In particular embodiments, a fusion polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 42-51. In particular embodiments, a fusion polypeptide comprises the sequence set forth in SEQ ID NO: 50.

In particular embodiments, a fusion polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 52-61. In particular embodiments, a fusion polypeptide comprises the sequence set forth in SEQ ID NO: 60.

F. POLYNUCLEOTIDES

In particular embodiments, polynucleotides encoding CD33 VHH DARICs, CD33 VHH DARIC binding components, CD33 DARIC signaling components, anti-CD33 VHH CARs and fragments thereof are provided. As used herein, the terms "polynucleotide" or "nucleic acid" refer to deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and DNA/RNA hybrids. Polynucleotides may be single-stranded or double-stranded and either recombinant, synthetic, or isolated. Polynucleotides include, but are not limited to: pre-messenger RNA (pre-mRNA), messenger RNA (mRNA), RNA, synthetic RNA, synthetic mRNA, genomic DNA (gDNA), PCR amplified DNA, complementary DNA (cDNA), synthetic DNA, or recombinant DNA. Polynucleotides refer to a polymeric form of nucleotides of at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, at least 100, at least 200, at least 300, at least 400, at least 500, at least 1000, at least 5000, at least 10000, or at least 15000 or more nucleotides in length, either ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide, as well as all intermediate lengths. It will be readily understood that "intermediate lengths," in this context, means any length between the quoted values, such as 6, 7, 8, 9, *etc.*, 101, 102, 103, *etc.*; 151, 152, 153, *etc.*; 201, 202, 203, *etc.* In particular embodiments, polynucleotides or variants have at least or about 50%, 55%, 60%, 65%, 70%, 71%, 72%, 73%, 74%, 75%,76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%,85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a reference sequence.

As used herein, "isolated polynucleotide" refers to a polynucleotide that has been purified from the sequences which flank it in a naturally-occurring state, *e.g.*, a DNA fragment that has been removed from the sequences that are normally adjacent to the

fragment. In particular embodiments, an "isolated polynucleotide" also refers to a complementary DNA (cDNA), a recombinant DNA, or other polynucleotide that does not exist in nature and that has been made by the hand of man. In particular embodiments, an isolated polynucleotide is a synthetic polynucleotide, a semi-synthetic polynucleotide, or a polynucleotide obtained or derived from a recombinant source.

In various embodiments, a polynucleotide comprises an mRNA encoding a polypeptide contemplated herein. In certain embodiments, the mRNA comprises a cap, one or more nucleotides, and a poly(A) tail.

In particular embodiments, polynucleotides encoding one or more CD33 VHH DARIC components may be codon-optimized. As used herein, the term "codon-optimized" refers to substituting codons in a polynucleotide encoding a polypeptide in order to increase the expression, stability and/or activity of the polypeptide. Factors that influence codon optimization include, but are not limited to one or more of: (i) variation of codon biases between two or more organisms or genes or synthetically constructed bias tables, (ii) variation in the degree of codon bias within an organism, gene, or set of genes, (iii) systematic variation of codons including context, (iv) variation of codons according to their decoding tRNAs, (v) variation of codons according to GC %, either overall or in one position of the triplet, (vi) variation in degree of similarity to a reference sequence for example a naturally occurring sequence, (vii) variation in the codon frequency cutoff, (viii) structural properties of mRNAs transcribed from the DNA sequence, (ix) prior knowledge about the function of the DNA sequences upon which design of the codon substitution set is to be based, (x) systematic variation of codon sets for each amino acid, and/or (xi) isolated removal of spurious translation initiation sites.

As used herein the term "nucleotide" refers to a heterocyclic nitrogenous base in N-glycosidic linkage with a phosphorylated sugar. Nucleotides are understood to include natural bases, and a wide variety of art-recognized modified bases. Such bases are generally located at the 1′ position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. In ribonucleic acid (RNA), the sugar is a ribose, and in deoxyribonucleic acid (DNA) the sugar is a deoxyribose, *i.e.*, a sugar lacking a hydroxyl group that is present in ribose.

Illustrative examples of polynucleotides include, but are not limited to, polynucleotides encoding polypeptides set forth in SEQ ID NOs: 2-82.

In various illustrative embodiments, polynucleotides contemplated herein include, but are not limited to polynucleotides encoding one or more CD33 VHH DARIC components, CD33 VHH DARIC receptors, anti-CD33 VHH CARs, fusion polypeptides, and expression vectors, viral vectors, and transfer plasmids comprising polynucleotides contemplated herein.

As used herein, the terms "polynucleotide variant" and "variant" and the like refer to polynucleotides displaying substantial sequence identity with a reference polynucleotide sequence or polynucleotides that hybridize with a reference sequence under stringent conditions that are defined hereinafter. These terms also encompass polynucleotides that are distinguished from a reference polynucleotide by the addition, deletion, substitution, or modification of at least one nucleotide. Accordingly, the terms "polynucleotide variant" and "variant" include polynucleotides in which one or more nucleotides have been added or deleted, or modified, or replaced with different nucleotides. In this regard, it is well understood in the art that certain alterations inclusive of mutations, additions, deletions and substitutions can be made to a reference polynucleotide whereby the altered polynucleotide retains the biological function or activity of the reference polynucleotide.

The recitations "sequence identity" or, for example, comprising a "sequence 50% identical to," as used herein, refer to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" may be calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.,* A, T, C, G, I) or the identical amino acid residue (*e.g.,* Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. Included are nucleotides and polypeptides having at least about 50%, 55%, 60%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%,

91%, 92%, 93%, 94%, 95%, 86%, 97%, 98%, or 99% sequence identity to any of the reference sequences described herein.

The polynucleotides contemplated herein, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters and/or enhancers, untranslated regions (UTRs), signal sequences, Kozak sequences, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, internal ribosomal entry sites (IRES), recombinase recognition sites (*e.g.,* LoxP, FRT, and Att sites), termination codons, transcriptional termination signals, and polynucleotides encoding self-cleaving polypeptides, epitope tags, as disclosed elsewhere herein or as known in the art, such that their overall length may vary considerably. It is therefore contemplated that a polynucleotide fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

Polynucleotides can be prepared, manipulated, expressed and/or delivered using any of a variety of well-established techniques known and available in the art. In order to express a desired polypeptide, a nucleotide sequence encoding the polypeptide, can be inserted into appropriate vector.

Illustrative examples of vectors include, but are not limited to plasmid, autonomously replicating sequences, and transposable elements, *e.g.,* Sleeping Beauty, PiggyBac.

Additional Illustrative examples of vectors include, without limitation, plasmids, phagemids, cosmids, artificial chromosomes such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC), bacteriophages such as lambda phage or M13 phage, and animal viruses.

Illustrative examples of viruses useful as vectors include, without limitation, retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (*e.g.,* herpes simplex virus), poxvirus, baculovirus, papillomavirus, and papovavirus (*e.g.,* SV40).

Illustrative examples of expression vectors include, but are not limited to, pClneo vectors (Promega) for expression in mammalian cells; pLenti4/V5-DEST™, pLenti6/V5-DEST™, and pLenti6.2/V5-GW/lacZ (Invitrogen) for lentivirus-mediated gene transfer and expression in mammalian cells. In particular embodiments, coding sequences of polypeptides

disclosed herein can be ligated into such expression vectors for the expression of the polypeptides in mammalian cells.

In particular embodiments, the vector is an episomal vector or a vector that is maintained extrachromosomally. As used herein, the term "episomal" refers to a vector that is able to replicate without integration into host's chromosomal DNA and without gradual loss from a dividing host cell also meaning that said vector replicates extrachromosomally or episomally.

"Expression control sequences," "control elements," or "regulatory sequences" present in an expression vector are those non-translated regions of the vector including an origin of replication, selection cassettes, promoters, enhancers, translation initiation signals (Shine Dalgarno sequence or Kozak sequence) introns, a polyadenylation sequence, 5' and 3' untranslated regions, all of which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including ubiquitous promoters and inducible promoters may be used.

In particular embodiments, a polynucleotide comprises a vector, including but not limited to expression vectors and viral vectors. A vector may comprise one or more exogenous, endogenous, or heterologous control sequences such as promoters and/or enhancers. An "endogenous control sequence" is one which is naturally linked with a given gene in the genome. An "exogenous control sequence" is one which is placed in juxtaposition to a gene by means of genetic manipulation (*i.e.*, molecular biological techniques) such that transcription of that gene is directed by the linked enhancer/promoter. A "heterologous control sequence" is an exogenous sequence that is from a different species than the cell being genetically manipulated. A "synthetic" control sequence may comprise elements of one more endogenous and/or exogenous sequences, and/or sequences determined *in vitro* or *in silico* that provide optimal promoter and/or enhancer activity for the particular therapy.

The term "promoter" as used herein refers to a recognition site of a polynucleotide (DNA or RNA) to which an RNA polymerase binds. An RNA polymerase initiates and transcribes polynucleotides operably linked to the promoter. In particular embodiments, promoters operative in mammalian cells comprise an AT-rich region located approximately

25 to 30 bases upstream from the site where transcription is initiated and/or another sequence found 70 to 80 bases upstream from the start of transcription, a CNCAAT region where N may be any nucleotide.

The term "enhancer" refers to a segment of DNA which contains sequences capable of providing enhanced transcription and in some instances can function independent of their orientation relative to another control sequence. An enhancer can function cooperatively or additively with promoters and/or other enhancer elements. The term "promoter/enhancer" refers to a segment of DNA which contains sequences capable of providing both promoter and enhancer functions.

The term "operably linked", refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. In one embodiment, the term refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, and/or enhancer) and a second polynucleotide sequence, e.g., a polynucleotide-of-interest, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

As used herein, the term "constitutive expression control sequence" refers to a promoter, enhancer, or promoter/enhancer that continually or continuously allows for transcription of an operably linked sequence. A constitutive expression control sequence may be a "ubiquitous" promoter, enhancer, or promoter/enhancer that allows expression in a wide variety of cell and tissue types or a "cell specific," "cell type specific," "cell lineage specific," or "tissue specific" promoter, enhancer, or promoter/enhancer that allows expression in a restricted variety of cell and tissue types, respectively.

Illustrative ubiquitous expression control sequences suitable for use in particular embodiments include, but are not limited to, a cytomegalovirus (CMV) immediate early promoter, a viral simian virus 40 (SV40) (*e.g.,* early or late), a Moloney murine leukemia virus (MoMLV) LTR promoter, a Rous sarcoma virus (RSV) LTR, a herpes simplex virus (HSV) (thymidine kinase) promoter, H5, P7.5, and P11 promoters from vaccinia virus, an elongation factor 1-alpha (EF1a) promoter, early growth response 1 (EGR1), ferritin H (FerH), ferritin L (FerL), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), eukaryotic translation initiation factor 4A1 (EIF4A1), heat shock 70kDa protein 5

(HSPA5), heat shock protein 90kDa beta, member 1 (HSP90B1), heat shock protein 70kDa (HSP70), β-kinesin (β-KIN), the human ROSA 26 locus (Irions *et al.*, *Nature Biotechnology* 25, 1477 - 1482 (2007)), a Ubiquitin C promoter (UBC), a phosphoglycerate kinase-1 (PGK) promoter, a cytomegalovirus enhancer/chicken β-actin (CAG) promoter, a β-actin promoter and a myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted (MND) U3 promoter (Haas *et al. Journal of Virology.* 2003;77(17): 9439-9450).

In one embodiment, a vector comprises an MNDU3 promoter.

In one embodiment, a vector comprises an EF1a promoter comprising the first intron of the human EF1a gene.

In one embodiment, a vector comprises an EF1a promoter that lacks the first intron of the human EF1a gene.

In a particular embodiment, it may be desirable to use a cell, cell type, cell lineage or tissue specific expression control sequence to achieve cell type specific, lineage specific, or tissue specific expression of a desired polynucleotide sequence (e.g., to express a particular nucleic acid encoding a polypeptide in only a subset of cell types, cell lineages, or tissues or during specific stages of development).

In a particular embodiment, it may be desirable to express a polynucleotide a T cell specific promoter.

As used herein, "conditional expression" may refer to any type of conditional expression including, but not limited to, inducible expression; repressible expression; expression in cells or tissues having a particular physiological, biological, or disease state, *etc.* This definition is not intended to exclude cell type or tissue specific expression. Certain embodiments provide conditional expression of a polynucleotide-of-interest, *e.g.*, expression is controlled by subjecting a cell, tissue, organism, *etc.*, to a treatment or condition that causes the polynucleotide to be expressed or that causes an increase or decrease in expression of the polynucleotide encoded by the polynucleotide-of-interest.

Illustrative examples of inducible promoters/systems include, but are not limited to, steroid-inducible promoters such as promoters for genes encoding glucocorticoid or estrogen receptors (inducible by treatment with the corresponding hormone),

metallothionine promoter (inducible by treatment with various heavy metals), MX-1 promoter (inducible by interferon), the "GeneSwitch" mifepristone-regulatable system (Sirin *et al.*, 2003, *Gene*, 323:67), the cumate inducible gene switch (WO 2002/088346), tetracycline-dependent regulatory systems, *etc.* Inducer agents include, but are not limited to glucocorticoids, estrogens, mifepristone (RU486), metals, interferons, small molecules, cumate, tetracycline, doxycycline, and variants thereof.

As used herein, an "internal ribosome entry site" or "IRES" refers to an element that promotes direct internal ribosome entry to the initiation codon, such as ATG, of a cistron (a protein encoding region), thereby leading to the cap-independent translation of the gene. *See, e.g.,* Jackson *et al.*, 1990. *Trends Biochem Sci* 15(12):477-83) and Jackson and Kaminski. 1995. *RNA* 1(10):985-1000. Examples of IRES generally employed by those of skill in the art include those described in U.S. Pat. No. 6,692,736. Further examples of "IRES" known in the art include, but are not limited to IRES obtainable from picornavirus (Jackson *et al.*, 1990) and IRES obtainable from viral or cellular mRNA sources, such as for example, immunoglobulin heavy-chain binding protein (BiP), the vascular endothelial growth factor (VEGF) (Huez *et al.* 1998. *Mol. Cell. Biol.* 18(11):6178-6190), the fibroblast growth factor 2 (FGF-2), and insulin-like growth factor (IGFII), the translational initiation factor eIF4G and yeast transcription factors TFIID and HAP4, the encephelomycarditis virus (EMCV) which is commercially available from Novagen (Duke *et al.*, 1992. J. Virol 66(3):1602-9) and the VEGF IRES (Huez *et al.*, 1998. Mol Cell Biol 18(11):6178-90). IRES have also been reported in viral genomes of Picornaviridae, Dicistroviridae and Flaviviridae species and in HCV, Friend murine leukemia virus (FrMLV) and Moloney murine leukemia virus (MoMLV).

In one embodiment, the IRES used in polynucleotides contemplated herein is an EMCV IRES.

In particular embodiments, the polynucleotides a consensus Kozak sequence. As used herein, the term "Kozak sequence" refers to a short nucleotide sequence that greatly facilitates the initial binding of mRNA to the small subunit of the ribosome and increases translation. The consensus Kozak sequence is (GCC)RCCATGG (SEQ ID NO: 83), where R is a purine (A or G) (Kozak, 1986. *Cell.* 44(2):283-92, and Kozak, 1987. *Nucleic Acids Res.* 15(20):8125-48).

Elements directing the efficient termination and polyadenylation of the heterologous nucleic acid transcripts increases heterologous gene expression. Transcription termination signals are generally found downstream of the polyadenylation signal. In particular embodiments, vectors comprise a polyadenylation sequence 3′ of a polynucleotide encoding a polypeptide to be expressed. The term "polyA site" or "polyA sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript by RNA polymerase II. Polyadenylation sequences can promote mRNA stability by addition of a polyA tail to the 3′ end of the coding sequence and thus, contribute to increased translational efficiency. Cleavage and polyadenylation are directed by a poly(A) sequence in the RNA. The core poly(A) sequence for mammalian pre-mRNAs has two recognition elements flanking a cleavage-polyadenylation site. Typically, an almost invariant AAUAAA hexamer lies 20-50 nucleotides upstream of a more variable element rich in U or GU residues. Cleavage of the nascent transcript occurs between these two elements and is coupled to the addition of up to 250 adenosines to the 5' cleavage product. In particular embodiments, the core poly(A) sequence is an ideal polyA sequence (*e.g.*, AATAAA, ATTAAA, AGTAAA). In particular embodiments, the poly(A) sequence is an SV40 polyA sequence, a bovine growth hormone polyA sequence (BGHpA), a rabbit β-globin polyA sequence (rβgpA), variants thereof, or another suitable heterologous or endogenous polyA sequence known in the art. In particular embodiments, the poly(A) sequence is synthetic.

In particular embodiments, polynucleotides encoding one or more polypeptides, or fusion polypeptides may be introduced into immune effector cells, *e.g.,* T cells, by both non-viral and viral methods. In particular embodiments, delivery of one or more polynucleotides may be provided by the same method or by different methods, and/or by the same vector or by different vectors.

The term "vector" is used herein to refer to a nucleic acid molecule capable transferring or transporting another nucleic acid molecule. The transferred nucleic acid is generally linked to, *e.g.,* inserted into, the vector nucleic acid molecule. A vector may include sequences that direct autonomous replication in a cell, or may include sequences sufficient to allow integration into host cell DNA. In particular embodiments, non-viral vectors are used to deliver one or more polynucleotides contemplated herein to a T cell.

Illustrative examples of non-viral vectors include, but are not limited to plasmids (*e.g.,* DNA plasmids or RNA plasmids), transposons, cosmids, and bacterial artificial chromosomes.

Illustrative methods of non-viral delivery of polynucleotides contemplated in particular embodiments include, but are not limited to: electroporation, sonoporation, lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, nanoparticles, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, DEAE-dextran-mediated transfer, gene gun, and heat-shock.

Illustrative examples of polynucleotide delivery systems suitable for use in particular embodiments contemplated in particular embodiments include, but are not limited to those provided by Amaxa Biosystems, Maxcyte, Inc., BTX Molecular Delivery Systems, and Copernicus Therapeutics Inc. Lipofection reagents are sold commercially (*e.g.,* Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides have been described in the literature. See *e.g.,* Liu *et al.* (2003) *Gene Therapy.* 10:180–187; and Balazs *et al.* (2011) *Journal of Drug Delivery.* 2011:1-12. Antibody-targeted, bacterially derived, non-living nanocell-based delivery is also contemplated in particular embodiments.

Viral vectors comprising polynucleotides contemplated in particular embodiments can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (*e.g.,* intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (*e.g.,* mobilized peripheral blood, lymphocytes, bone marrow aspirates, tissue biopsy, *etc.*) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient.

In one embodiment, viral vectors comprising polynucleotides contemplated herein are administered directly to an organism for transduction of cells *in vivo*. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells including, but not limited to, injection, infusion, topical application and electroporation. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and,

although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

Illustrative examples of viral vector systems suitable for use in particular embodiments contemplated in particular embodiments include, but are not limited to, adeno-associated virus (AAV), retrovirus, herpes simplex virus, adenovirus, and vaccinia virus vectors.

In various embodiments, one or more polynucleotides encoding one or more CD33 VHH DARIC components and/or other polypeptides contemplated herein are introduced into an immune effector cell, *e.g.,* T cell, by transducing the cell with a recombinant adeno-associated virus (rAAV), comprising the one or more polynucleotides.

AAV is a small (~26 nm) replication-defective, primarily episomal, non-enveloped virus. AAV can infect both dividing and non-dividing cells and may incorporate its genome into that of the host cell. Recombinant AAV (rAAV) are typically composed of, at a minimum, a transgene and its regulatory sequences, and 5' and 3' AAV inverted terminal repeats (ITRs). The ITR sequences are about 145 bp in length. In particular embodiments, the rAAV comprises ITRs and capsid sequences isolated from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10.

In some embodiments, a chimeric rAAV is used the ITR sequences are isolated from one AAV serotype and the capsid sequences are isolated from a different AAV serotype. For example, a rAAV with ITR sequences derived from AAV2 and capsid sequences derived from AAV6 is referred to as AAV2/AAV6. In particular embodiments, the rAAV vector may comprise ITRs from AAV2, and capsid proteins from any one of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10. In a preferred embodiment, the rAAV comprises ITR sequences derived from AAV2 and capsid sequences derived from AAV6. In a preferred embodiment, the rAAV comprises ITR sequences derived from AAV2 and capsid sequences derived from AAV2.

In some embodiments, engineering and selection methods can be applied to AAV capsids to make them more likely to transduce cells of interest.

Construction of rAAV vectors, production, and purification thereof have been disclosed, *e.g.,* in U.S. Patent Nos. 9,169,494; 9,169,492; 9,012,224; 8,889,641; 8,809,058; and 8,784,799, each of which is incorporated by reference herein, in its entirety.

In various embodiments, one or more polynucleotides encoding one or more CD33 VHH DARIC components and/or other polypeptides contemplated herein are introduced into an immune effector cell, *e.g.,* T cell, by transducing the cell with a retrovirus, *e.g.,* lentivirus, comprising the one or more polynucleotides.

As used herein, the term "retrovirus" refers to an RNA virus that reverse transcribes its genomic RNA into a linear double-stranded DNA copy and subsequently covalently integrates its genomic DNA into a host genome. Illustrative retroviruses suitable for use in particular embodiments, include, but are not limited to: Moloney murine leukemia virus (M-MuLV), Moloney murine sarcoma virus (MoMSV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), feline leukemia virus (FLV), spumavirus, Friend murine leukemia virus, Murine Stem Cell Virus (MSCV) and Rous Sarcoma Virus (RSV)) and lentivirus.

As used herein, the term "lentivirus" refers to a group (or genus) of complex retroviruses. Illustrative lentiviruses include, but are not limited to, HIV (human immunodeficiency virus; including HIV type 1, and HIV type 2); visna-maedi virus (VMV) virus; the caprine arthritis-encephalitis virus (CAEV); equine infectious anemia virus (EIAV); feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV). In one embodiment, HIV based vector backbones (*i.e.,* HIV cis-acting sequence elements) are preferred.

In various embodiments, a lentiviral vector contemplated herein comprises one or more LTRs, and one or more, or all, of the following accessory elements: a cPPT/FLAP, a Psi (Ψ) packaging signal, an export element, poly (A) sequences, and may optionally comprise a WPRE or HPRE, an insulator element, a selectable marker, and a cell suicide gene, as discussed elsewhere herein.

In particular embodiments, lentiviral vectors contemplated herein may be integrative or non-integrating or integration defective lentivirus. As used herein, the term "integration defective lentivirus" or "IDLV" refers to a lentivirus having an integrase that lacks the capacity

to integrate the viral genome into the genome of the host cells. Integration-incompetent viral vectors have been described in patent application WO 2006/010834, which is herein incorporated by reference in its entirety.

Illustrative mutations in the HIV-1 pol gene suitable to reduce integrase activity include, but are not limited to: H12N, H12C, H16C, H16V, S81 R, D41A, K42A, H51A, Q53C, D55V, D64E, D64V, E69A, K71A, E85A, E87A, D116N, D1161, D116A, N120G, N1201, N120E, E152G, E152A, D35E, K156E, K156A, E157A, K159E, K159A, K160A, R166A, D167A, E170A, H171A, K173A, K186Q, K186T, K188T, E198A, R199c, R199T, R199A, D202A, K211A, Q214L, Q216L, Q221 L, W235F, W235E, K236S, K236A, K246A, G247W, D253A, R262A, R263A and K264H.

The term "long terminal repeat (LTR)" refers to domains of base pairs located at the ends of retroviral DNAs which, in their natural sequence context, are direct repeats and contain U3, R and U5 regions.

As used herein, the term "FLAP element" or "cPPT/FLAP" refers to a nucleic acid whose sequence includes the central polypurine tract and central termination sequences (cPPT and CTS) of a retrovirus, *e.g.,* HIV-1 or HIV-2. Suitable FLAP elements are described in U.S. Pat. No. 6,682,907 and in Zennou, *et al.,* 2000, *Cell*, 101:173.

As used herein, the term "packaging signal" or "packaging sequence" refers to psi [Ψ] sequences located within the retroviral genome which are required for insertion of the viral RNA into the viral capsid or particle, *see e.g.,* Clever *et al.,* 1995. *J. of Virology*, Vol. 69, No. 4; pp. 2101–2109.

The term "export element" refers to a cis-acting post-transcriptional regulatory element which regulates the transport of an RNA transcript from the nucleus to the cytoplasm of a cell. Examples of RNA export elements include, but are not limited to, the human immunodeficiency virus (HIV) rev response element (RRE) (*see e.g.,* Cullen *et al.*, 1991. *J. Virol.* 65: 1053; and Cullen *et al.,* 1991. *Cell* 58: 423), and the hepatitis B virus post-transcriptional regulatory element (HPRE).

In particular embodiments, expression of heterologous sequences in viral vectors is increased by incorporating posttranscriptional regulatory elements, efficient polyadenylation sites, and optionally, transcription termination signals into the vectors. A variety of

posttranscriptional regulatory elements can increase expression of a heterologous nucleic acid at the protein, *e.g.,* woodchuck hepatitis virus posttranscriptional regulatory element (WPRE; Zufferey *et al.*, 1999, *J. Virol.*, 73:2886); the posttranscriptional regulatory element present in hepatitis B virus (HPRE) (Huang *et al., Mol. Cell. Biol.*, 5:3864); and the like (Liu *et al.,* 1995, *Genes Dev.*, 9:1766).

Lentiviral vectors preferably contain several safety enhancements as a result of modifying the LTRs. "Self-inactivating" (SIN) vectors refers to replication-defective vectors, *e.g.,* retroviral or lentiviral vectors, in which the right (3') LTR enhancer-promoter region, known as the U3 region, has been modified (*e.g.,* by deletion or substitution) to prevent viral transcription beyond the first round of viral replication. Self-inactivation is preferably achieved through in the introduction of a deletion in the U3 region of the 3' LTR of the vector DNA, *i.e.*, the DNA used to produce the vector RNA. Thus, during reverse transcription, this deletion is transferred to the 5' LTR of the proviral DNA. In particular embodiments, it is desirable to eliminate enough of the U3 sequence to greatly diminish or abolish altogether the transcriptional activity of the LTR, thereby greatly diminishing or abolishing the production of full-length vector RNA in transduced cells. In the case of HIV based lentivectors, it has been discovered that such vectors tolerate significant U3 deletions, including the removal of the LTR TATA box (*e.g.*, deletions from -418 to -18), without significant reductions in vector titers.

An additional safety enhancement is provided by replacing the U3 region of the 5' LTR with a heterologous promoter to drive transcription of the viral genome during production of viral particles. Examples of heterologous promoters which can be used include, for example, viral simian virus 40 (SV40) (*e.g.,* early or late), cytomegalovirus (CMV) (*e.g.,* immediate early), Moloney murine leukemia virus (MoMLV), Rous sarcoma virus (RSV), and herpes simplex virus (HSV) (thymidine kinase) promoters.

The terms "pseudotype" or "pseudotyping" as used herein, refer to a virus whose viral envelope proteins have been substituted with those of another virus possessing preferable characteristics. For example, HIV can be pseudotyped with vesicular stomatitis virus G-protein (VSV-G) envelope proteins, which allows HIV to infect a wider range of

cells because HIV envelope proteins (encoded by the env gene) normally target the virus to $CD4^+$ presenting cells.

In certain embodiments, lentiviral vectors are produced according to known methods. *See e.g.,* Kutner *et al., BMC Biotechnol.* 2009;9:10. doi: 10.1186/1472-6750-9-10; Kutner *et al.* *Nat. Protoc.* 2009;4(4):495–505. doi: 10.1038/nprot.2009.22.

According to certain specific embodiments contemplated herein, most or all of the viral vector backbone sequences are derived from a lentivirus, *e.g.,* HIV-1. However, it is to be understood that many different sources of retroviral and/or lentiviral sequences can be used, or combined and numerous substitutions and alterations in certain of the lentiviral sequences may be accommodated without impairing the ability of a transfer vector to perform the functions described herein. Moreover, a variety of lentiviral vectors are known in the art, *see* Naldini *et al.,* (1996a, 1996b, and 1998); Zufferey *et al.*, (1997); Dull *et al.*, 1998, U.S. Pat. Nos. 6,013,516; and 5,994,136, many of which may be adapted to produce a viral vector or transfer plasmid contemplated herein.

In various embodiments, one or more polynucleotides encoding one or more CD33 VHH DARIC components and/or other polypeptides contemplated herein are introduced into an immune effector cell, by transducing the cell with an adenovirus comprising the one or more polynucleotides.

Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and high levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Most adenovirus vectors are engineered such that a transgene replaces the Ad E1a, E1b, and/or E3 genes; subsequently the replication defective vector is propagated in human 293 cells that supply deleted gene function in trans. Ad vectors can transduce multiple types of tissues *in vivo*, including non-dividing, differentiated cells such as those found in liver, kidney and muscle. Conventional Ad vectors have a large carrying capacity.

Generation and propagation of the current adenovirus vectors, which are replication deficient, may utilize a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus

genome (Jones & Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham & Prevec, 1991). Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus & Horwitz, 1992; Graham & Prevec, 1992). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.,* 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz & Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993). An example of the use of an Ad vector in a clinical trial involved polynucleotide therapy for antitumor immunization with intramuscular injection (Sterman *et al., Hum. Gene Ther.* 7:1083-9 (1998)).

In various embodiments, one or more polynucleotides encoding one or more CD33 VHH DARIC components and/or other polypeptides contemplated herein are introduced into an immune effector cell by transducing the cell with a herpes simplex virus, *e.g.,* HSV-1, HSV-2, comprising the one or more polynucleotides.

The mature HSV virion consists of an enveloped icosahedral capsid with a viral genome consisting of a linear double-stranded DNA molecule that is 152 kb. In one embodiment, the HSV based viral vector is deficient in one or more essential or non-essential HSV genes. In one embodiment, the HSV based viral vector is replication deficient. Most replication deficient HSV vectors contain a deletion to remove one or more intermediate-early, early, or late HSV genes to prevent replication. For example, the HSV vector may be deficient in an immediate early gene selected from the group consisting of: ICP4, ICP22, ICP27, ICP47, and a combination thereof. Advantages of the HSV vector are its ability to enter a latent stage that can result in long-term DNA expression and its large viral DNA genome that can accommodate exogenous DNA inserts of up to 25 kb. HSV-based vectors are described in, for example, U.S. Pat. Nos. 5,837,532, 5,846,782, and 5,804,413, and International Patent Applications WO 91/02788, WO 96/04394, WO 98/15637, and WO 99/06583, each of which are incorporated by reference herein in its entirety.

G. GENETICALLY MODIFIED CELLS

In various embodiments, cells are modified to express a CD33 VHH DARIC, one or more CD33 VHH DARIC components, an anti-CD33 VHH CAR, and/or fusion proteins contemplated herein, for use in the treatment of cancer. Cells may be non-genetically modified to express one or more of the polypeptides contemplated herein, or in particular preferred embodiments, cells may be genetically modified to express one or more of the polypeptides contemplated herein. As used herein, the term "genetically engineered" or "genetically modified" refers to the addition of extra genetic material in the form of DNA or RNA into the total genetic material in a cell. The terms, "genetically modified cells," "modified cells," and "redirected cells," are used interchangeably in particular embodiments.

In particular embodiments, one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein are introduced and expressed in immune effector cells to improve the efficacy of the immune effector cells.

An "immune effector cell," is any cell of the immune system that has one or more effector functions (*e.g.*, cytotoxic cell killing activity, secretion of cytokines, induction of ADCC and/or CDC). The illustrative immune effector cells contemplated herein are T lymphocytes, including but not limited to cytotoxic T cells (CTLs; $CD8^+$ T cells), TILs, and helper T cells (HTLs; $CD4^+$ T cells). In a particular embodiment, the cells comprise αβ T cells. In a particular embodiment, the cells comprise γδ T cells. In one embodiment, immune effector cells include natural killer (NK) cells. In one embodiment, immune effector cells include natural killer T (NKT) cells. Immune effector cells can be autologous/autogeneic ("self") or non-autologous ("non-self," *e.g.,* allogeneic, syngeneic or xenogeneic).

"Autologous," as used herein, refers to cells from the same subject. "Allogeneic," as used herein, refers to cells of the same species that differ genetically to the cell in comparison. "Syngeneic," as used herein, refers to cells of a different subject that are genetically identical to the cell in comparison. "Xenogeneic," as used herein, refers to cells of a different species to the cell in comparison. In preferred embodiments, the cells are human autologous immune effector cells.

Illustrative immune effector cells suitable for introducing one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein include T lymphocytes. The terms "T cell" or "T lymphocyte" are art-recognized and are intended to include thymocytes, immature T lymphocytes, mature T lymphocytes, resting T lymphocytes, or activated T lymphocytes. A T cell can be a T helper (Th) cell, for example a T helper 1 (Th1) or a T helper 2 (Th2) cell. The T cell can be a helper T cell (HTL; $CD4^+$ T cell), a cytotoxic T cell (CTL; $CD8^+$ T cell), $CD4^+CD8^+$ T cell, $CD4^-CD8^-$ T cell, or any other subset of T cells. In particular embodiments, the T cell expresses a T cell receptor. T cell receptors comprise two subunits, an alpha chain and a beta chain subunit (αβTCR), or a gamma chain and a delta chain subunit (γδTCR), each of which is a unique protein produced by recombination event in each T cell's genome. In particular embodiments, a T cell is an αβTCR T cell (an αβ T cell). In particular embodiments, a T cell is a γδTCR T cell (a γδ T cell). Other illustrative populations of T cells suitable for use in particular embodiments include naïve T cells and memory T cells.

As would be understood by the skilled person, other cells may also be used as immune effector cells comprising one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein. In particular embodiments, immune effector cells also include NK cells, NKT cells, neutrophils, and macrophages. Immune effector cells also include progenitors of effector cells wherein such progenitor cells can be induced to differentiate into immune effector cells *in vivo* or *in vitro*. Thus, in particular embodiments, immune effector cells include progenitors of immune effectors cells such as hematopoietic stem cells (HSCs) contained within the $CD34^+$ population of cells derived from cord blood, bone marrow or mobilized peripheral blood which upon administration in a subject differentiate into mature immune effector cells, or which can be induced *in vitro* to differentiate into mature immune effector cells.

The term, "$CD34^+$ cell," as used herein refers to a cell expressing the CD34 protein on its cell surface. "CD34," as used herein refers to a cell surface glycoprotein (*e.g.*, sialomucin protein) that often acts as a cell-cell adhesion factor and is involved in T cell entrance into lymph nodes. The $CD34^+$ cell population contains hematopoietic stem cells (HSC), which upon administration to a patient differentiate and contribute to all

hematopoietic lineages, including T cells, NK cells, NKT cells, neutrophils and cells of the monocyte/macrophage lineage.

Methods for making the immune effector cells which express one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein are provided in particular embodiments. In one embodiment, the method comprises transfecting or transducing immune effector cells isolated from an individual such that the immune effector cells with one or more nucleic acids and/or vectors, *e.g.*, a lentiviral vector comprising a nucleic acid encoding one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein. In one embodiment, the method comprises transfecting or transducing immune effector cells isolated from an individual such that the immune effector cells express one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein. In certain embodiments, the immune effector cells are isolated from an individual and genetically modified without further manipulation *in vitro*. Such cells can then be directly re-administered into the individual. In further embodiments, the immune effector cells are first activated and stimulated to proliferate *in vitro* prior to being genetically modified. In this regard, the immune effector cells may be cultured before and/or after being genetically modified.

In particular embodiments, prior to *in vitro* manipulation or genetic modification of the immune effector cells described herein, the source of cells is obtained from a subject. In particular embodiments, the modified immune effector cells comprise T cells.

T cells can be obtained from a number of sources including, but not limited to, peripheral blood mononuclear cells, bone marrow, lymph nodes tissue, cord blood, thymus issue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled person, such as sedimentation, *e.g.*, FICOLL™ separation.

In other embodiments, an isolated or purified population of T cells is used. In some embodiments, after isolation of PBMC, both cytotoxic and helper T lymphocytes can be sorted into naïve, memory, and effector T cell subpopulations either before or after activation, expansion, and/or genetic modification.

In one embodiment, an isolated or purified population of T cells expresses one or more of the markers including, but not limited to a $CD3^+$, $CD4^+$, $CD8^+$, or a combination thereof.

In certain embodiments, the T cells are isolated from an individual and first activated and stimulated to proliferate *in vitro* prior to being modified to express one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR.

In order to achieve sufficient therapeutic doses of T cell compositions, T cells are often subjected to one or more rounds of stimulation, activation and/or expansion. In particular embodiments, T cells can be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; and 6,867,041, each of which is incorporated herein by reference in its entirety. In particular embodiments, T cells are activated and expanded for about 6 hours, about 12 hours, about 18 hours or about 24 hours prior to introduction of vectors or polynucleotides encoding one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein.

H. COMPOSITIONS AND FORMULATIONS

The compositions contemplated herein may comprise one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR, polynucleotides encoding one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR, vectors comprising same, genetically modified immune effector cells, bridging factors, *etc.* Compositions include, but are not limited to, pharmaceutical compositions. A "pharmaceutical composition" refers to a composition formulated in pharmaceutically-acceptable or physiologically-acceptable solutions for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy. It will also be understood that, if desired, the compositions may be administered in combination with other agents as well, such as, *e.g.*, cytokines, growth factors, hormones, small molecules, chemotherapeutics, pro-drugs, drugs, antibodies, or other various pharmaceutically-active agents. There is virtually no limit to other components that may also be included in the compositions,

provided that the additional agents do not adversely affect the ability of the composition to deliver the intended therapy.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The term "pharmaceutically acceptable carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the bridging factors, polypeptides, polynucleotides, vectors comprising same, or genetically modified immune effector cells are administered. Illustrative examples of pharmaceutical carriers can be sterile liquids, such as cell culture media, 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. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients in particular embodiments, 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. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

In one embodiment, a composition comprising a pharmaceutically acceptable carrier is suitable for administration to a subject. In particular embodiments, a composition comprising a carrier is suitable for parenteral administration, *e.g.*, intravascular (intravenous or intraarterial), intraperitoneal or intramuscular administration. In particular embodiments, a composition comprising a pharmaceutically acceptable carrier is suitable for intraventricular, intraspinal, or intrathecal administration. Pharmaceutically acceptable carriers include sterile aqueous solutions, cell culture media, or dispersions. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the bridging factors, polypeptides, polynucleotides, vectors comprising

same, or genetically modified immune effector cells, use thereof in the pharmaceutical compositions is contemplated.

In particular embodiments, compositions contemplated herein comprise genetically modified T cells and a pharmaceutically acceptable carrier. A composition comprising a cell-based composition contemplated herein can be administered separately by enteral or parenteral administration methods or in combination with other suitable compounds to effect the desired treatment goals.

In particular embodiments, compositions contemplated herein comprise a bridging factor and a pharmaceutically acceptable carrier.

The pharmaceutically acceptable carrier must be of sufficiently high purity and of sufficiently low toxicity to render it suitable for administration to the human subject being treated. It further should maintain or increase the stability of the composition. The pharmaceutically acceptable carrier can be liquid or solid and is selected, with the planned manner of administration in mind, to provide for the desired bulk, consistency, *etc.*, when combined with other components of the composition. For example, the pharmaceutically acceptable carrier can be, without limitation, a binding agent (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*), a filler (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates, calcium hydrogen phosphate, *etc.*), a lubricant (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*), a disintegrant (*e.g.*, starch, sodium starch glycolate, *etc.*), or a wetting agent (*e.g.*, sodium lauryl sulfate, *etc.*). Other suitable pharmaceutically acceptable carriers for the compositions contemplated herein include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatins, amyloses, magnesium stearates, talcs, silicic acids, viscous paraffins, hydroxymethylcelluloses, polyvinylpyrrolidones and the like.

Such carrier solutions also can contain buffers, diluents and other suitable additives. The term “buffer” as used herein refers to a solution or liquid whose chemical makeup neutralizes acids or bases without a significant change in pH. Examples of buffers contemplated herein include, but are not limited to, Dulbecco´s phosphate buffered

saline (PBS), Ringer´s solution, 5% dextrose in water (D5W), normal/physiologic saline (0.9% NaCl).

The pharmaceutically acceptable carriers may be present in amounts sufficient to maintain a pH of the composition of about 7. Alternatively, the composition has a pH in a range from about 6.8 to about 7.4, *e.g.*, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, and 7.4. In still another embodiment, the composition has a pH of about 7.4.

Compositions contemplated herein may comprise a nontoxic pharmaceutically acceptable medium. The compositions may be a suspension. The term "suspension" as used herein refers to non-adherent conditions in which cells are not attached to a solid support. For example, cells maintained as a suspension may be stirred or agitated and are not adhered to a support, such as a culture dish.

In particular embodiments, compositions contemplated herein are formulated in a suspension, where the modified T cells are dispersed within an acceptable liquid medium or solution, *e.g.*, saline or serum-free medium, in an intravenous (IV) bag or the like. Acceptable diluents include, but are not limited to water, PlasmaLyte, Ringer´s solution, isotonic sodium chloride (saline) solution, serum-free cell culture medium, and medium suitable for cryogenic storage, *e.g.*, Cryostor® medium.

In certain embodiments, a pharmaceutically acceptable carrier is substantially free of natural proteins of human or animal origin, and suitable for storing a composition comprising a population of modified T cells. The therapeutic composition is intended to be administered into a human patient, and thus is substantially free of cell culture components such as bovine serum albumin, horse serum, and fetal bovine serum.

In some embodiments, compositions are formulated in a pharmaceutically acceptable cell culture medium. Such compositions are suitable for administration to human subjects. In particular embodiments, the pharmaceutically acceptable cell culture medium is a serum free medium.

Serum-free medium has several advantages over serum containing medium, including a simplified and better-defined composition, a reduced degree of contaminants, elimination of a potential source of infectious agents, and lower cost. In various embodiments, the serum-free medium is animal-free, and may optionally be protein-free.

Optionally, the medium may contain biopharmaceutically acceptable recombinant proteins. "Animal-free" medium refers to medium wherein the components are derived from non-animal sources. Recombinant proteins replace native animal proteins in animal-free medium and the nutrients are obtained from synthetic, plant or microbial sources. "Protein-free" medium, in contrast, is defined as substantially free of protein.

Illustrative examples of serum-free media used in particular compositions includes, but is not limited to, QBSF-60 (Quality Biological, Inc.), StemPro-34 (Life Technologies), and X-VIVO 10.

In one embodiment, the compositions comprising modified T cells are formulated in PlasmaLyte.

In various embodiments, compositions comprising modified T cells are formulated in a cryopreservation medium. For example, cryopreservation media with cryopreservation agents may be used to maintain a high cell viability outcome post-thaw. Illustrative examples of cryopreservation media used in particular compositions includes, but is not limited to, CryoStor CS10, CryoStor CS5, and CryoStor CS2.

In one embodiment, the compositions are formulated in a solution comprising 50:50 PlasmaLyte A to CryoStor CS10.

In particular embodiments, the composition is substantially free of mycoplasma, endotoxin, and microbial contamination. By "substantially free" with respect to endotoxin is meant that there is less endotoxin per dose of cells than is allowed by the FDA for a biologic, which is a total endotoxin of 5 EU/kg body weight per day, which for an average 70 kg person is 350 EU per total dose of cells. In particular embodiments, compositions contemplated herein contain about 0.5 EU/ml to about 5.0 EU/ml, or about 0.5 EU/ml, 1.0 EU/ml, 1.5 EU/ml, 2.0 EU/ml, 2.5 EU/ml, 3.0 EU/ml, 3.5 EU/ml, 4.0 EU/ml, 4.5 EU/ml, or 5.0 EU/ml.

In particular embodiments, formulation of pharmaceutically-acceptable carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, enteral and parenteral, *e.g.*, intravascular, intravenous, intrarterial, intraosseously, intraventricular, intracerebral, intracranial,

intraspinal, intrathecal, and intramedullary administration and formulation. It would be understood by the skilled artisan that particular embodiments contemplated herein may comprise other formulations, such as those that are well known in the pharmaceutical art, and are described, for example, in *Remington: The Science and Practice of Pharmacy*, volume I and volume II. 22nd Edition. Edited by Loyd V. Allen Jr. Philadelphia, PA: Pharmaceutical Press; 2012, which is incorporated by reference herein, in its entirety.

In particular embodiments, compositions comprise an amount of immune effector cells comprising a polynucleotide encoding one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein. In particular embodiments, compositions comprise an amount of immune effector cells that express one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein. As used herein, the term "amount" refers to "an amount effective" or "an effective amount" of cells comprising one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein, *etc.*, to achieve a beneficial or desired prophylactic or therapeutic result in the presence of a bridging factor, including clinical results.

A "prophylactically effective amount" refers to an amount of cells comprising one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein, *etc.*, effective to achieve the desired prophylactic result in the presence of a bridging factor. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount is less than the therapeutically effective amount.

A "therapeutically effective amount" refers to an amount of cells comprising one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein that is effective to "treat" a subject (*e.g.,* a patient) in the presence of a bridging factor. When a therapeutic amount is indicated, the precise amount of the compositions to be administered, cells, bridging factor, etc, can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject).

It can generally be stated that a pharmaceutical composition comprising the immune effector cells described herein may be administered at a dosage of 10^2 to

10^{10} cells/kg body weight, preferably 10^5 to 10^6 cells/kg body weight, including all integer values within those ranges. The number of cells will depend upon the ultimate use for which the composition is intended as will the type of cells included therein. For uses provided herein, the cells are generally in a volume of a liter or less, can be 500 mls or less, even 250 mls or 100 mls or less. Hence the density of the desired cells is typically greater than 10^6 cells/ml and generally is greater than 10^7 cells/ml, generally 10^8 cells/ml or greater. The clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, or 10^{12} cells. In some embodiments, particularly since all the infused cells will be redirected to a particular target antigen, lower numbers of cells, in the range of 10^6/kilogram (10^6-10^{11} per patient) may be administered.

If desired, the treatment may also include administration of mitogens (*e.g.,* PHA) or lymphokines, cytokines, and/or chemokines (*e.g.,* IFN-γ, IL-2, IL-12, TNF-alpha, IL-18, and TNF-beta, GM-CSF, IL-4, IL-13, Flt3-L, RANTES, MIP1α, *etc.*) as described herein to enhance induction of the immune response.

Generally, compositions comprising the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised. In particular, compositions contemplated herein are used in the treatment of cancer. In particular embodiments, the immune effector cells may be administered either alone, or as a pharmaceutical composition in combination with carriers, diluents, excipients, and/or with other components such as IL-2 or other cytokines or cell populations.

In particular embodiments, pharmaceutical compositions comprise an amount of genetically modified T cells, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients.

In particular embodiments, pharmaceutical compositions comprise an amount of bridging factor, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients.

In a particular embodiment, compositions comprise an effective amount of immune effector cells comprising one or more CD33 VHH DARIC components or an anti-CD33

VHH CAR contemplated herein, alone or in combination with a bridging factor and/or one or more therapeutic agents, such as radiation therapy, chemotherapy, transplantation, immunotherapy, hormone therapy, photodynamic therapy, *etc.* The compositions may also be administered in combination with antibiotics. Such therapeutic agents may be accepted in the art as a standard treatment for a particular disease state as described herein, such as a particular cancer. Exemplary therapeutic agents contemplated include cytokines, growth factors, steroids, NSAIDs, DMARDs, anti-inflammatories, chemotherapeutics, radiotherapeutics, therapeutic antibodies, or other active and ancillary agents.

In a particular embodiment, a composition comprising an effective amount of immune effector cells comprising a polynucleotide encoding one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein is administered to a subject, and a composition comprising an effective amount of a bridging factor is administered to the subject, before, during, in combination with or subsequently to the cellular composition, and optionally repetitively administered to the subject.

In certain embodiments, compositions comprising immune effector cells comprising a polynucleotide encoding one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein may be administered in conjunction with any number of anti-inflammatory agents, chemotherapeutic agents, or therapeutic antibodies, and the like.

I. THERAPEUTIC METHODS

Immune effector cells modified to express a polynucleotide encoding one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR contemplated herein provide improved methods of adoptive immunotherapy for use in the prevention, treatment, and amelioration of, or for preventing, treating, or ameliorating at least one symptom associated with an immune disorder, *e.g.,* cancer.

Immune effector cells comprising a CD33 DARIC signaling component, a CD33 VHH DARIC binding component, or an anti-CD33 VHH CAR provide improved methods of adoptive immunotherapy for use in the prevention, treatment, and amelioration of, or for

preventing, treating, or ameliorating at least one symptom associated with an immune disorder, *e.g.,* cancer.

In particular embodiments, immune effector cells modified to express a CD33 VHH DARIC provide improved methods of adoptive immunotherapy to fine-tune the safety and efficacy of a cytotoxic response against target cells, *e.g.,* tumor cells, expressing target antigens while decreasing the risk of on-target antigen, off-target cell cytotoxicity (recognizing the target antigen on a normal, non-target cell).

In particular embodiments, a method of preventing, treating, or ameliorating at least one symptom of a cancer comprises administering the subject an effective amount of modified immune effector cells or T cells comprising one or more components of a CD33 VHH DARIC or an anti-CD33 VHH CAR to redirect the cells to a target cell. The genetically modified cells are a more efficacious and safe cellular immunotherapy by virtue of transducing a chemically regulatable immunostimulatory signal.

In particular embodiments, one or more immune effector cells, *e.g.,* T cells, are modified to express both a CD33 VHH DARIC binding component and a CD33 DARIC signaling component. In this case, the modified cells are administered to a subject in need thereof and home to the target cells via the interaction of the CD33 VHH binding component expressed on the immune effector cell and CD33 expressed on the target cell. A bridging factor is administered to the subject before the modified cells, about the same time as the modified cells, or after the modified cells have been administered to the subject. In the presence of the bridging factor, a ternary complex forms between the CD33 VHH DARIC binding component, the bridging factor, and the CD33 DARIC signaling component. Upon formation of the ternary complex, the CD33 VHH DARIC transduces an immunostimulatory signal to the immune effector cell that in turn, elicits a cytotoxic response from the immune effector cell against the target cell.

In particular embodiments, one or more immune effector cells, *e.g.,* T cells, are modified to express a CD33 DARIC signaling component. In this case, the modified cells are administered to a subject in need thereof. A CD33 VHH DARIC binding component can be administered to the subject before the modified cells, about the same time as the modified cells, or after the modified cells have been administered to the subject. In addition, the CD33 VHH

DARIC binding component can be administered to the subject in a preformed complex with the bridging factor; at the same time as the bridging factor, but in a separate composition; or at a different time than the bridging factor. The CD33 VHH binding component binds CD33 expressed on the target cell, either in the presence or absence of the bridging factor. In the presence of the bridging factor, a ternary complex forms between the CD33 VHH DARIC binding component, the bridging factor, and the CD33 DARIC signaling component. Upon formation of the ternary complex, the CD33 VHH DARIC transduces an immunostimulatory signal to the immune effector cell that in turn, elicits a cytotoxic response from the immune effector cell against the target cell.

In particular embodiments, one or more immune effector cells, *e.g.,* T cells, are modified to express the CD33 DARIC signaling component. In this case, the modified cells are administered to a subject in need thereof. A CD33 VHH DARIC binding component can be administered to the subject before the modified cells, about the same time as the modified cells, or after the modified cells have been administered to the subject. In addition, the CD33 VHH DARIC binding component can be administered to the subject in a preformed complex with the bridging factor; at the same time as the bridging factor, but in a separate composition; or at a different time than the bridging factor. The CD33 binding component binds the target antigen expressed on the target cell, either in the presence or absence of the bridging factor. In the presence of the bridging factor, a ternary complex forms between the CD33 VHH DARIC binding component, the bridging factor, and the CD33 DARIC signaling component. Upon formation of the ternary complex, the CD33 VHH DARIC transduces an immunostimulatory signal to the immune effector cell that in turn, elicits a cytotoxic response from the immune effector cell against the target cell. In particular embodiments, CD33 VHH DARIC activation can be induced in cases where remission or regression is incomplete and the condition relapses or becomes refractory to treatment.

In particular preferred embodiments, the specificity of a primary T cell is redirected to tumor or cancer cells that express CD33 by genetically modifying a T cell, *e.g.,* a primary T cell, with one or more CD33 VHH DARIC components.

In particular preferred embodiments, the specificity of a primary T cell is redirected to tumor or cancer cells that express CD33 by genetically modifying a T cell, *e.g.,* a primary T

cell, with an engineered antigen receptor directed to the target antigen and one or more CD33 VHH DARIC components.

In particular embodiments, the modified immune effector cells contemplated herein are used in the treatment of solid tumors or cancers.

In particular embodiments, the modified immune effector cells contemplated herein are used in the treatment of solid tumors or cancers including, but not limited to: adrenal cancer, adrenocortical carcinoma, anal cancer, appendix cancer, astrocytoma, atypical teratoid/rhabdoid tumor, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, brain/CNS cancer, breast cancer, bronchial tumors, cardiac tumors, cervical cancer, cholangiocarcinoma, chondrosarcoma, chordoma, colon cancer, colorectal cancer, craniopharyngioma, ductal carcinoma in situ (DCIS) endometrial cancer, ependymoma, esophageal cancer, esthesioneuroblastoma, Ewing's sarcoma, extracranial germ cell tumor, extragonadal germ cell tumor, eye cancer, fallopian tube cancer, fibrous histiosarcoma, fibrosarcoma, gallbladder cancer, gastric cancer, gastrointestinal carcinoid tumors, gastrointestinal stromal tumor (GIST), germ cell tumors, glioma, glioblastoma, head and neck cancer, hemangioblastoma, hepatocellular cancer, hypopharyngeal cancer, intraocular melanoma, kaposi sarcoma, kidney cancer, laryngeal cancer, leiomyosarcoma, lip cancer, liposarcoma, liver cancer, lung cancer, non-small cell lung cancer, lung carcinoid tumor, malignant mesothelioma, medullary carcinoma, medulloblastoma, menangioma, melanoma, Merkel cell carcinoma, midline tract carcinoma, mouth cancer, myxosarcoma, myelodysplastic syndrome, myeloproliferative neoplasms, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, oligodendroglioma, oral cancer, oral cavity cancer, oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic islet cell tumors, papillary carcinoma, paraganglioma, parathyroid cancer, penile cancer, pharyngeal cancer, pheochromocytoma, pinealoma, pituitary tumor, pleuropulmonary blastoma, primary peritoneal cancer, prostate cancer, rectal cancer, retinoblastoma, renal cell carcinoma, renal pelvis and ureter cancer, rhabdomyosarcoma, salivary gland cancer, sebaceous gland carcinoma, skin cancer, soft tissue sarcoma, squamous cell carcinoma, small cell lung cancer, small intestine cancer, stomach cancer, sweat gland carcinoma, synovioma, testicular cancer,

throat cancer, thymus cancer, thyroid cancer, urethral cancer, uterine cancer, uterine sarcoma, vaginal cancer, vascular cancer, vulvar cancer, and Wilms Tumor.

In particular embodiments, the modified immune effector cells contemplated herein are used in the treatment of solid tumors or cancers including, without limitation, non-small cell lung carcinoma, head and neck squamous cell carcinoma, colorectal cancer, pancreatic cancer, breast cancer, thyroid cancer, bladder cancer, cervical cancer, esophageal cancer, ovarian cancer, gastric cancer endometrial cancer, gliomas, glioblastomas, and oligodendroglioma.

In particular embodiments, the modified immune effector cells contemplated herein are used in the treatment of solid tumors or cancers including, without limitation, non-small-cell lung cancer, metastatic colorectal cancer, glioblastoma, head and neck cancer, pancreatic cancer, and breast cancer.

In particular embodiments, the modified immune effector cells contemplated herein are used in the treatment of glioblastoma.

In particular embodiments, the modified immune effector cells contemplated herein are used in the treatment of liquid cancers or hematological cancers.

In particular embodiments, the modified immune effector cells contemplated herein are used in the treatment of B-cell malignancies, including but not limited to: leukemias, lymphomas, and multiple myeloma.

In particular embodiments, the modified immune effector cells contemplated herein are used in the treatment of liquid cancers including, but not limited to leukemias, lymphomas, and multiple myelomas: acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia, hairy cell leukemia (HCL), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML), chronic myelomonocytic leukemia (CMML) and polycythemia vera, Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma, Burkitt lymphoma, small lymphocytic lymphoma (SLL), diffuse large B-cell lymphoma, follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, mantle cell lymphoma, marginal zone lymphoma, mycosis fungoides, anaplastic large cell lymphoma, Sézary syndrome, precursor T-lymphoblastic lymphoma, multiple myeloma, overt multiple myeloma, smoldering multiple

myeloma, plasma cell leukemia, non-secretory myeloma, IgD myeloma, osteosclerotic myeloma, solitary plasmacytoma of bone, and extramedullary plasmacytoma.

In particular embodiments, the modified immune effector cells contemplated herein are used in the treatment of acute myeloid leukemia (AML).

Preferred cells for use in the methods contemplated herein include autologous/autogeneic ("self") cells, preferably hematopoietic cells, more preferably T cells, and more preferably immune effector cells.

In particular embodiments, a method comprises administering a therapeutically effective amount of modified immune effector cells that express one or more CD33 VHH DARIC components, to a patient in need thereof, and also administering a bridging factor to the subject. In certain embodiments, the cells are used in the treatment of patients at risk for developing an immune disorder. Thus, particular embodiments comprise the treatment or prevention or amelioration of at least one symptom of an immune disorder, *e.g.,* cancer, comprising administering to a subject in need thereof, a therapeutically effective amount of the modified immune effector cells contemplated herein and a bridging factor.

In particular embodiments, a method comprises administering a therapeutically effective amount of modified immune effector cells that express an anti-CD33 VHH CAR to a patient in need thereof. In certain embodiments, the cells are used in the treatment of patients at risk for developing an immune disorder. Thus, particular embodiments comprise the treatment or prevention or amelioration of at least one symptom of an immune disorder, *e.g.,* cancer, comprising administering to a subject in need thereof, a therapeutically effective amount of the modified immune effector cells contemplated herein and a bridging factor.

In particular embodiments, a method comprises administering a therapeutically effective amount of modified immune effector cells that express a CD33 DARIC signaling component or a composition comprising the same, to a patient in need thereof, and also administering a CD33 VHH DARIC binding component and a bridging factor, optionally wherein the CD33 VHH DARIC binding component is bound to the bridging factor prior to administration, to the subject. In certain embodiments, the cells are used in the treatment of patients at risk for developing an immune disorder. Thus, particular embodiments comprise the treatment or prevention or amelioration of at least one symptom of an immune disorder,

e.g., cancer, comprising administering to a subject in need thereof, a therapeutically effective amount of the modified immune effector cells that express a CD33 DARIC signaling component and optionally and engineered antigen receptor or another DARIC binding component, a CD33 VHH DARIC binding component, and a bridging factor.

The quantity and frequency of administration of modified immune effector cells, CD33 DARIC VHH binding components, and/or bridging factor will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages and dose schedules may be determined by clinical trials.

In one illustrative embodiment, the effective amount of modified immune effector cells provided to a subject is at least 2 x 10^6 cells/kg, at least 3 x 10^6 cells/kg, at least 4 x 10^6 cells/kg, at least 5 x 10^6 cells/kg, at least 6 x 10^6 cells/kg, at least 7 x 10^6 cells/kg, at least 8 x 10^6 cells/kg, at least 9 x 10^6 cells/kg, or at least 10 x 10^6 cells/kg, or more cells/kg, including all intervening doses of cells.

In another illustrative embodiment, the effective amount of modified immune effector cells provided to a subject is about 2 x 10^6 cells/kg, about 3 x 10^6 cells/kg, about 4 x 10^6 cells/kg, about 5 x 10^6 cells/kg, about 6 x 10^6 cells/kg, about 7 x 10^6 cells/kg, about 8 x 10^6 cells/kg, about 9 x 10^6 cells/kg, or about 10 x 10^6 cells/kg, or more cells/kg, including all intervening doses of cells.

In another illustrative embodiment, the effective amount of modified immune effector cells provided to a subject is from about 2 x 10^6 cells/kg to about 10 x 10^6 cells/kg, about 3 x 10^6 cells/kg to about 10 x 10^6 cells/kg, about 4 x 10^6 cells/kg to about 10 x 10^6 cells/kg, about 5 x 10^6 cells/kg to about 10 x 10^6 cells/kg, 2 x 10^6 cells/kg to about 6 x 10^6 cells/kg, 2 x 10^6 cells/kg to about 7 x 10^6 cells/kg, 2 x 10^6 cells/kg to about 8 x 10^6 cells/kg, 3 x 10^6 cells/kg to about 6 x 10^6 cells/kg, 3 x 10^6 cells/kg to about 7 x 10^6 cells/kg, 3 x 10^6 cells/kg to about 8 x 10^6 cells/kg, 4 x 10^6 cells/kg to about 6 x 10^6 cells/kg, 4 x 10^6 cells/kg to about 7 x 10^6 cells/kg, 4 x 10^6 cells/kg to about 8 x 10^6 cells/kg, 5 x 10^6 cells/kg to about 6 x 10^6 cells/kg, 5 x 10^6 cells/kg to about 7 x 10^6 cells/kg, 5 x 10^6 cells/kg to about 8 x 10^6 cells/kg, or 6 x 10^6 cells/kg to about 8 x 10^6 cells/kg, including all intervening doses of cells.

One of ordinary skill in the art would recognize that multiple administrations of the compositions contemplated in particular embodiments may be required to effect the desired therapy. For example, a composition may be administered 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more times over a span of 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 1 year, 2 years, 5, years, 10 years, or more. Modified immune effector cells, CD33 VHH DARIC components, and bridging factor may be administered in the same or different compositions; in one or more compositions at the same time; or more than one composition at different times. Modified immune effector cells, CD33 VHH DARIC components, and bridging factor may be administered through the same route of administration or different routes.

In certain embodiments, it may be desirable to administer activated T cells to a subject and then subsequently redraw blood (or have an apheresis performed), activate T cells therefrom, and reinfuse the patient with these activated and expanded T cells. This process can be carried out multiple times every few weeks. In certain embodiments, T cells can be activated from blood draws of from 10cc to 400cc. In certain embodiments, T cells are activated from blood draws of 20cc, 30cc, 40cc, 50cc, 60cc, 70cc, 80cc, 90cc, 100cc, 150cc, 200cc, 250cc, 300cc, 350cc, or 400cc or more. Not to be bound by theory, using this multiple blood draw/multiple reinfusion protocol may serve to select out certain populations of T cells.

In one embodiment, a method of treating a subject diagnosed with a cancer, comprises removing immune effector cells from the subject, modifying the immune effector cells by introducing one or more vectors encoding one or more CD33 VHH DARIC components into the cell and producing a population of modified immune effector cells, and administering the population of modified immune effector cells to the same subject. In a preferred embodiment, the immune effector cells comprise T cells.

In one embodiment, a method of treating a subject diagnosed with a cancer, comprises removing immune effector cells from the subject, modifying the immune effector cells by introducing one or more vectors encoding an anti-CD33 VHH CAR into the cell and producing a population of modified immune effector cells, and administering the population of modified immune effector cells to the same subject. In a preferred embodiment, the immune effector cells comprise T cells.

The methods for administering the cell compositions contemplated in particular embodiments include any method which is effective to result in reintroduction of *ex vivo* modified immune effector cells or reintroduction of modified progenitors of immune effector cells that upon introduction into a subject differentiate into mature immune effector cells. One method comprises modifying peripheral blood T cells *ex vivo* by introducing one or more vectors encoding one or more CD33 VHH DARIC components or an anti-CD33 VHH CAR into the cell and returning the transduced cells into the subject.

All publications, patent applications, and issued patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or issued patent were specifically and individually indicated to be incorporated by reference.

Although the foregoing embodiments have been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings contemplated herein that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified in particular embodiments to yield essentially similar results.

EXAMPLES

EXAMPLE 1

CD33 VHH DARIC T CELLS EXHIBIT ANTI-TUMOR RESPONSES

Anti-CD33 VHH DARIC binding and signaling components were designed, constructed, and verified. CD33 specific VHH DARIC lentiviral vectors were constructed comprising an MNDU3 promoter operably linked to a polynucleotide encoding: a DARIC signaling component (CD8α-signal peptide, an FRB variant (T82L), a CD8α transmembrane domain, an intracellular 4-1BB costimulatory domain, and a CD3 zeta signaling domain); a P2A sequence; and a DARIC binding component (an Igκ-signal peptide, a CD33 specific VHH binding domain (camelid or humanized), a G4S linker, an FKBP12 domain, and a CD4 derived transmembrane domain with a truncated intracellular domain (Figure 1B). *See, e.g.,* SEQ ID NOs: 32-41. T cells transduced with anti-CD33 DARIC lentiviral vectors express the membrane bound polypeptides shown in Figure 1A. An anti-CD33 scFv CAR or DARIC design was used as a control.

T cells from three donors were each transduced with LVVs encoding different CD33 specific VHH DARICs, an anti-CD33 scFv DARIC, or anti-CD33 scFv CAR and expanded for 10 days. Untransduced T cells, T cells transduced with anti-CD33 scFv control constructs or anti-CD33 VHH DARIC T cells were stained with recombinant CD33-Fc reagent. Only control CAR and DARIC T cells were positively stained with the CD33-Fc staining (Figure 2A, bottom panel, Figure 2B). However, most of the anti-CD33 VHH DARIC T cells, but not control CAR or DARIC T cells, stained positively when analyzed with a monoclonal antibody specific for the VHH domain (Figure 2A, top panel). Both control CAR and DARIC T cells and anti-CD33 VHH DARIC T cells had a similar T cell phenotype, as determined, in part, by CD62L and CD45RA staining (Figure 3A and Figure 3B).

Untransduced T cells, T cells transduced with anti-CD33 scFv control constructs or anti-CD33 VHH DARIC T cells were co-cultured with $CD33^+$ THP-1 cells at an E:T ratio of 1:1 in the presence or absence of AP21967 for 24 hours. Anti-CD33 scFv CAR control

cells had strong cytokine production both in the presence or absence of rapalog. Anti-CD33 scFv DARIC T cells and anti-CD33 VHH DARIC T cells exhibited a robust cytokine response only when cultured with THP-1 cell in the presence of AP21967 (Figure 4A and Figure 4B). Minimal cytokine production was detected in untransduced controls.

Additionally, the specificity of VHH9 and VHH10 DARICs were assessed against full-length CD33 (CD33M) as well as a splice variant that expresses a shorter, truncated CD33 (CD33m). Human 293T cells were electroporated with mRNA encoding either full length CD33M or the splice variant CD33m (Figure 4C). DARIC T cells were co-cultured with the modified 293T cells an E:T ratio of 1:1 in the presence or absence of AP21967 for 24 hours and assessed for activation as measured by cytokine secretion (Figure 4C). The VHH9 DARIC T cells exhibited robust cytokine response in response to either CD33M or CD33m 293T cells whereas the VHH10 DARIC T cells were only activated in the presence of CD33M.

EXAMPLE 2

CD33 VHH DARIC T CELLS SPECIFICALLY RESPOND TO CD33 ANTIGEN

Anti-CD33 VHH DARIC T cells were generated as described in Example 1. T cells from three donors were each transduced with LVVs encoding different anti-CD33 specific VHH DARICs and expanded for 10 days. Controls included untransduced (UTD) T cells and T cells transduced with a CD33 CAR. The AML cell line MV4-11 normally expresses CD33. MV4-11 cells were engineered to knock out the CD33 gene (CD33-KO cells). The resultant CD33-KO cell line lacked CD33 expression on the cell surface. Figure 5A. Anti-CD33 VHH DARIC T cells were co-cultured with MV4-11 cells or CD33-KO cells at an E:T ratio of 1:1 in the presence or absence of dimerizing drug for 24 hours. Anti-CD33 VHH DARIC T cells produced cytokine in the presence of MV4-11 target cells but not in the presence of CD33-KO cells. Figure 5B.

The CD33-KO cell line was modified to express a CD33m splice variant (CD33-KO-C2). MV4-11 cells and CD33-KO-C2 cells were co-cultured with UTD cells, anti-CD33 CAR T cells, or anti-CD33 VHH DARIC T cells in the presence or absence of

dimerizing drug and cytokine production analyzed after 24 hr. Anti-CD33 VHH9 DARIC recognized both normal CD33 and the CD33m splice variant and produced cytokine when co-cultured with MV4-11 cells or CD33-KO-C2 cells (Figure 5C). Anti-CD33 CAR T cells or anti-CD33VHH2 DARIC control T cells were only active against MV4-11 target cells.

EXAMPLE 3

CD33 VHH DARIC T CELLS ARE NOT INHIBITED BY SOLUBLE CD33 PROTEIN

Anti-CD33 VHH DARIC T cells were generated as described in Example 1. T cells from three donors were each transduced with LVVs encoding different anti-CD33 specific VHH DARICs and expanded for 10 days. Anti-CD33 VHH DARIC T cells were co-cultured with $CD33^{+}$ THP-1 cells at an E:T ratio of 1:1 in the presence or absence of rapamycin for 24 hours. Various amounts of recombinant CD33-Fc protein were added during the co-culture period. Anti-CD33 VHH DARIC T cells exhibited a robust cytokine response in the presence of rapamycin in the presence and absence of recombinant soluble CD33 protein. Figure 6.

EXAMPLE 4

CD33 VHH DARIC T CELLS RESPOND TO LOW LEVELS OF CD33 ANTIGEN

Anti-CD33 VHH DARIC T cells were generated as described in Example 1. T cells from three donors were each transduced with LVVs encoding different anti-CD33 specific VHH DARICs and expanded for 10 days. Anti-CD33 VHH DARIC T cells were co-cultured with AP21967 dimerizing agent and $CD33^{neg}$ 293T cells transfected with different amounts of mRNA encoding CD33. Supernatant was collected after 24hr and cytokine production was analyzed. Anti-CD33 VHH DARIC T cells showed dose-dependent increases in IFNγ production following co-culture with CD33-transfected target cells, even at very low mRNA concentrations (Figure 7).

EXAMPLE 5

CD33 VHH DARIC T CELLS CONTROL TUMOR GROWTH *IN VIVO*

Anti-CD33 VHH DARIC T cells were generated as described in Example 1. CD33 expressing tumors were established in immunodeficient NSG mice by inoculating the mice with HL60 AML tumor cells expressing a luciferase reporter. Tumor growth was monitored by luminescence. After 10 days, mice were administered 10 x 10^6 anti-CD33 VHH DARIC T cell co-administered with vehicle or rapamycin. Controls included mice that received rapamycin alone or untransduced (UTD) T cells. Tumor growth was comparable among both treatment and control groups. Figure 8A. Mice treated with anti-CD33 VHH DARIC T cells and rapamycin showed increased tumor control compared to mice treated with UTD T cells and rapamycin. Figure 8B.

In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

1. A non-natural cell comprising:

(a) a first polypeptide comprising: an FRB multimerization domain polypeptide or variant thereof; a CD8α transmembrane domain or a CD4 transmembrane domain; a CD137 co-stimulatory domain; and/or a CD3ζ primary signaling domain; and

(b) a second polypeptide comprising: an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21; an FKBP multimerization domain polypeptide or variant thereof; and a CD4 transmembrane domain or a CD8α transmembrane domain;

wherein a bridging factor promotes the formation of a polypeptide complex on the non-natural cell surface with the bridging factor associated with and disposed between the multimerization domains of the first and second polypeptides.

2. The non-natural cell of claim 1, wherein the FKBP multimerization domain is FKBP12.

3. The non-natural cell of claim 1 or claim 2, wherein the FRB polypeptide is FRB T2098L.

4. The non-natural cell of any one of claims 1 to 3, wherein the bridging factor is selected from the group consisting of: AP21967, sirolimus, everolimus, novolimus, pimecrolimus, ridaforolimus, tacrolimus, temsirolimus, umirolimus, and zotarolimus.

5. The non-natural cell of any one of claims 1 to 4, wherein the first polypeptide comprises a signal peptide, a CD8α transmembrane domain; a CD137 co-stimulatory domain; and a CD3ζ primary signaling domain.

6. The non-natural cell of any one of claims 1 to 5, wherein the second polypeptide comprises a signal peptide and a CD4 transmembrane domain.

7. The non-natural cell of any one of claims 1 to 6, wherein the second polypeptide comprises a costimulatory domain.

8. The non-natural cell of claim 7, wherein the costimulatory domain of the second polypeptide is selected from a costimulatory molecule selected from the group consisting of: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNFRS14, TNFRS18, TNRFS25, and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

9. The non-natural cell of claim 7 or claim 8, wherein the costimulatory domain of the second polypeptide is a costimulatory domain isolated from OX40 or TNFR2.

10. The non-natural cell of any one of claims 1 to 9, wherein the second polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 22-31.

11. A non-natural cell comprising a polypeptide complex that comprises:

(a) a first polypeptide comprising: an FRB multimerization domain polypeptide or variant thereof; a CD8α transmembrane domain or a CD4 transmembrane domain; a CD137 co-stimulatory domain; and/or a CD3ζ primary signaling domain;

(b) a second polypeptide comprising: an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21; an FKBP multimerization domain

polypeptide or variant thereof; and a CD4 transmembrane domain or a CD8α transmembrane domain; and

(c) a bridging factor associated with and disposed between the multimerization domains of the first and second polypeptides.

12. The non-natural cell of claim 11, wherein the FKBP multimerization domain is FKBP12.

13. The non-natural cell of claim 11 or claim 12, wherein the FRB polypeptide is FRB T2098L.

14. The non-natural cell of any one of claims 11 to 13, wherein the bridging factor is selected from the group consisting of: AP21967, sirolimus, everolimus, novolimus, pimecrolimus, ridaforolimus, tacrolimus, temsirolimus, umirolimus, and zotarolimus.

15. The non-natural cell of any one of claims 11 to 14, wherein the first polypeptide comprises a signal peptide, a CD8α transmembrane domain; a CD137 co-stimulatory domain; and a CD3ζ primary signaling domain.

16. The non-natural cell of any one of claims 11 to 15, wherein the second polypeptide comprises a signal peptide and a CD4 transmembrane domain.

17. The non-natural cell of any one of claims 11 to 16, wherein the second polypeptide comprises a costimulatory domain.

18. The non-natural cell of claim 17, wherein the costimulatory domain of the second polypeptide is selected from a costimulatory molecule selected from the group consisting of: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28, CD30,

CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNFRS14, TNFRS18, TNRFS25, and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

19. The non-natural cell of claim 17 or claim 18, wherein the costimulatory domain of the second polypeptide is a costimulatory domain isolated from OX40 or TNFR2.

20. The non-natural cell of any one of claims 11 to 19, wherein the second polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 22-31.

21. The non-natural cell of any one of claims 1 to 20, wherein the cell is a hematopoietic cell.

22. The non-natural cell of any one of claims 1 to 21, wherein the cell is a T cell, an αβ T cell, or a γδ T cell.

23. The non-natural cell of any one of claims 1 to 22, wherein the cell is a $CD3^+$, $CD4^+$, and/or $CD8^+$ cell.

24. The non-natural cell of any one of claims 1 to 23, wherein the cell is an immune effector cell.

25. The non-natural cell of any one of claims 1 to 24, wherein the cell is a cytotoxic T lymphocytes (CTLs), a tumor infiltrating lymphocytes (TILs), or a helper T cell.

26. The non-natural cell of any one of claims 1 to 25, wherein the cell is a natural killer (NK) cell or natural killer T (NKT) cell.

27. The non-natural cell of any one of claims 1 to 26, wherein the source of the cell is peripheral blood mononuclear cells, bone marrow, lymph nodes tissue, cord blood, thymus issue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, or tumors.

28. The non-natural cell of any one of claims 1 to 27, wherein the FRB multimerization domain and the FKBP multimerization domain localize extracellularly when of the first polypeptide and the second polypeptide are expressed.

29. A fusion polypeptide comprising:

(a) a first polypeptide comprising: an FRB multimerization domain polypeptide or variant thereof; a CD8α transmembrane domain or a CD4 transmembrane domain; a CD137 co-stimulatory domain; and/or a CD3ζ primary signaling domain;

(b) a polypeptide cleavage signal; and

(c) a second polypeptide comprising: an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21; an FKBP multimerization domain polypeptide or variant thereof; and a CD4 transmembrane domain or a CD8α transmembrane domain.

30. The fusion polypeptide of claim 29, wherein the FKBP multimerization domain is FKBP12.

31. The fusion polypeptide of claim 29 or claim 30, wherein the FRB polypeptide is FRB T2098L.

32. The fusion polypeptide of any one of claims 29 to 31, wherein the bridging factor is selected from the group consisting of: AP21967, sirolimus, everolimus, novolimus, pimecrolimus, ridaforolimus, tacrolimus, temsirolimus, umirolimus, and zotarolimus.

33. The fusion polypeptide of any one of claims 29 to 32, wherein the first polypeptide comprises a signal peptide, a CD8α transmembrane domain; a CD137 co-stimulatory domain; and a CD3ζ primary signaling domain.

34. The fusion polypeptide of any one of claims 29 to 33, wherein the second polypeptide comprises a signal peptide and a CD4 transmembrane domain.

35. The non-natural cell of any one of claims 29 to 34, wherein the fusion polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 32-41.

36. The fusion polypeptide of any one of claims 29 to 35, wherein the second polypeptide comprises a costimulatory domain.

37. The fusion polypeptide of claim 36, wherein the costimulatory domain of the second polypeptide is selected from a costimulatory molecule selected from the group consisting of: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNFRS14, TNFRS18, TNRFS25, and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

38. The fusion polypeptide of claim 36 or claim 37, wherein the costimulatory domain of the second polypeptide is a costimulatory domain isolated from OX40 or TNFR2.

39. The fusion polypeptide of any one of claims 29 to 38, wherein the polypeptide cleavage signal is a viral self-cleaving polypeptide.

40. The fusion polypeptide of any one of claims 29 to 39, wherein the polypeptide cleavage signal is a viral self-cleaving 2A polypeptide.

41. The fusion polypeptide of any one of claims 29 to 40, wherein the polypeptide cleavage signal is a viral self-cleaving polypeptide selected from the group consisting of: a foot-and-mouth disease virus (FMDV) (F2A) peptide, an equine rhinitis A virus (ERAV) (E2A) peptide, a Thosea asigna virus (TaV) (T2A) peptide, a porcine teschovirus-1 (PTV-1) (P2A) peptide, a Theilovirus 2A peptide, and an encephalomyocarditis virus 2A peptide.

42. The non-natural cell of any one of claims 29 to 41, wherein the fusion polypeptide comprises the sequence set forth in any one of SEQ ID NOs: 42-61.

43. The fusion polypeptide of any one of claims 29 to 42, wherein the FRB multimerization domain and the FKBP multimerization domain localize extracellularly when of the first polypeptide and the second polypeptide are expressed.

44. A polypeptide complex comprising:

(a) a first polypeptide comprising: an FRB multimerization domain polypeptide or variant thereof; a CD8α transmembrane domain or a CD4 transmembrane domain; a CD137 co-stimulatory domain; and/or a CD3ζ primary signaling domain;

(b) a second polypeptide comprising: an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21; an FKBP multimerization domain polypeptide or variant thereof; and a CD4 transmembrane domain or a CD8α transmembrane domain; and

(c) a bridging factor associated with and disposed between the multimerization domains of the first and second polypeptides.

45. The polypeptide complex of claim 44, wherein the FKBP multimerization domain is FKBP12.

46. The polypeptide complex of claim 44 or claim 45, wherein the FRB polypeptide is FRB T2098L.

47. The polypeptide complex of any one of claims 44 to 46, wherein the bridging factor is selected from the group consisting of: AP21967, sirolimus, everolimus, novolimus, pimecrolimus, ridaforolimus, tacrolimus, temsirolimus, umirolimus, and zotarolimus.

48. The polypeptide complex of any one of claims 44 to 47, wherein the first polypeptide comprises a CD8α transmembrane domain; a CD137 co-stimulatory domain; and a CD3ζ primary signaling domain.

49. The polypeptide complex of any one of claims 44 to 48, wherein the second polypeptide comprises a CD4 transmembrane domain.

50. The polypeptide complex of any one of claims 44 to 49, wherein the second polypeptide comprises a costimulatory domain.

51. The polypeptide complex of claim 50, wherein the costimulatory domain of the second polypeptide is selected from a costimulatory molecule selected from the group consisting of: Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, caspase recruitment domain family member 11 (CARD11), CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD94, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DNAX-Activation Protein 10 (DAP10), Linker for activation of T-cells family member 1 (LAT), SH2 Domain-Containing Leukocyte Protein Of 76 kD (SLP76), T cell receptor associated transmembrane adaptor 1 (TRAT1), TNFR2, TNFRS14, TNFRS18, TNRFS25, and zeta chain of T cell receptor associated protein kinase 70 (ZAP70).

52. The polypeptide complex of claim 50 or claim 51, wherein the costimulatory domain of the second polypeptide is a costimulatory domain isolated from OX40 or TNFR2.

53. The polypeptide complex of any one of claims 44 to 52, wherein the cell is a hematopoietic cell.

54. The polypeptide complex of any one of claims 44 to 53, wherein the cell is a T cell, an αβ T cell, or a γδ T cell.

55. The polypeptide complex of any one of claims 44 to 54, wherein the cell is a $CD3^+$, $CD4^+$, and/or $CD8^+$ cell.

56. The polypeptide complex of any one of claims 44 to 55, wherein the cell is an immune effector cell.

57. The polypeptide complex of any one of claims 44 to 56, wherein the cell is a cytotoxic T lymphocytes (CTLs), a tumor infiltrating lymphocytes (TILs), or a helper T cell.

58. The polypeptide complex of any one of claims 44 to 57, wherein the cell is a natural killer (NK) cell or natural killer T (NKT) cell.

59. The polypeptide complex of any one of claims 44 to 58, wherein the source of the cell is peripheral blood mononuclear cells, bone marrow, lymph nodes tissue, cord blood, thymus issue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, or tumors.

60. The polypeptide complex of any one of claims 44 to 59, wherein the FRB multimerization domain and the FKBP multimerization domain localize extracellularly when of the first polypeptide and the second polypeptide are expressed.

61. A chimeric antigen receptor (CAR) comprising:

a) an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21;

b) a hinge domain;

c) a transmembrane domain;

d) one or more intracellular costimulatory signaling domains; and/or

e) a primary signaling domain.

62. The CAR of claim 61, wherein the CAR comprises from 5′ to 3′:

a) an anti-CD33 VHH antibody that has an amino acid sequence set forth in any one of SEQ ID NOs: 2-21;

b) a hinge domain;

c) a transmembrane domain;

d) one or more intracellular costimulatory signaling domains; and/or

e) a primary signaling domain.

63. The CAR of claim 61 or claim 62, wherein the hinge domain and transmembrane domain are isolated from CD8α, CD27, CD28, CD33, CD37, CD45, CD64, CD71, CD80, CD86, CD 134, CD137, CD152, CD154, AMN, and PD1.

64. The CAR of any one of claims 61 to 63, wherein the one or more costimulatory signaling domains are isolated from a costimulatory molecule selected from the group consisting of: CD28, CD134, CD137, and CD278.

65. The CAR of any one of 61 to 64, wherein the CAR comprises a CD8α signal peptide, a CD8α hinge and transmembrane domain, a CD134 costimulatory domain, and a CD3ζ primary signaling domain.

66. A CAR comprising the amino acid sequence set forth in any one of SEQ ID NOs: 62-81.

67. A polynucleotide encoding the first or second polypeptide of any one of claims 1 to 28, the fusion polypeptide of any one of claims 29 to 43, or the CAR of any one of claims 61-66.

68. A cDNA encoding the first or second polypeptide of any one of claims 1 to 28, the fusion polypeptide of any one of claims 29 to 43, or the CAR of any one of claims 61-66.

69. An RNA encoding the first or second polypeptide of any one of claims 1 to 28, the fusion polypeptide of any one of claims 29 to 43, or the CAR of any one of claims 61-66.

70. A vector comprising the polynucleotide of any one of claims 67 to 69.

71. The vector of claim 70, wherein the vector is an expression vector.

72. The vector of claim 70, wherein the vector is a transposon.

73. The vector of claim 72, wherein the vector is a piggyBAC transposon or a Sleeping Beauty transposon.

74. The vector of claim 70, wherein the vector is a viral vector.

75. The vector of claim 74, wherein the vector is an adenoviral vector, an adeno-associated viral (AAV) vector, a herpes virus vector, a vaccinia virus vector, or a retroviral vector.

76. The vector of claim 75, wherein the retroviral vector is a lentiviral vector.

77. The vector of claim 76, wherein the lentiviral vector is selected from the group consisting of: human immunodeficiency virus 1 (HIV-1); human immunodeficiency virus 2

(HIV-2), visna-maedi virus (VMV) virus; caprine arthritis-encephalitis virus (CAEV); equine infectious anemia virus (EIAV); feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV).

78. A cell comprising the first and second polypeptide of any one of claims 1 to 28, the fusion polypeptide of any one of claims 29 to 43, or the CAR of any one of claims 61-66.

79. The cell of claim 78, wherein the cell is a hematopoietic cell.

80. The cell of claim 78 or 79, wherein the cell is an immune effector cell.

81. The cell of any one of claims 78 to 80, wherein the cell is a T cell, an αβ T cell, or a γδ T cell.

82. The cell of any one of claims 78 to 81, wherein the cell expresses $CD3^+$, $CD4^+$, $CD8^+$, or a combination thereof.

83. The cell of any one of claims 78 to 82, wherein the cell is a cytotoxic T lymphocyte (CTL), a tumor infiltrating lymphocyte (TIL), or a helper T cell.

84. The cell of any one of claims 78 to 83, wherein the cell is a natural killer (NK) cell or natural killer T (NKT) cell.

85. A composition comprising a cell according to any one of claims 1-28 and 78 to 84.

86. A composition comprising a physiologically acceptable carrier and a cell according to any one of claims 1-28 and 78 to 84.

87. A method of treating a subject in need thereof comprising administering the subject an effective amount of the composition of claim 85 or claim 86.

88. A method of treating, preventing, or ameliorating at least one symptom of a cancer, infectious disease, autoimmune disease, inflammatory disease, and immunodeficiency, or condition associated therewith, comprising administering to the subject an effective amount of the composition of claim 85 or claim 86.

89. A method of treating a solid cancer comprising administering to the subject an effective amount of the composition of claim 85 or claim 86.

90. The method of claim 89, wherein the solid cancer is selected from the group consisting of: lung cancer, squamous cell carcinoma, colorectal cancer, pancreatic cancer, breast cancer, thyroid cancer, bladder cancer, cervical cancer, esophageal cancer, ovarian cancer, gastric cancer endometrial cancer, or brain cancer.

91. The method of claim 89 or claim 90, wherein the solid cancer is a non-small cell lung carcinoma, head and neck squamous cell carcinoma, colorectal cancer, pancreatic cancer, breast cancer, thyroid cancer, bladder cancer, cervical cancer, esophageal cancer, ovarian cancer, gastric cancer endometrial cancer, gliomas, glioblastomas, or oligodendroglioma.

92. A method of treating a hematological malignancy comprising administering to the subject an effective amount of the composition of claim 85 or claim 86.

93. The method of claim 92, wherein the hematological malignancy is a leukemia, lymphoma, or multiple myeloma.

94. The method of claim 92, wherein the hematological malignancy is acute myelogenous leukemia (AML).

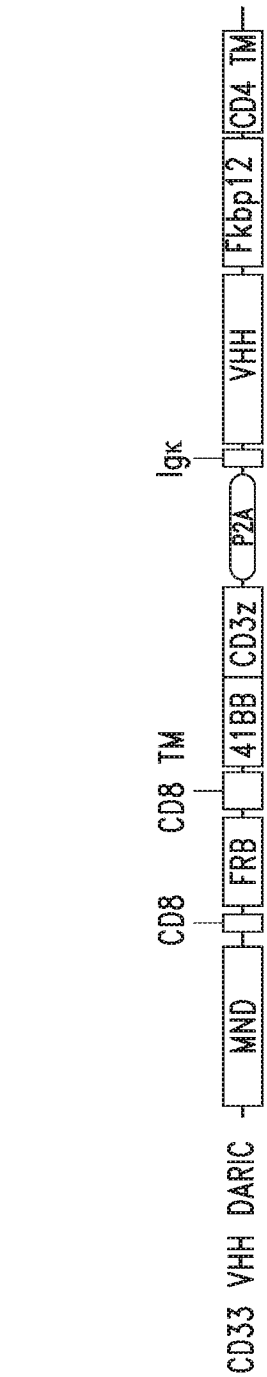
CD33 VHH DARIC
MND
CD8
FRB
CD8 TM
41BB
CD3z
P2A
IgK
VHH
Fkbp12
CD4 TM

FIG. 1B

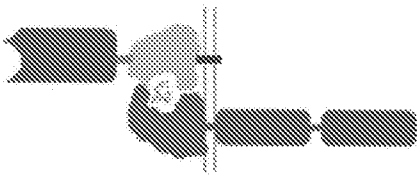

FIG. 1A

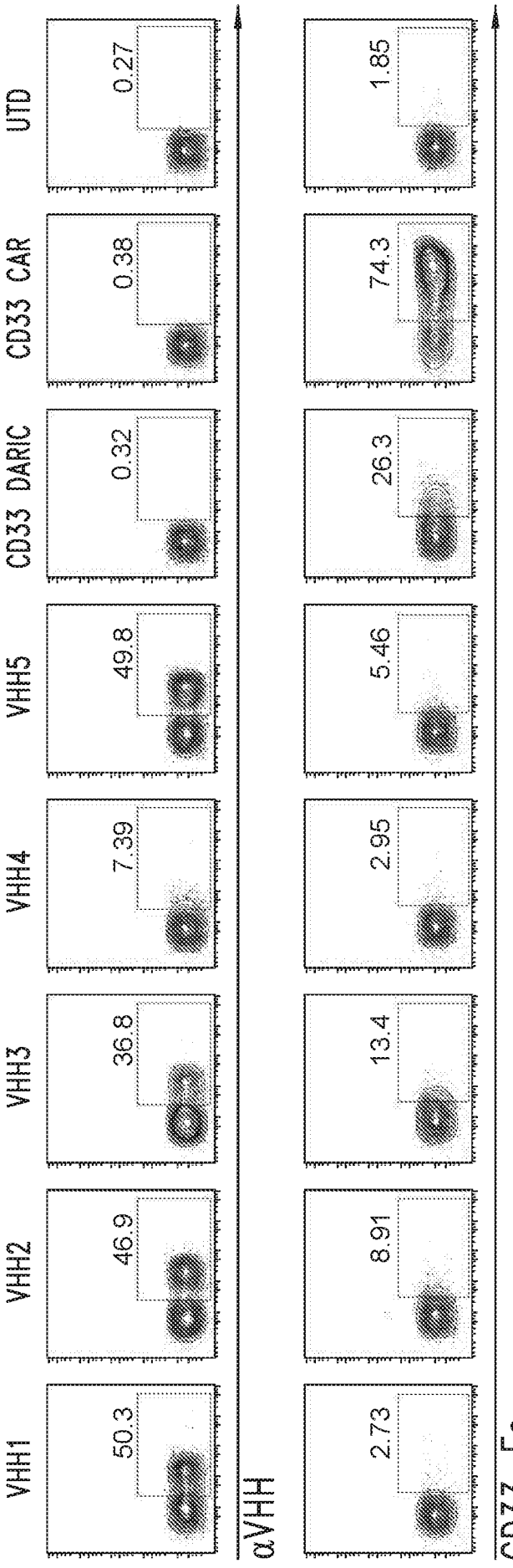
VHH1
VHH2
VHH3
VHH4
VHH5
CD33 DARIC
CD33 CAR
UTD
50.3
46.9
36.8
7.39
49.8
0.32
0.38
0.27
2.73
8.91
13.4
2.95
5.46
26.3
74.3
1.85
αVHH
CD33-Fc

FIG. 2A

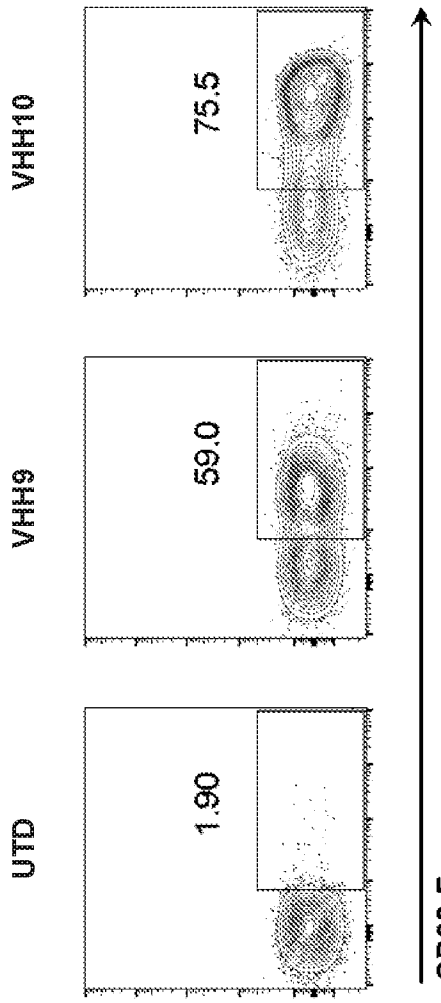

FIG. 2B

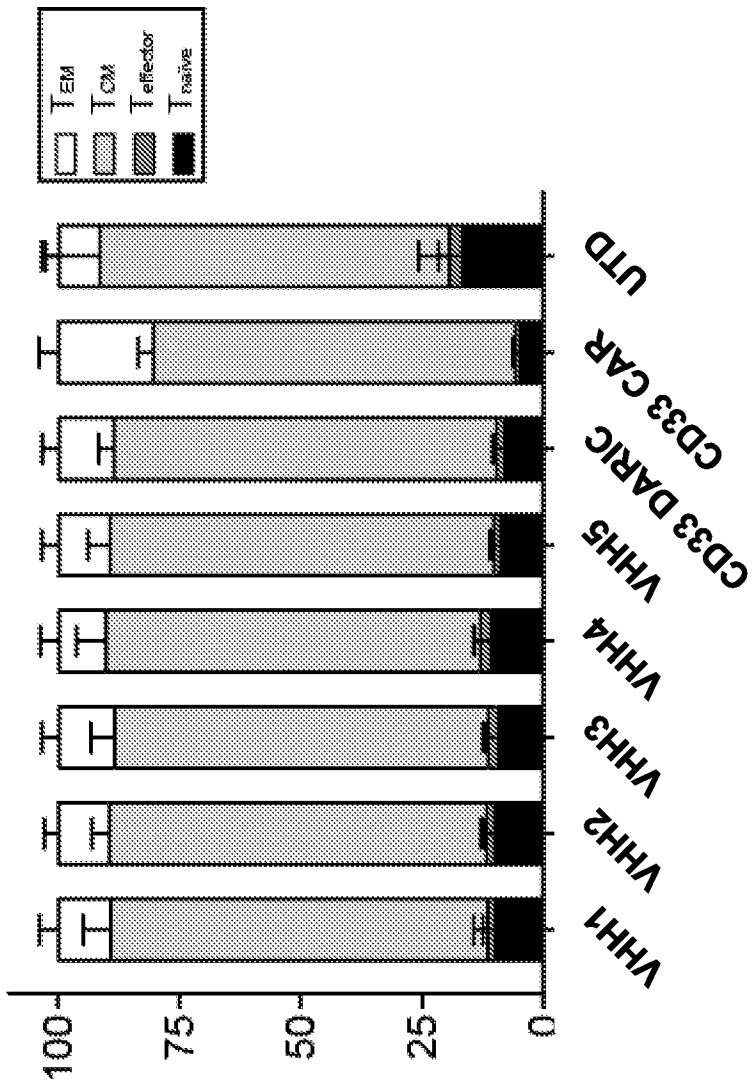
TEM
TCM
Teffector
Tnaive
100
75
50
25
0
VHH1
VHH2
VHH3
VHH4
VHH5
CD33 DARIC
CD33 CAR
UTD

FIG. 3A

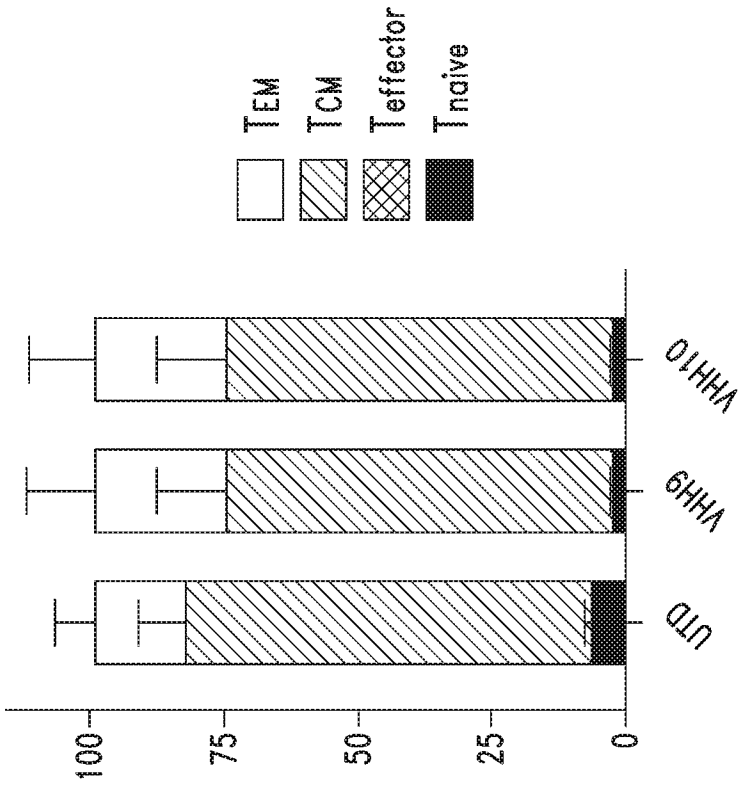

FIG. 3B

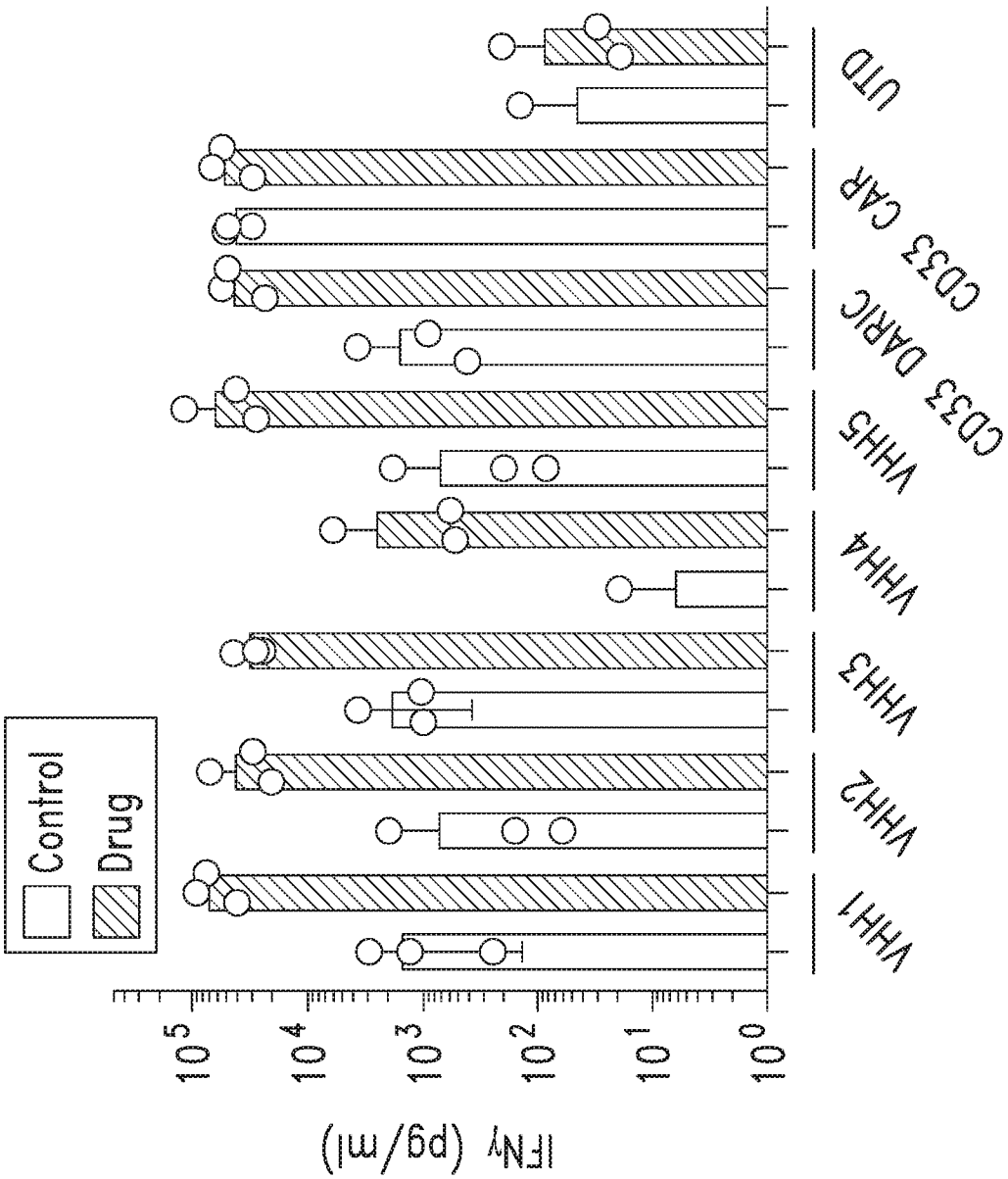
Control
Drug
IFNγ (pg/ml)
10^5
10^4
10^3
10^2
10^1
10^0
VHH1
VHH2
VHH3
VHH4
VHH5
CD33 DARIC
CD33 CAR
UTD

FIG. 4A

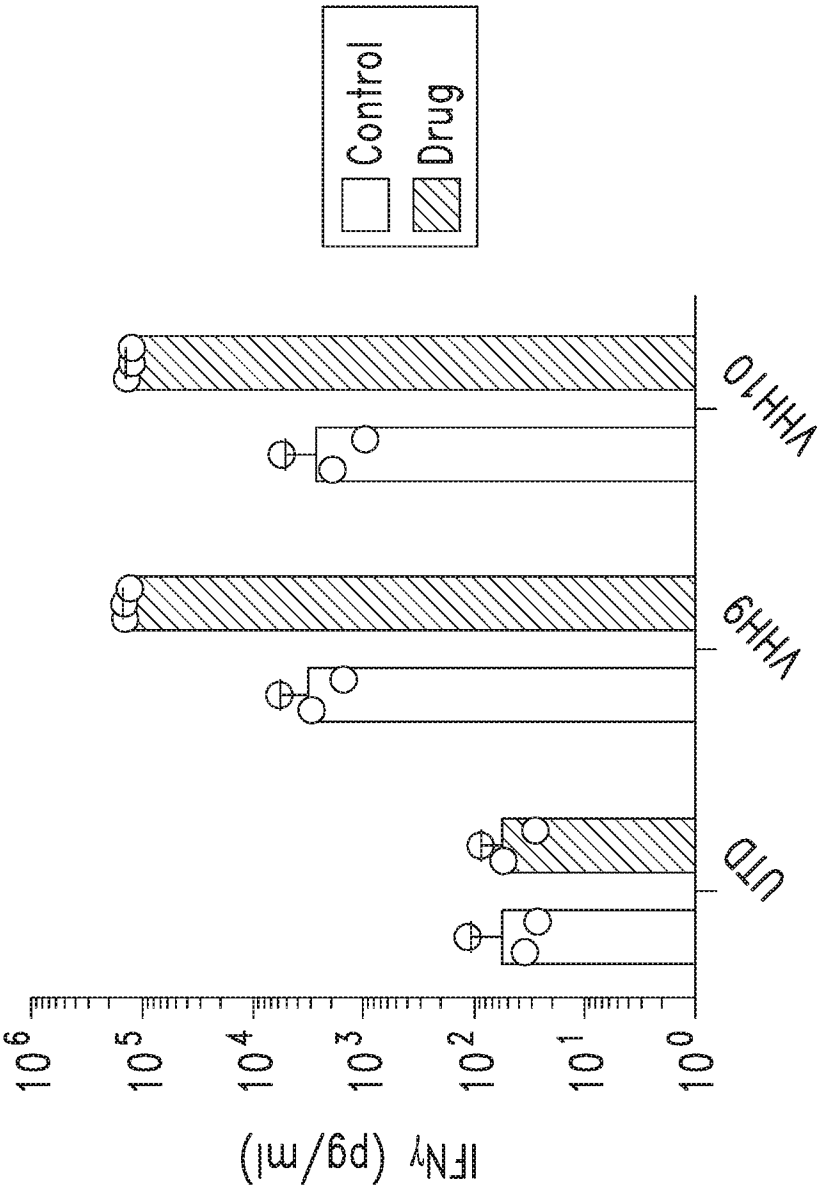
Control
Drug
VHH10
6HHA
UTD
10^6
10^5
10^4
10^3
10^2
10^1
10^0
IFNγ (pg/ml)

FIG. 4B

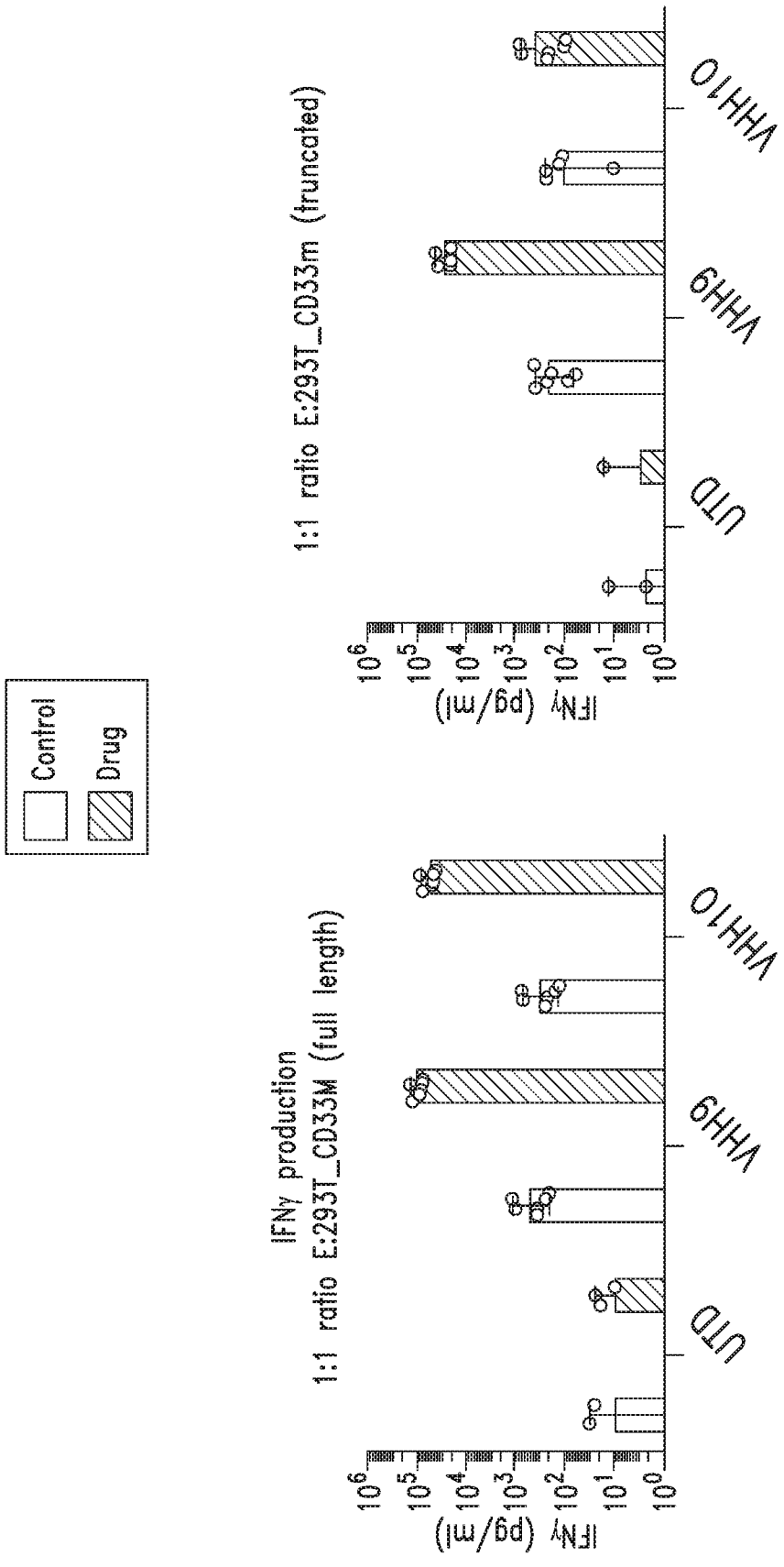
Control
Drug
IFNγ production
1:1 ratio E:293T_CD33M (full length)
IFNγ (pg/ml)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
UTD
6HHA
VHH10
1:1 ratio E:293T_CD33m (truncated)
IFNγ (pg/ml)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
UTD
6HHA
VHH10

FIG. 4C

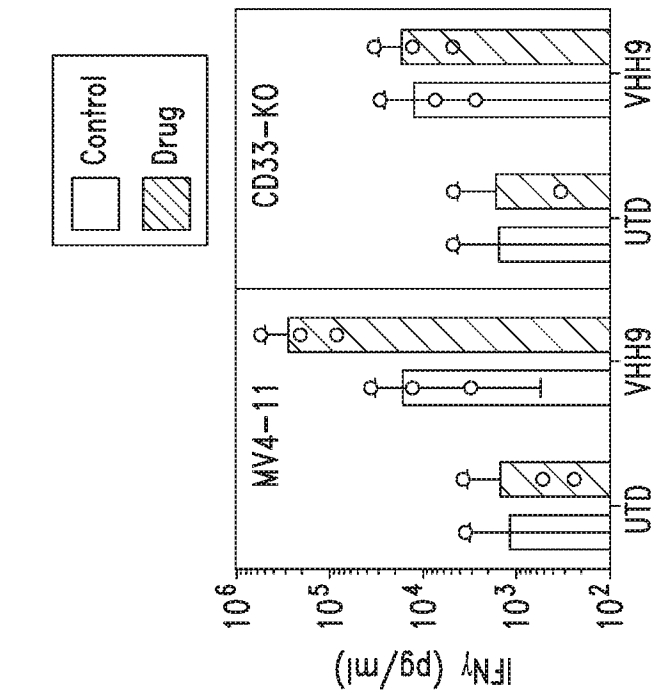
Control
Drug
CD33-KO
MV4-11
UTD
VHH9
UTD
VHH9
IFNγ (pg/ml)
10^6
10^5
10^4
10^3
10^2

FIG. 5B

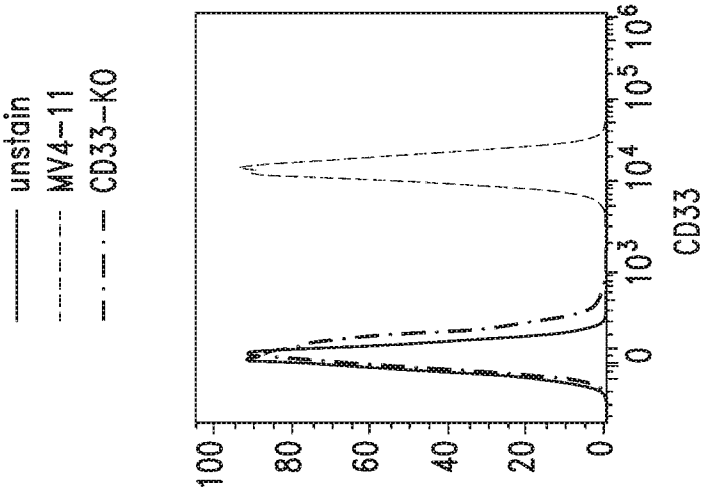
unstain
MV4-11
CD33-KO
100
80
60
40
20
0
0
10^3
10^4
10^5
10^6
CD33

FIG. 5A

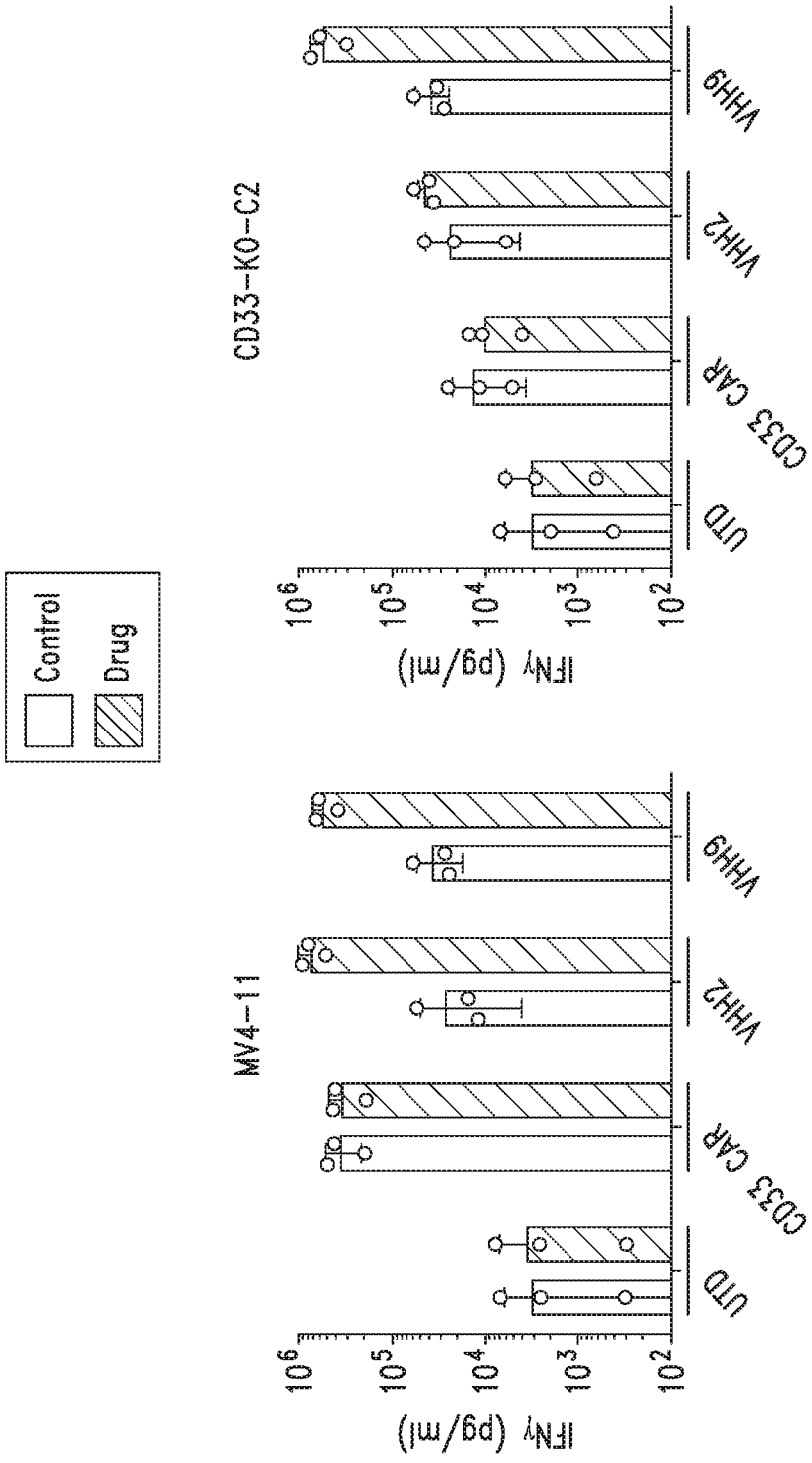
Control
Drug
MV4-11
CD33-KO-C2
IFNγ (pg/ml)
10^2
10^3
10^4
10^5
10^6
UTD
CD33 CAR
VHH2
VHH9

FIG. 5C

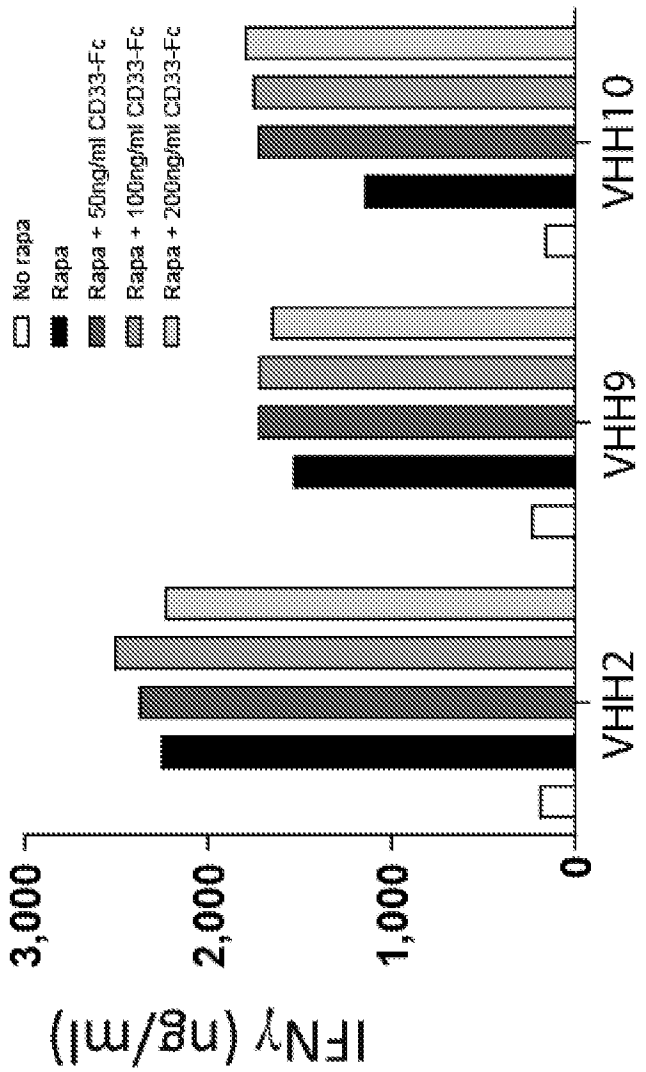
No rapa
Rapa
Rapa + 50ng/ml CD33-Fc
Rapa + 100ng/ml CD33-Fc
Rapa + 200ng/ml CD33-Fc
IFNγ (ng/ml)
3,000
2,000
1,000
0
VHH2
VHH9
VHH10

FIG. 6

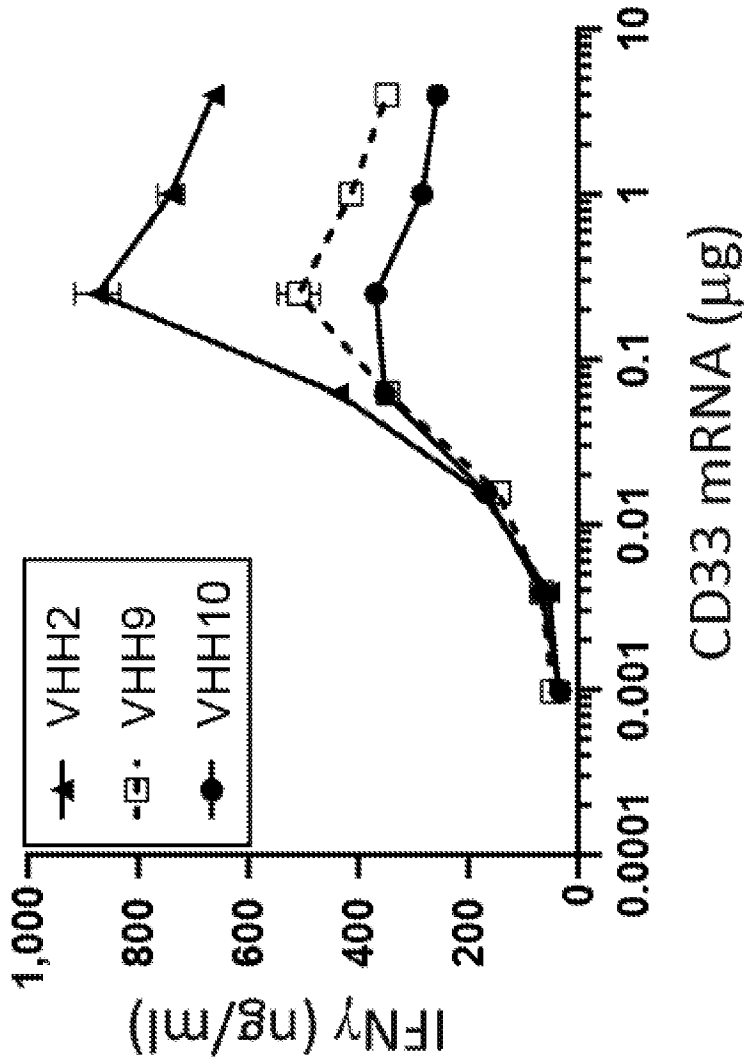
IFNγ (ng/ml)
1,000
800
600
400
200
0
0.0001
0.001
0.01
0.1
1
10
CD33 mRNA (µg)
VHH2
VHH9
VHH10

FIG. 7

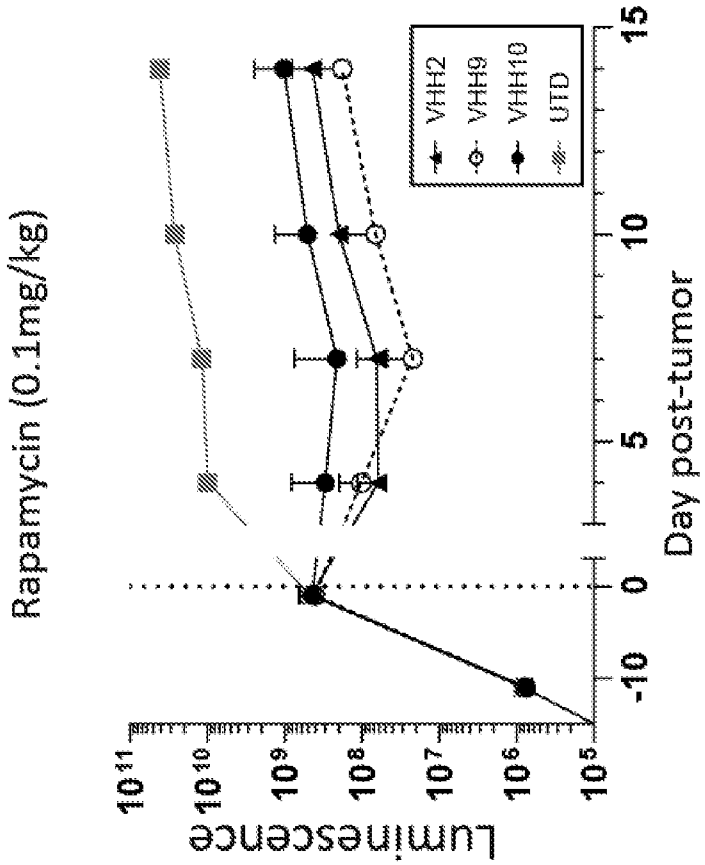
Rapamycin (0.1mg/kg)
Luminescence
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
-10
0
5
10
15
Day post-tumor
VHH2
VHH9
VHH10
UTD

FIG. 8B

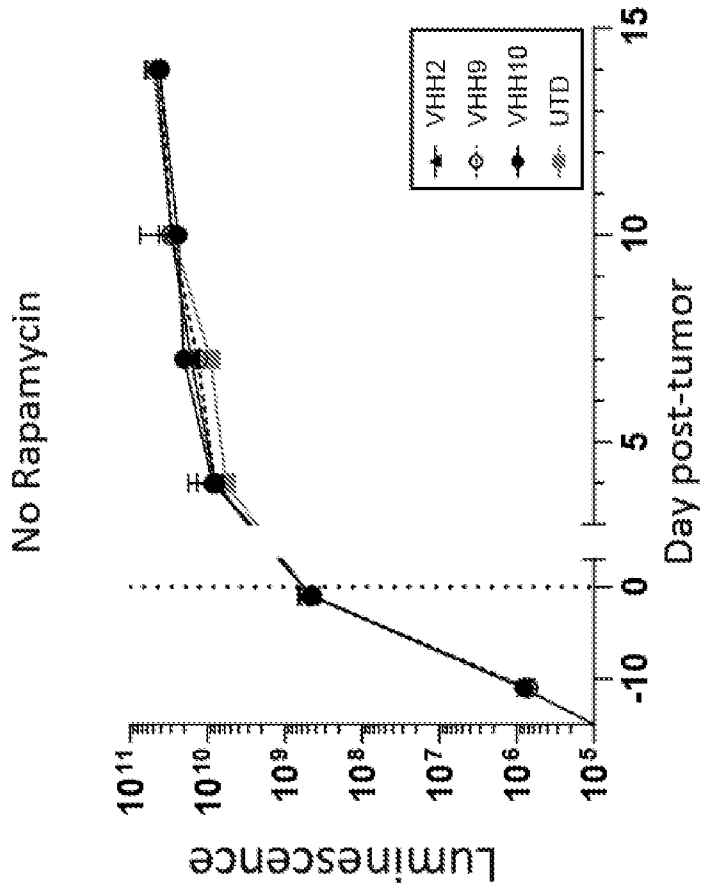
No Rapamycin
Luminescence
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
-10
0
5
10
15
Day post-tumor
VHH2
VHH9
VHH10
UTD

FIG. 8A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/31780

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07K 16/28, C07K 14/705, A61K 38/17, A61K 39/395 (2020.01)

CPC - C07K 16/2851, C07K 14/705, A61K 31/436, A61K 35/28, C07K 2319/00, C07K 2319/03, C07K 2319/70, A61K 38/1774

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)
See Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2016/0311901 A1 (Bluebird Bio, Inc.) 27 October 2016 (27.10.2016) Claim 1, Claim 4, Claim 17, Claim 20, claim 40, Claim 95, para [0269], [0313], [0314]	1-3, 11-13, 29-31, 44-46
A	US 2018/0237533 A1 (Cellectis) 23 August 2018 (23.08.2018) abstract, para [0044], [0369], [0194]	1-3, 11-13, 29-31, 44-46
A	US 2010/0003253 A1 (Laeremans et al.) 07 January 2010 (07.01.2010) para [0034], SEQ ID NO: 10	1-3, 11-13, 29-31, 44-46

☐ 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
"D" document cited by the applicant in the international application
"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
21 September 2020	01 OCT 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/31780

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.: 4-10, 14-28, 32-43, 47-60, 64-65, 67-94
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 extra sheet for Box No. III Observations where unity of invention is lacking -

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 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.:
1-3, 11-13, 29-31, 44-46 limited to SEQ ID NO: 2

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.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US 20/31780

Continuation of:
Box No. III. Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Groups I+: Claims 1-3, 11-13, 29-31, 44-46, drawn to a non-natural cell or a fusion polypeptide comprising: (a) a first polypeptide comprising: an FRB multimerization domain polypeptide; and (b) a second polypeptide comprising: an anti-CD33 VHH antibody and an FKBP multimerization domain polypeptide. The non-natural cell/fusion polypeptide will be searched to the extent that the anti-CD33 VHH encompasses SEQ ID NO: 2. It is believed that claims 1-3, 11-13, 29-31, 44-46 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 2. Additional anti-CD33 VHH(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected anti-CD33 VHH(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be an anti-CD33 VHH encompasses SEQ ID NO: 3 (Claims 1-3, 11-13, 29-31, 44-46).

Groups II+: Claims 61-63, 66, drawn to a chimeric antigen receptor (CAR) comprising: an anti-CD33 VHH antibody. Group II+ will be searched upon payment of additional fees. The CAR may be searched, for example, to the extent that the anti-CD33 VHH encompasses SEQ ID NO: 2 and the CAR encompasses SEQ ID NO: 62, for an additional fee and election as such. It is believed that claims 61-63, 66 read on this exemplary invention. Additional anti-CD33 VHH(s) and CAR(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected anti-CD33 VHH(s) and CAR(s). Failure to clearly identify how any paid additional invention fees are to be applied to the"+" group(s) will result in only the first claimed invention to be searched. Another exemplary election would be an anti-CD33 VHH encompasses SEQ ID NO: 3 and a CAR encompasses SEQ ID NO: 63 (Claims 61-63, 66).

The inventions listed as Groups I+ and II+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Groups I+ include the special technical feature of a first and a second polypeptide that are associated by bridging factor-induced multimerization, not required by Groups II+.

Groups II+ include the special technical feature of a chimeric antigen receptor comprising: a hinge domain, not required by Groups I+.

No technical features are shared between the amino acid sequences of Groups I+ and II+, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ and II+ were considered to share the technical features of including: a non-natural cell, a fusion peptide or a CAR, these shared technical features are previously disclosed (see below).

Common Technical Features

The inventions of Groups I+ and II+ share the technical feature of a fusion peptide comprising anti-CD33 VHH, a transmembrane domain, an intracellular costimulatory signaling domains; and/or a primary signaling domain.

However, these shared technical features do not represent a contribution over prior art in view of US 2016/0311901 A to Bluebird Bio, Inc. (hereinafter "Bluebird") and US 2018/0237533 A1 (Cellectis).

Bluebird teaches (instant claim 1) a non-natural cell comprising (Claim 1, A non-natural cell):
(a) a first polypeptide comprising: an FRB multimerization domain polypeptide or variant thereof; a CD8alpha transmembrane domain or a CD4 transmembrane domain; a CD137 costimulatory domain; and/or a CD3zeta primary signaling domain (Claim 1, (a) a first nucleic acid molecule encoding a first fusion protein comprising a first multimerization domain, a hydrophobic domain, and an actuator domain, wherein the first multimerization domain localizes extracellularly when the first fusion protein is expressed.; Claim 4, wherein the first and second multimerization domains are a pair selected from FKBP and FRB.; Claim 17, wherein the transmembrane domain is a CD4, CD8 or CD28 transmembrane domain.; para [0269], "sub-threshold signaling domain".....CD137; Claim 20, wherein the actuator domain comprises.....CD3zeta.); and
(b) a second polypeptide comprising: an FKBP multimerization domain polypeptide or variant thereof; and a CD4 transmembrane domain or a CD8a transmembrane domain (para [0313], A series of additional DARIC molecules, in which the antigen binding component was maintained on the T cell surface rather than released into the extracellular space, were tested (see, e.g., FIG. 1I). Several protein regions and transmembrane domains were used to anchor the binding domain to the T cell surface.; [0314], The tethered DARIC binding component containing the CD8 hinge/CD8 transmembrane domain (SEQ ID NO.:53).);
wherein a bridging factor promotes the formation of a polypeptide complex on the nonnatural cell surface with the bridging factor associated with and disposed between the multimerization domains of the first and second polypeptides (Claim 1, wherein a bridging factor promotes the formation of a polypeptide complex on the non-natural cell surface with the bridging factor associated with and disposed between the multimerization domains of the first and second fusion proteins.). Bluebird further teaches a fusion polypeptide (Claim 95, A fusion polypeptide.) and the binding domain is specifically binds to a target selected form CD33 (Claim 40, wherein the binding domain specifically binds to a target selected from.....CD33).

----continued on next sheet----

Continuation of:
Box No. III. Observations where unity of invention is lacking

Cellectis teaches a chemically regulated signaling molecules targeting CD33 (abstract, chimeric antigen receptor which comprise in at least one ectodomain a molecular switch turning the antigen binding function of the receptor from an off to on state, and vice versa.; para [0044], the first multimerizing ligand-binding domain and the second multimerizing ligand binding domain are derived from a chemical induced dimerization (CID) system.; [0369], To design an integrated system to switch the scFv/antigen interaction between on/off states, either the FRB, the FKBP12, or fusion of the FRB and FKBP12 were inserted between the CD8a hinge and the scFv domains (FIG. 1B). As a starting experiment, primary T cell with mRNAs encoding each chain of the multichain CAR (mcCAR) were transfected. Upon addition of rapamycin, changes in the detection of the extracellular hinge domain were monitored by tracking the Fab'2 domain of CD19-targeting scFv (100 nM, 20 h).; see Fig. 1B, scFv-FKBP-CD8a-TM+intracel.; [0194], the extracellular antigen binding domain may be directed against CD33. Such extracellular antigen binding domain may be a scFV derived from a CD33 monoclonal antibody.). Given that polypeptides facilitating chemical-induced multimerization has been used to target CD33, as taught by Cellectis, it would have been obvious to one of ordinary skill in the art to have directed the non-natural cells to target CD33 expressing cells by incorporating the anti-CD33 antibody into the fusion polypeptides of Bluebird.

The inventions of Groups II+ share the technical feature of a CAR, which is disclosed by US 2016/0096892 A1 to Brogdon et al. (hereinafter "Brogdon").

Brogdon teaches (instant claim 61) a chimeric antigen receptor (CAR) comprising:
a) an anti-CD33 VHH antibody;
b) a hinge domain;
c) a transmembrane domain;
d) one or more intracellular costimulatory signaling domains; and/or
e) a primary signaling domain (Claim 25, An isolated chimeric antigen receptor (CAR) polypeptide, wherein the CAR comprises a CD33 binding domain, a transmembrane domain, and an intracellular signaling domain comprising a costimulatory domain and/or a primary signaling domain.; para [0013], the encoded CD33 binding domain is connected to the transmembrane domain by a hinge region.; [0323], The antigen binding domain can be any protein that binds to the antigen including but not limited to a monoclonal antibody,.....a variable domain (VHH) of camelid derived nanobody.).

As said technical features were known in the art at the time of the invention, these cannot be considered special technical features that would otherwise unify the groups.

Groups I+ and II+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note, Claims 4-10, 14-28, 32-43, 47-60, 64-65, 67-94 are improper multiple dependent claims because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).