

What is claimed is:

1. A method of making a population of cells that express a chimeric antigen receptor (CAR), the method comprising:

(i) contacting a population of cells with a multispecific binding molecule comprising (A) an anti-CD3 binding domain, and (B) a costimulatory molecule binding domain;

(ii) contacting the population of cells with a nucleic acid molecule encoding the CAR, thereby providing a population of cells comprising the nucleic acid molecule, and

(iii) harvesting the population of cells for storage or administration, wherein the population of cells from step (iii) are not expanded, or expanded by no more than 40%, compared to the population of cells at the beginning of step (i)

2. The method of claim 1, wherein:

(i) the anti-CD3 binding domain, is situated N-terminal of the costimulatory molecule binding domain; or

(ii) the anti-CD3 binding domain, is situated C-terminal of the costimulatory molecule binding domain, optionally wherein:

(a) an Fc region is situated between the anti-CD3 binding domain and the costimulatory molecule binding domain; or

(b) the multispecific binding molecule comprises a CH2, and the anti-CD3 binding domain is situated N-terminal of the CH2;

optionally wherein the anti-CD3 binding domain comprises an scFv and the costimulatory molecule binding domain is part of a Fab fragment.

3. The method of claim 1 or 2, wherein the multispecific binding molecule comprises:

(A) (i) a first polypeptide comprising from N-terminal to C-terminal: VH of the anti-CD3 binding domain, VL of the anti-CD3 binding domain, VH of the costimulatory molecule binding domain, CH1, CH2, and CH3; and

(ii) a second polypeptide comprising from N-terminal to C-terminal: VL of the costimulatory molecule binding domain and CL;

(B) (i) a first polypeptide comprising from N-terminal to C-terminal: VH of the costimulatory molecule binding domain, CH1, CH2, CH3, VH of the anti-CD3 binding domain, and VL of the anti-CD3 binding domain; and

(ii) a second polypeptide comprising from N-terminal to C-terminal: VL of the costimulatory molecule binding domain and CL; or

(C) (i) a first polypeptide comprising from N-terminal to C-terminal: VH of the costimulatory molecule binding domain, CH1, VH of the anti-CD3 binding domain, VL of the anti-CD3 binding domain, CH2, and CH3; and

(ii) a second polypeptide comprising from N-terminal to C-terminal: VL of the costimulatory molecule binding domain and CL

4. The method of any one of claims 1-3, wherein the anti-CD3 binding domain comprises:

(i) a variable heavy chain region (VH) comprising a heavy chain complementarity determining region 1 (HCDR1), a HCDR2, and a HCDR3, and a light chain variable region (VL) comprising a light chain complementarity determining region 1 (LCDR1), a LCDR2, and a LCDR3 of an anti-CD3 antibody molecule of Table 27 (for example the anti-CD3 (1), anti-CD3 (2), anti-CD3 (3), or anti-CD3 (4)); and/or

(ii) the amino acid sequence of any VH and/or VL region of an anti-CD3 antibody molecule provided in Table 27 (for example the anti-CD3 (1), anti-CD3 (2), anti-CD3 (3), or anti-CD3 (4)), or an amino acid sequence at least 95% identical thereto.

5. The method of any one of claims 1-4, wherein the costimulatory molecule binding domain is:

(A) an anti-CD2 binding domain, optionally wherein the anti-CD2 binding domain comprises:

(i) a VH comprising a HCDR1, a HCDR2, and a HCDR3, and a VL comprising a LCDR1, a LCDR2, and a LCDR3 of an anti-CD2 antibody molecule of Table 27 (for example the anti-CD2 (1)); and/or

(ii) the amino acid sequence of any VH and/or VL region of an anti-CD2 antibody molecule provided in Table 27 (for example the anti-CD2 (1)), or an amino acid sequence at least 95% identical thereto;

(B) an anti-CD28 binding domain, optionally wherein the anti-CD28 binding domain comprises:

(i) a VH comprising a HCDR1, a HCDR2, and a HCDR3, and a VL comprising a LCDR1, a LCDR2, and a LCDR3 of an anti-CD28 antibody molecule of Table 27 (for example the anti-CD28 (1) or anti-CD28 (2)); and/or

(ii) the amino acid sequence of any VH and/or VL region of an anti-CD28 antibody molecule provided in Table 27 (for example the anti-CD28 (1) or anti-CD28 (2)), or an amino acid sequence at least 95% identical thereto; and/or

(C) part of a Fab fragment, e.g., a Fab fragment that is part of a polypeptide sequence that comprises an Fc domain, optionally wherein the Fc domain comprises an amino acid sequence provided in Table 28, or a sequence with at least 95% sequence identity to an amino acid sequence of an Fc domain provided in Table 28.

6. The method of any one of claims 1-5, wherein the anti-CD3 binding domain comprises:

(i) an scFv;

(ii) a VH linked to a VL by a peptide linker, e.g., a glycine-serine linker, e.g., a (G₄S)₄ linker; or

(iii) a VH and a VL, wherein the VH is N-terminal of the VL.

7. The method of any one of claims 1-6, wherein:

(a) the anti-CD3 binding domain is situated N-terminal of the costimulatory molecule binding domain, optionally wherein the anti-CD3 binding domain is linked to the costimulatory molecule binding domain by a peptide linker, e.g., a glycine-serine linker, e.g., a (G₄S)₄ linker; or

(b) the anti-CD3 binding domain is situated C-terminal of the costimulatory molecule binding domain.

8. The method of claim 7, wherein:

(i) an Fc region is situated between the anti-CD3 binding domain and the costimulatory molecule binding domain; and/or

(ii) the multispecific binding molecule comprises one or both of a CH2 and a CH3, optionally wherein the anti-CD3 binding domain is linked to the CH3 by a peptide linker, e.g., a glycine-serine linker, e.g., a (G₄S)₄ linker;

(iii) the multispecific binding molecule comprises a CH2, and the anti-CD3 binding domain is situated N-terminal of the CH2;

(iv) the anti-CD3 binding domain is linked to a CH1 by a peptide linker, e.g., a glycine-serine linker, e.g., a (G₄S)₂ linker; and/or

(v) the anti-CD3 binding domain is linked to a CH2 by a peptide linker, e.g., a glycine-serine linker, e.g., a (G₄S)₄ linker.

9. The method of any one of claims 1-8, wherein the multispecific binding molecule comprises:

(i) the amino acid sequence of any heavy chain provided in Table 28, or an amino acid sequence with at least 95% sequence identity thereto; and/or

(ii) the amino acid sequence of any light chain provided in Table 28, or an amino acid sequence with at least 95% sequence identity thereto.

10. The method of any one of claims 1-9, wherein:

(a) step (ii) is performed together with step (i) or no later than 20 hours after the beginning of step (i), and

step (iii) is performed no later than 30 hours after the beginning of step (i); or

step (iii) is performed no later than 30 hours after the beginning of step (ii);

(b) the nucleic acid molecule in step (ii) is on a viral vector;

(c) the population of cells comprises a population of T cells, optionally wherein the population of T cells is isolated from a frozen or fresh leukapheresis product; and/or

(d) the storage of step (iii) comprises reformulating the population of cells in cryopreservation media.

11. The method of any one of claims 1-10, wherein:

(I) (a) the percentage of naïve cells in the population of cells from step (iii) is the same as or differs by no more than 5 or 10% from the percentage of naïve cells in the population of cells at the beginning of step (i); or

(b) the percentage of naïve cells in the population of cells from step (iii) does not decrease, or decreases by no more than 5 or 10%, as compared to the percentage of naïve cells in the population of cells at the beginning of step (i); and/or

(II) (a) the percentage of central memory cells in the population of cells from step (iii) is the same as or differs by no more than 5 or 10% from the percentage of central memory cells in the population of cells at the beginning of step (i); or

(b) the percentage of central memory cells in the population of cells from step (iii) does not increase, or increases by no more than 5 or 10%, as compared to the percentage of central memory cells in the population of cells at the beginning of step (i);

optionally wherein said naïve cells are naïve T cells or CD45RA⁺ CD45RO⁻ CCR7⁺ T cells; or said central memory cells are central memory T cells, CD95⁺ central memory T cells, or CCR7⁺CD45RO⁺ cells.

12. The method of any one of claims 1-9, wherein:

(I) step (i) increases the percentage of CAR-expressing cells in the population of cells from step (iii), for example, the population of cells from step (iii) shows a higher percentage of CAR-expressing cells (for example, at least 10, 20, 30, 40, 50, or 60% higher), compared with cells made by an otherwise similar method without step (i); and/or

(II) a) the percentage of naïve cells, in the population of cells from step (iii) is increased by, for example, at least 1.2-fold, as compared to the percentage of naïve cells in the population of cells at the beginning of step (i); or

(b) the percentage of CAR-expressing naïve T cells in the population of cells increases during the duration of step (ii), or between 18-24 hours after the beginning of step (ii); and/or

(III) (a) the population of cells from step (iii) shows a higher percentage of naïve cells compared with cells made by an otherwise similar method in which step (iii) is performed more than 26 hours after the beginning of step (i);

(b) the percentage of naïve cells in the population of cells from step (iii) is higher than the percentage of naïve cells in cells made by an otherwise similar method in which step (iii) is performed more than 26 hours after the beginning of step (i);

(c) the percentage of CAR-expressing naïve T cells in the population of cells from step (iii) is higher than the percentage of CAR-expressing naïve T cells in cells made by an

otherwise similar method in which step (iii) is performed more than 26 hours after the beginning of step (i);

(d) the population of cells from step (iii) shows a higher percentage of naïve cells, for example, naïve T cells, for example, CD45RA⁺ CD45RO⁻ CCR7⁺ T cells, compared with cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days, for example, for 5, 6, 7, 8 or 9 days;

(e) the percentage of naïve cells in the population of cells from step (iii) is higher than the percentage of naïve cells in cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days; or

(f) the percentage of CAR-expressing naïve T cells in the population of cells from step (iii) is higher than the percentage of CAR-expressing naïve T cells in cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days; and/or

(IV) (a) the percentage of central memory cells in the population of cells from step (iii) is reduced by at least 20%, as compared to the percentage of central memory cells in the population of cells at the beginning of step (i); or

(b) the percentage of CAR-expressing central memory T cells decreases during the duration of step (ii), or between 18-24 hours after the beginning of step (ii); and/or

(V) (a) the population of cells from step (iii) shows a lower percentage of central memory cells compared with cells made by an otherwise similar method in which step (iii) is performed more than 26 hours after the beginning of step (i);

(b) the percentage of central memory cells in the population of cells from step (iii) is lower than the percentage of central memory cells in cells made by an otherwise similar method in which step (iii) is performed more than 26 hours after the beginning of step (i);

(c) the percentage of CAR-expressing central memory T cells in the population of cells from step (iii) is lower than the percentage of CAR-expressing central memory T cells in cells made by an otherwise similar method in which step (iii) is performed more than 26 hours after the beginning of step (i);

(d) the population of cells from step (iii) shows a lower percentage of central memory cells compared with cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days;

(e) the percentage of central memory cells in the population of cells from step (iii) is lower than the percentage of central memory cells in cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days; or

(f) the percentage of CAR-expressing central memory T cells in the population of cells from step (iii) is lower than the percentage of CAR-expressing central memory T cells in cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days; and/or

(VI) (a) the percentage of stem memory T cells in the population of cells from step (iii) is increased, as compared to the percentage of stem memory T cells in the population of cells at the beginning of step (i);

(b) the percentage of CAR-expressing stem memory T cells in the population of cells from step (iii) is increased, as compared to the percentage of CAR-expressing stem memory T cells in the population of cells at the beginning of step (i);

(c) the percentage of stem memory T cells in the population of cells from step (iii) is higher than the percentage of stem memory T cells in cells made by an otherwise similar method in which step (iii) is performed more than 26 hours after the beginning of step (i); or

(d) the percentage of CAR-expressing stem memory T cells in the population of cells from step (iii) is higher than the percentage of CAR-expressing stem memory T cells in cells made by an otherwise similar method in which step (iii) is performed more than 26 hours after the beginning of step (i);

(e) the percentage of stem memory T cells in the population of cells from step (iii) is higher than the percentage of stem memory T cells in cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days; or

(f) the percentage of CAR-expressing stem memory T cells in the population of cells from step (iii) is higher than the percentage of CAR-expressing stem memory T cells in cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days;

optionally wherein said naïve cells are naïve T cells or CD45RA⁺ CD45RO⁻ CCR7⁺ T cells;

said central memory cells are central memory T cells, CD95⁺ central memory T cells, or CCR7⁺CD45RO⁺ cells; and/or

said stem memory T cells are CD45RA⁺CD95⁺IL-2 receptor β +CCR7⁺CD62L⁺ T cell.

13. The method of any one of claims 1-12, wherein:

(a) the population of cells from step (iii), after being incubated with a cell expressing an antigen recognized by the CAR, secretes IL-2 at a higher level than cells made by an otherwise similar method in which step (iii) is performed more than 26 hours after the beginning of step (i), or cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days;

(b) the population of cells from step (iii), after being administered in vivo, persists longer or expands at a higher level, compared with cells made by an otherwise similar method in which step (iii) is performed more than 26 hours after the beginning of step (i), or compared with cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days;

(c) the population of cells from step (iii), after being administered in vivo, shows a stronger anti-tumor activity than cells made by an otherwise similar method in which step (iii) is performed more than

26 hours after the beginning of step (i), or cells made by an otherwise similar method which further comprises, after step (ii) and prior to step (iii), expanding the population of cells in vitro for more than 3 days;

(d) the population of cells from step (iii) are expanded by no more than 5, 10, 15, 20, 25, 30, 35, or 40%, compared to the population of cells at the beginning of step (i);

(e) the population of cells from step (iii) are expanded by no more than 10%, compared to the population of cells at the beginning of step (i);

(f) the population of cells from step (iii) are not expanded, or expanded by no more than 5, 10, 15, 20, 25, 30, 35, or 40%, as assessed by the number of living cells, compared to the population of cells at the beginning of step (i);

(g) the population of cells from step (iii) are expanded by no more than 10% compared to the population of cells at the beginning of step (i);

(h) the number of living cells in the population of cells from step (iii) decreases from the number of living cells in the population of cells at the beginning of step (i);

(i) the population of cells from step (iii) are not expanded, or expanded by less than 2 hours, for example, less than 1 or 1.5 hours, compared to the population of cells at the beginning of step (i);

(j) the population of cells from step (iii) are not expanded; and/or

(k) the population of cells from step (iii) are not expanded, as assessed by the number of living cells, compared to the population of cells at the beginning of step (i).

14. The method of any one of claims 1-13, wherein:

(a) steps (i) and/or (ii) are performed in cell media comprising IL-2, IL-15, IL-7, IL-21, IL-6, a LSD1 inhibitor, a MALT1 inhibitor, or a combination thereof, optionally wherein said IL-15 is hetIL-15 or IL15/sIL15Ra and/or said IL-6 is IL-6/IL-6Ra;

(b) steps (i) and/or (ii) are performed in serum-free media comprising IL-2, IL-15, IL-7, IL-21, IL-6, a LSD1 inhibitor, a MALT1 inhibitor, or a combination thereof, optionally wherein said IL-15 is hetIL-15 or IL15/sIL15Ra and/or said IL-6 is IL-6/IL-6Ra;

(c) steps (i) and/or (ii) are performed in serum-free cell media comprising a serum replacement; and/or

(d) the serum replacement is CTS™ Immune Cell Serum Replacement (ICSR).

15. The method of any one of claims 1-14, further comprising prior to step (i):

(l) (iv) optionally receiving a fresh leukapheresis product (or an alternative source of hematopoietic tissue such as a fresh whole blood product, a fresh bone marrow product, or a fresh tumor or organ biopsy or removal, or a fresh product from thymectomy from an entity, for example, a laboratory, hospital, or healthcare provider, and

(v) isolating the population of cells contacted in step (i) from a fresh leukapheresis product or an alternative source of hematopoietic tissue such as a fresh whole blood product, a fresh bone marrow product, a fresh tumor or organ biopsy or removal, or a fresh product from thymectomy, optionally wherein:

step (iii) is performed no later than 35 hours after the beginning of step (v), or

the population of cells from step (iii) are not expanded, or expanded by no more than 40% compared to the population of cells at the end of step (v); or

(II) receiving cryopreserved T cells isolated from a leukapheresis product or an alternative source of hematopoietic tissue such as cryopreserved T cells isolated from whole blood, bone marrow, tumor or organ biopsy or removal, or thymectomy from an entity, for example, a laboratory, hospital, or healthcare provider; or

(III) (iv) optionally receiving a cryopreserved leukapheresis product or an alternative source of hematopoietic tissue such as a cryopreserved whole blood product, a cryopreserved bone marrow product, a cryopreserved tumor or organ biopsy or removal, or a cryopreserved product from thymectomy from an entity, for example, a laboratory, hospital, or healthcare provider, and

(v) isolating the population of cells contacted in step (i) from a cryopreserved leukapheresis product or an alternative source of hematopoietic tissue such as a cryopreserved whole blood product, a cryopreserved bone marrow product, a cryopreserved tumor or organ biopsy or removal, or a cryopreserved product from thymectomy, optionally wherein:

step (iii) is performed no later than 35 hours after the beginning of step (v), or

the population of cells from step (iii) are not expanded, or expanded by no more than 40% compared to the population of cells at the end of step (v).

16. The method of any one of claims 1-15, further comprising step (vi):

culturing a portion of the population of cells from step (iii) for at least 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, or 7 days, for example, at least 2 days and no more than 7 days, and measuring CAR expression level in the portion, optionally wherein:

step (iii) comprises harvesting and freezing the population of cells and step (vi) comprises thawing a portion of the population of cells from step (iii), culturing the portion for at least 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, or 7 days, and measuring CAR expression level in the portion.

17. The method of any one of claims 1-16, wherein:

(a) step (ii) further comprises adding F108 during transduction and/or contacting the population of cells with an shRNA that targets Tet2;

(b) the population of cells at the beginning of step (i) has been enriched for IL6R-expressing cells (optionally, cells that are positive for IL6R α and/or IL6R β);

(c) the population of cells at the beginning of step (i) comprises no less than 50, 60, or 70% of IL6R-expressing cells (optionally, cells that are positive for IL6R α and/or IL6R β); and/or

(d) steps (i) and (ii) are performed in cell media comprising IL-15 and/or hetIL-15 (IL15/sIL-15Ra)), optionally wherein IL-15 increases the ability of the population of cells to expand, and/or increases the percentage of IL6R β -expressing cells in the population of cells.

18. The method of any one of claims 1-17, wherein the CAR comprises an antigen binding domain, a transmembrane domain, and an intracellular signaling domain, optionally wherein:

(I) the antigen binding domain binds to an antigen chosen from: CD19, CD20, CD22, BCMA, mesothelin, EGFRvIII, GD2, Tn antigen, sTn antigen, Tn-O-Glycopeptides, sTn-O-Glycopeptides, PSMA, CD97, TAG72, CD44v6, CEA, EPCAM, KIT, IL-13Ra2, leguman, GD3, CD171, IL-11Ra, PSCA, MAD-CT-1, MAD-CT-2, VEGFR2, LewisY, CD24, PDGFR-beta, SSEA-4, folate receptor alpha, ERBBs (for example, ERBB2), Her2/neu, MUC1, EGFR, NCAM, Ephrin B2, CAIX, LMP2, sLe, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, FAP, Legumain, HPV E6 or E7, ML-IAP, CLDN6, TSHR, GPRC5D, ALK, Polysialic acid, Fos-related antigen, neutrophil elastase, TRP-2, CYP1B1, sperm protein 17, beta human chorionic gonadotropin, AFP, thyroglobulin, PLAC1, globoH, RAGE1, MN-CA IX, human telomerase reverse transcriptase, intestinal carboxyl esterase, mut hsp 70-2, NA-17, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, NY-ESO-1, GPR20, Ly6k, OR51E2, TARP, GFR α 4, or a peptide of any of these antigens presented on MHC; and/or

(II) the antigen binding domain comprises a VH and a VL, wherein the VH and VL are connected by a linker, optionally wherein the linker comprises the amino acid sequence of SEQ ID NO: 63 or 104; and/or

(III) (a) the transmembrane domain comprises a transmembrane domain of a protein chosen from the alpha, beta or zeta chain of T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154,

(b) the transmembrane domain comprises a transmembrane domain of CD8,

(c) the transmembrane domain comprises the amino acid sequence of SEQ ID NO: 6, or an amino acid sequence having at least about 85%, 90%, 95%, or 99% sequence identity thereof, or

(d) the nucleic acid molecule comprises a nucleic acid sequence encoding the transmembrane domain, wherein the nucleic acid sequence comprises the nucleic acid sequence of SEQ ID NO: 17, or a nucleic acid sequence having at least about 85%, 90%, 95%, or 99% sequence identity thereof; and/or

(IV) the antigen binding domain is connected to the transmembrane domain by a hinge region, optionally wherein:

(a) the hinge region comprises the amino acid sequence of SEQ ID NO: 2, 3, or 4, or an amino acid sequence having at least about 85%, 90%, 95%, or 99% sequence identity thereof, or

(b) the nucleic acid molecule comprises a nucleic acid sequence encoding the hinge region, wherein the nucleic acid sequence comprises the nucleic acid sequence of SEQ ID NO: 13, 14, or 15, or a nucleic acid sequence having at least about 85%, 90%, 95%, or 99% sequence identity thereof; and/or

(V) the intracellular signaling domain comprises a primary signaling domain, optionally wherein the primary signaling domain comprises a functional signaling domain derived from CD3 zeta, TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, CD278 (ICOS), FcεRI, DAP10, DAP12, or CD66d, optionally wherein:

(a) the primary signaling domain comprises a functional signaling domain derived from CD3 zeta,

(b) the primary signaling domain comprises the amino acid sequence of SEQ ID NO: 9 or 10, or an amino acid sequence having at least about 85%, 90%, 95%, or 99% sequence identity thereof, or

(c) the nucleic acid molecule comprises a nucleic acid sequence encoding the primary signaling domain, wherein the nucleic acid sequence comprises the nucleic acid sequence of SEQ ID NO: 20 or 21, or a nucleic acid sequence having at least about 85%, 90%, 95%, or 99% sequence identity thereof; and/or

(VI) the intracellular signaling domain comprises a costimulatory signaling domain, optionally wherein the costimulatory signaling domain comprises a functional signaling domain derived from a MHC class I molecule, a TNF receptor protein, an Immunoglobulin-like protein, a cytokine receptor, an integrin, a signaling lymphocytic activation molecule (SLAM protein), an activating NK cell receptor, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, 4-1BB (CD137), B7-H3, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, CD28-OX40, CD28-4-1BB, or a ligand that specifically binds with CD83, optionally wherein:

(a) the costimulatory signaling domain comprises a functional signaling domain derived from 4-1BB,

(b) the costimulatory signaling domain comprises the amino acid sequence of SEQ ID NO: 7, or an amino acid sequence having at least about 85%, 90%, 95%, or 99% sequence identity thereof, or

(c) the nucleic acid molecule comprises a nucleic acid sequence encoding the costimulatory signaling domain, wherein the nucleic acid sequence comprises the nucleic acid sequence of SEQ ID NO: 18, or a nucleic acid sequence having at least about 85%, 90%, 95%, or 99% sequence identity thereof; and/or

(VII) the intracellular signaling domain comprises a functional signaling domain derived from 4-1BB and a functional signaling domain derived from CD3 zeta, optionally wherein the intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 7 (or an amino acid sequence having at least about 85%, 90%, 95%, or 99% sequence identity thereof) and the amino acid sequence of SEQ ID NO: 9 or 10 (or an amino acid sequence having at least about 85%, 90%, 95%, or 99% sequence identity

thereof), optionally wherein the intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 7 and the amino acid sequence of SEQ ID NO: 9 or 10; and/or

(VIII) the CAR further comprises a leader sequence comprising the amino acid sequence of SEQ ID NO: 1.

19. The method of claim 18, wherein the antigen binding domain comprises a complementarity determining region (CDR), heavy chain variable region (VH), light chain variable region (VL), scFv or CAR sequence, wherein:

(a) the antigen binding domain binds to BCMA and comprises a CDR, VH, VL, scFv or CAR sequence disclosed in Tables 3-15, or a sequence having at least 80%, 85%, 90%, 95%, or 99% identity thereto;

(b) the antigen binding domain binds to CD19 and comprises a CDR, VH, VL, scFv or CAR sequence disclosed in Table 2, or a sequence having at least 80%, 85%, 90%, 95%, or 99% identity thereto;

(c) the antigen binding domain binds to CD20 and comprises a CDR, VH, VL, scFv or CAR sequence disclosed herein, or a sequence having at least 80%, 85%, 90%, 95%, or 99% identity thereto; or

(d) the antigen binding domain binds to CD22 and comprises a CDR, VH, VL, scFv or CAR sequence disclosed herein, or a sequence having at least 80%, 85%, 90%, 95%, or 99% identity thereto.

20. The method of any one of claims 1-19, wherein the CAR comprises:

(I) an antigen binding domain, a transmembrane domain, and an intracellular signaling domain, wherein the antigen binding domain binds to CD19 and comprises a heavy chain complementarity determining region 1 (HC CDR1), an HC CDR2, an HC CDR3, a light chain complementarity determining region 1 (LC CDR 1), an LC CDR2, and an LC CDR3, wherein:

(a) the HC CDR1 comprises the amino acid sequence of SEQ ID NO: 295;

(b) the HC CDR2 comprising the amino acid sequence of SEQ ID NO: 296;

(c) the HC CDR3 comprising the amino acid sequence of SEQ ID NO: 297;

(d) the LC CDR1 comprising the amino acid sequence of SEQ ID NO: 298;

(e) the LC CDR2 comprising the amino acid sequence of SEQ ID NO: 299; and

(f) the LC CDR3 comprising the amino acid sequence of SEQ ID NO: 300;

optionally wherein the CAR comprises the amino acid sequence of SEQ ID NO: 301, or a sequence having at least 80%, 85%, 90%, 95%, or 99% identity thereto; and/or the nucleic acid molecule encoding the CAR comprises the nucleotide sequence of SEQ ID NO: 302, or a sequence having at least 80%, 85%, 90%, 95%, or 99% identity thereto; or

(II) an antigen binding domain, a transmembrane domain, and an intracellular signaling domain, wherein the antigen binding domain binds to BCMA and comprises a HC CDR1, an HC CDR2, an HC CDR3, a LC CDR 1, an LC CDR2, and an LC CDR3, wherein:

(i) the HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2 and LC CDR3 sequences comprise the amino acid sequences of SEQ ID NO: 86, 87, 88, 95, 96, and 97, respectively;

(ii) the HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2 and LC CDR3 sequences comprise the amino acid sequences of SEQ ID NO: 47, 89, 88, 98, 99, and 100, respectively;

(iii) the HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2 and LC CDR3 sequences comprise the amino acid sequences of SEQ ID NO: 90, 91, 92, 101, 99, and 97, respectively;

optionally wherein, the CAR comprises the amino acid sequence of SEQ ID NO: 107, or a sequence having at least 80%, 85%, 90%, 95%, or 99% identity thereto; and/or the nucleic acid molecule encoding the CAR comprises the nucleotide sequence of SEQ ID NO: 108, or a sequence having at least 80%, 85%, 90%, 95%, or 99% identity thereto.

21. A population of CAR-expressing cells made by the method of any one of claims 1-20, optionally wherein the population of CAR-expressing cells are autologous or allogeneic CAR-expressing T cells or NK cells.

22. A pharmaceutical composition comprising the population of CAR-expressing cells of claim 21 and a pharmaceutically acceptable carrier.

23. Use of the population of CAR-expressing cells of claim 21 or the pharmaceutical composition of claim 22, in the manufacture of a medicament for:

(a) increasing an immune response in a subject; and/or

(b) treating a cancer in a subject, optionally wherein:

(i) the cancer is a solid cancer, optionally wherein the solid cancer is chosen from: one or more of mesothelioma, malignant pleural mesothelioma, non-small cell lung cancer, small cell lung cancer, squamous cell lung cancer, large cell lung cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, esophageal adenocarcinoma, breast cancer, glioblastoma, ovarian cancer, colorectal cancer, prostate cancer, cervical cancer, skin cancer, melanoma, renal cancer, liver cancer, brain cancer, thymoma, sarcoma, carcinoma, uterine cancer, kidney cancer, gastrointestinal cancer, urothelial cancer, pharynx cancer, head and neck cancer, rectal cancer, esophagus cancer, or bladder cancer, or a metastasis thereof;

(ii) the cancer is a liquid cancer, optionally wherein the liquid cancer is chosen from: chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), multiple myeloma, acute lymphoid leukemia (ALL), Hodgkin lymphoma, B-cell acute lymphoid leukemia (BALL), T-cell acute lymphoid leukemia (TALL), small lymphocytic lymphoma (SLL), B cell prolymphocytic

leukemia, blastic plasmacytoid dendritic cell neoplasm, Burkitt's lymphoma, diffuse large B cell lymphoma (DLBCL), DLBCL associated with chronic inflammation, chronic myeloid leukemia, myeloproliferative neoplasms, follicular lymphoma, pediatric follicular lymphoma, hairy cell leukemia, small cell- or a large cell-follicular lymphoma, malignant lymphoproliferative conditions, MALT lymphoma (extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue), Marginal zone lymphoma, myelodysplasia, myelodysplastic syndrome, non-Hodgkin lymphoma, plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, Waldenstrom macroglobulinemia, splenic marginal zone lymphoma, splenic lymphoma/leukemia, splenic diffuse red pulp small B-cell lymphoma, hairy cell leukemia-variant, lymphoplasmacytic lymphoma, a heavy chain disease, plasma cell myeloma, solitary plasmocytoma of bone, extraosseous plasmocytoma, nodal marginal zone lymphoma, pediatric nodal marginal zone lymphoma, primary cutaneous follicle center lymphoma, lymphomatoid granulomatosis, primary mediastinal (thymic) large B-cell lymphoma, intravascular large B-cell lymphoma, ALK+ large B-cell lymphoma, large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease, primary effusion lymphoma, B-cell lymphoma, acute myeloid leukemia (AML), or unclassifiable lymphoma; or
 (iii) the cancer is selected from CLL, SLL, DLBCL, or relapsed and/or refractory DLBCL.

24. The use of claim 23, wherein:

- (i) the use further comprising use of a second therapeutic agent to the subject; and/or
- (ii) the population of CAR-expressing cells is formulated for administration at a dose determined based on the percentage of CAR-expressing cells measured in claim 16.

25. An antibody molecule that binds CD28, comprising a heavy chain variable region (VH) comprising a heavy chain complementarity determining region 1 (HCDR1), a HCDR2, and a HCDR3, and a light chain variable region (VL) comprising a light chain complementarity determining region 1 (LCDR1), a LCDR2, and a LCDR3, wherein:

- (i) the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprise the amino acid sequences of SEQ ID NOs: 538, 539, 540, 530, 531, and 532, respectively;
- (ii) the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprise the amino acid sequences of SEQ ID NOs: 541, 539, 540, 530, 531, and 532, respectively;
- (iii) the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprise the amino acid sequences of SEQ ID NOs: 542, 543, 540, 533, 534, and 535, respectively; or
- (iv) the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 comprise the amino acid sequences of SEQ ID NOs: 544, 545, 546, 536, 534, and 532, respectively.

26. The antibody molecule of claim 25, comprising:

(i) a VH comprising the amino acid sequence of SEQ ID NO: 547 or 548, or a sequence with at least 95% sequence identity to SEQ ID NO: 547 or 548;

(ii) a VL comprising the amino acid sequence of SEQ ID NO: 537, or a sequence with at least 95% sequence identity thereto;

(iii) a VH comprising the amino acid sequence of SEQ ID NO: 547 or a sequence with at least 95% sequence identity thereto, and a VL comprising the amino acid sequence of SEQ ID NO: 537, or a sequence with at least 95% sequence identity thereto; or

(iv) a VH comprising the amino acid sequence of SEQ ID NO: 548 or a sequence with at least 95% sequence identity thereto, and a VL comprising the amino acid sequence of SEQ ID NO: 537, or a sequence with at least 95% sequence identity thereto.

27. The antibody molecule of claim 25 or 26, which is a full length antibody, a bispecific antibody, Fab, F(ab')₂, Fv, or a single chain Fv fragment (scFv).

28. The antibody molecule of any one of claims 25-27, comprising a heavy chain constant region selected from IgG1, IgG2, IgG3, and IgG4, and a light chain constant region chosen from the light chain constant regions of kappa or lambda.

29. An antibody molecule that:

(i) competes for binding to CD28 with the antibody molecule of any one of claims 25-28; and/or

(ii) binds to the same epitope as, substantially the same epitope as, an epitope that overlaps with, or an epitope that substantially overlaps with, the epitope of the antibody molecule of any one of claims 25-28.

30. An isolated nucleic acid molecule encoding the antibody molecule of any one of claims 25-29.

31. A multispecific binding molecule comprising:

(i) an anti-CD3 binding domain and an anti-CD28 binding domain comprising the antibody molecule of any one of claims 25-29, and

(ii) a first binding domain and a second binding domain, wherein the multispecific binding molecule comprises a first polypeptide comprising from N-terminal to C-terminal: VH of the first binding domain, VL of the first binding domain, VH of the second binding domain, CH1, CH2, and CH3; and a second polypeptide comprising from N-terminal to C-terminal: VL of the second binding domain and CL;

(iii) a first binding domain and a second binding domain, wherein the multispecific binding molecule comprises a first polypeptide comprising from N-terminal to C-terminal: VH of the second binding domain, CH1, CH2, CH3, VH of the first binding domain, and VL of the first binding domain; and a

second polypeptide comprising from N-terminal to C-terminal: VL of the second binding domain and CL;
or

(iv) a first binding domain and a second binding domain, wherein the multispecific binding molecule comprises a first polypeptide comprising from N-terminal to C-terminal: VH of the second binding domain, CH1, VH of the first binding domain, VL of the first binding domain, CH2, and CH3; and a second polypeptide comprising from N-terminal to C-terminal: VL of the second binding domain and CL.

32. The multispecific binding molecule of claim 31, wherein:

(a) the first binding domain comprises an anti-CD3 binding domain and the second binding domain comprises a costimulatory molecule binding domain; or

(b) the first binding domain comprises a costimulatory molecule binding domain and the second binding domain comprises an anti-CD3 binding domain.

33. The multispecific binding molecule of claim 32, wherein the costimulatory molecule binding domain comprises an anti-CD2 binding domain or an anti-CD28 binding domain.

34. The multispecific binding molecule of any one of claims 31-33, wherein the anti-CD3 binding domain comprises:

(i) a HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 of an anti-CD3 antibody molecule of Table 27 (for example the anti-CD3 (1), anti-CD3 (2), anti-CD3 (3), or anti-CD3 (4)); or

(ii) the amino acid sequence of any VH and/or VL region of an anti-CD3 antibody molecule provided in Table 27 (for example the anti-CD3 (1), anti-CD3 (2), anti-CD3 (3), or anti-CD3 (4)), or an amino acid sequence at least 95% identical thereto.

35. A method of activating cells (e.g., immune effector cells, e.g., T cells), comprising contacting (for example, binding) a population of cells (for example, T cells, for example, T cells isolated from a frozen or fresh leukapheresis product) with the multispecific binding molecule of any one of claims 31-34, or the antibody molecule of any one of claims 25-29.

36. A method of transducing cells (e.g., immune effector cells, e.g., T cells), comprising contacting (for example, binding) a population of cells (for example, T cells, for example, T cells isolated from a frozen or fresh leukapheresis product) with (i) the multispecific binding molecule of any one of claims 31-34, or the antibody molecule of any one of claims 25-29 and (ii) a nucleic acid molecule, e.g., a nucleic acid molecule encoding a CAR.