

CLAIMS

1. A plasmid for use with clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) integration systems wherein the plasmid comprises in order a left homology arm, a polynucleotide sequence encoding a chimeric antigen receptor (CAR) polypeptide, and a right homology arm; wherein the left and right homology arms are each 30bp to 1000bp in length.
2. The plasmid of claim 1, wherein the CAR polypeptide comprises a transmembrane domain, a co-stimulatory domain, a CD3 ζ signaling domain, and a single-chain variable fragment (scFV) that specifically binds to a receptor on a target cell, wherein the transmembrane domain of the CAR polypeptide comprises a CD4 transmembrane domain, a CD8 transmembrane domain, a CD28 transmembrane domain, a CD3 ζ transmembrane domain, or an NKG2D transmembrane domain, and wherein the co-stimulatory domain of the CAR polypeptide comprises a 2B4 domain, a CD28 co-stimulatory domain, a 4-1 BB co-stimulatory domain, or any combination thereof.
3. The plasmid of claim 2, wherein the receptor comprises CD33.
4. The plasmid of claim 3, wherein scFV specifically binding to CD33 comprises a sequence at least 90% identical to SEQ ID NO: 29 or a fragment thereof.
5. The plasmid of any one of claims 1-4, wherein the homology arms specifically hybridize to the Adeno-Associated Virus Integration Site 1 (AAVS1) of chromosome 19 of humans.
6. An Adeno-associated viral (AAV) vector comprising the plasmid of any one of claims 1-5.
7. The AAV vector of claim 6, wherein the serotype of the AAV comprises AAV6.
8. The AAV vector of claim 7, wherein the vector further comprises a plasmid encoding a crRNA, tracer RNA (trcrRNA), and a CAS endonuclease.

9. The AAV vector of any of claims 6-8, wherein the vector is a single stranded AAV (ssAAV).
10. The AAV vector of any of claims 6-8, wherein the vector is a self-complimentary AAV (scAAV).
11. The AAV vector of any of claims 6-10 , wherein the vector comprises a sequence at least 90% identical to SEQ ID NO: 22 or SEQ ID NO: 23 or a fragment thereof.
12. A modified cell comprising the plasmid of any one of claims 1-5 or the AAV vector of any one of claims 6-11, wherein the modified cell is a natural killer (NK) cell or NK T cell.
13. The modified cell of claim 12, wherein the NK cell or NK T cell has been expanded in the presence of irradiated feeder cells, plasma membrane particles, or exosomes expressing membrane bound IL-21, membrane bound 4-1BBL, and/or membrane bound IL-15 or any combination thereof.
14. Use of a modified cell of claim 12 or 13 in the manufacture of a medicament for treating a cancer in a subject.
15. A method of genetically modifying a Natural Killer (NK) cell or a NK T cell, comprising
 - a) obtaining a ribonucleoprotein (RNP) complex comprising a class 2 CRISPR/Cas endonuclease complexed with a corresponding CRISPR/Cas guide RNA and an AAV vector comprising a plasmid comprising a polynucleotide sequence encoding a chimeric antigen receptor (CAR) polypeptide; wherein the polynucleotide sequence is flanked by homology arms; and wherein the homology arms are 800 bp in length or less; and
 - b) introducing the polynucleotide sequence encoding the CAR polypeptide and the RNP complex into the NK cell or NK T cell; wherein the polynucleotide sequence encoding the CAR polypeptide is introduced into the NK cell or NK T cell via infection with the AAV into the cell; wherein the RNP complex hybridizes to a target sequence

within the genomic DNA of the NK cell or NK T cells and the cell's DNA repair enzymes insert the polynucleotide sequence encoding the CAR polypeptide into the host genome at the target sequence within the genomic DNA of the NK cell or NK T cell thereby creating a modified NK cell or a modified NK T cell.

16. The method of claim 15, wherein the NK cell or NK T cell is a primary cell or an expanded cell, wherein the primary cell is incubated for about 4 to 10 days in the presence of IL-2 prior to infection or wherein the primary cell is expanded for about 4 to 10 days in the presence of irradiated feeder cells, plasma membrane particles, or exosomes prior to infection, wherein the irradiated feeder cells, plasma membrane particles, or exosomes express membrane bound 4-1BBL, membrane-bound IL-21, or membrane-bound IL-15, or any combination thereof.

17. The method of claim 15 or 16, wherein the CAR polypeptide comprises a transmembrane domain, a co-stimulatory domain, a CD3 ζ signaling domain, and a single-chain variable fragment (scFV) that specifically binds to a receptor on a target cell, wherein the transmembrane domain of the CAR polypeptide comprises a CD4 transmembrane domain, a CD8 transmembrane domain, a CD28 transmembrane domain, a CD3 ζ transmembrane domain, or an NKG2D transmembrane domain and wherein the co-stimulatory domain of the CAR polypeptide comprises a 2B4 domain, a CD28 co-stimulatory domain, a 4-1 BB co-stimulatory domain, or any combination thereof.

18. The method of claim 17, wherein the receptor comprises CD33.

19. The method of any one of claims 15-18, wherein the homology arms specifically hybridize to the Adeno-Associated Virus Integration Site 1 (AAVS1) of chromosome 19 of humans.

20. A method of creating a chimeric antigen receptor (CAR) natural killer (NK) cell or a CAR NK T cell comprising

a) obtaining a ribonucleoprotein (RNP) complex comprising a class 2 CRISPR/Cas endonuclease complexed with a corresponding CRISPR/Cas guide RNA and an AAV vector comprising a plasmid comprising a polynucleotide sequence encoding a chimeric antigenreceptor (CAR) polypeptide; wherein the polynucleotide sequence is flanked by

homology arms; and wherein the homology arms are 1000bp in length or less; and

b) introducing the polynucleotide sequence encoding the CAR polypeptide and the RNP complex into an NK cell or an NK T cell; wherein the polynucleotide sequence encoding the CAR polypeptide is introduced into the NK cell or NK T cell via infection with the AAV into the NK cell or NK T cell; wherein the RNP complex hybridizes to a target sequence within the genomic DNA of the NK cell or NK T cell and the DNA repair enzymes of the NK cell or NK T cell insert the polynucleotide sequence encoding the CAR polypeptide into the host genome at the target sequence within the genomic DNA of the cell thereby creating a CAR NK cell or CAR NK T cell.

21. The method of claim 20, wherein the NK cells or NK T cells are primary or expanded NK cells or NK T cells, and wherein the primary NK cells or NK T cells are incubated for about 4 to 10 days in the presence of IL-2 prior to infection or are expanded for about 4 to 10 days in the presence of irradiated feeder cells, plasma membrane particles, or exosomes prior to infection, wherein the irradiated feeder cells, plasma membrane particles, or exosomes express membrane bound 4-1BBL, membrane-bound IL-21, or membrane-bound IL-15 or any combination thereof.

22. The method of claim 20 or 21 , further comprising expanding the CAR NK cell or CAR NK T cell with IL-2 or with irradiated feeder cells, plasma membrane particles, or exosomes following infection, wherein the irradiated feeder cells, plasma membrane particles, or exosomes express membrane bound 4-1BBL, membrane-bound IL-21, or membrane-bound IL-15 or any combination thereof.

23. The method of any one of claims 20-22, wherein the NK cell or NK T cell is infected with about 5 to 500K MOI of the AAV.

24. The method of any one of claims 20-23, wherein the RNP complex is introduced into the NK cell or NK T cell via electroporation or via transfection; and wherein the RNP complex is encoded on the same or a different AAV from that which comprises the polynucleotide sequence.

25. The method of any one of claims 20-24, wherein the CAR polypeptide comprises a

transmembrane domain, a co-stimulatory domain, a CD3 ζ signaling domain, and a single-chain variable fragment (scFV) that specifically binds to a receptor on a target cell, wherein the transmembrane domain of the CAR polypeptide comprises a CD4 transmembrane domain, a CD8 transmembrane domain, a CD28 transmembrane domain, a CD3 ζ transmembrane domain, or a NKG2D transmembrane domain and/or wherein the co-stimulatory domain of the CAR polypeptide comprises a 2B4 domain, a CD28 co-stimulatory domain, a 4-1 BB co-stimulatory domain, or any combination thereof.

26. The method of claim 25, wherein the receptor comprises CD33.

27. The method of any one of claims 20-26, wherein the homology arms specifically hybridize to the Adeno-Associated Virus Integration Site 1 (AAVS1) of chromosome 19 of humans.

28. The method of any one of claims 20-27, wherein the serotype of the AAV comprises AAV6.

29. The method of any one of claims 20-28, wherein the vector is a single stranded AAV (ssAAV) or a self-complimentary AAV (scAAV).

30. The method of any one of claims 20-29, wherein the vector comprises a sequence at least 90% identical to SEQ ID NO: 22 or SEQ ID NO: 23 or a fragment thereof.

31. A natural killer (NK) cell or NK T cell for use in treating cancer, wherein the NK cell or NK T cell comprises a plasmid for use with clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) integration systems wherein the plasmid comprises in order a left homology arm, a polynucleotide sequence encoding a chimeric antigen receptor (CAR) polypeptide, and a right homology arm; wherein the left and right homology arms are each 30bp to 1000bp in length, wherein the CAR polypeptide comprises a transmembrane domain, a co-stimulatory domain, a CD3 ζ signaling domain, and a single-chain variable fragment (scFV) that specifically binds to a receptor on a target cell, wherein the transmembrane domain of the CAR polypeptide comprises a CD4 transmembrane domain, a CD8 transmembrane domain, a

CD28 transmembrane domain, a CD3 ζ transmembrane domain, or an NKG2D transmembrane domain, wherein the co-stimulatory domain of the CAR polypeptide comprises a 2B4 domain, a CD28 co-stimulatory domain, a 4-1 BB co-stimulatory domain, or any combination thereof, and wherein the receptor comprises CD33.

32. The NK cell or NK T cell of claim 31, wherein scFV specifically binding to CD33 comprises a sequence at least 90% identical to SEQ ID NO: 29 or a fragment thereof.

33. The NK cell or NK T cell of claim 31 or 32, wherein the homology arms specifically hybridize to the Adeno-Associated Virus Integration Site 1 (AAVS1) of chromosome 19 of humans.

34. A plasmid for use with clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) integration systems, wherein the plasmid comprises a polynucleotide sequence encoding a chimeric antigen receptor (CAR) polypeptide; wherein the polynucleotide sequence is adjacent to one protospacer adjacent motif (PAM) and one polynucleotide sequence encoding crispr RNA (crRNA) or flanked by two PAMs and two polynucleotide sequences that encode crRNAs.

35. The plasmid of claim 34, wherein the plasmid comprises in order a first PAM sequence and a first polynucleotide sequence that encodes crRNA, the polynucleotide sequence encoding the CAR polypeptide, and a second PAM sequence and a second polynucleotide sequence that encodes crRNA, wherein the CAR polypeptide comprises a transmembrane domain, a co-stimulatory domain, a CD3 ζ signaling domain, and a single-chain variable fragment (scFV) that specifically binds to a receptor on a target cell, wherein the transmembrane domain of the CAR polypeptide comprises a CD4 transmembrane domain, a CD8 transmembrane domain, a CD28 transmembrane domain, a CD3 ζ transmembrane domain, or an NKG2D transmembrane domain, and wherein the co-stimulatory domain of the CAR polypeptide comprises a 2B4 domain, a CD28 co-stimulatory domain, a 4-1 BB co-stimulatory domain, or any combination thereof.

36. The plasmid of claim 35, wherein the receptor comprises CD33.

37. An Adeno-associated viral (AAV) vector comprising the plasmid of any one of claims 34-36.
38. A modified cell comprising the plasmid of any one of claims 34-36 or the AAV vector of claim 37, wherein the modified cell is a natural killer (NK) cell or NK T cell.
39. The modified cell of claim 38, wherein the NK cell or NK T cell has been expanded in the presence of irradiated feeder cells, plasma membrane particles, or exosomes expressing membrane bound IL-21, membrane bound 4-1BBL, and/or membrane bound IL-15 or any combination thereof.
40. A method of creating a chimeric antigen receptor (CAR) natural killer (NK) cell or NK T cell comprising
- a) obtaining a ribonucleoprotein (RNP) complex comprising a class 2 CRISPR/Cas endonuclease complexed with a corresponding CRISPR/Cas guide RNA and an AAV vector comprising a plasmid comprising a polynucleotide sequence encoding a chimeric antigen receptor (CAR); wherein the polynucleotide sequence is adjacent to one protospacer adjacent motif (PAM) and one polynucleotide sequence encoding crispr RNA (crRNA) or flanked by two PAMs and two polynucleotide sequences that encode crRNAs; and
 - b) introducing the polynucleotide sequence encoding the CAR polypeptide and the RNP complex into the NK cell or NK T cell; wherein the plasmid is introduced into the cell via infection with the Adeno-associated virus (AAV) into a target cell; wherein in the ribonucleoprotein (RNP) complex hybridizes to a target sequence within the genomic DNA of the cell, and the cell's DNA repair enzymes insert the polynucleotide encoding the CAR into the host genome at the target sequence, thereby creating a CAR NK cell or CAR NK T cell.
41. A method of genetically modifying a natural killer (NK) cell or NK T cell comprising
- a) obtaining a ribonucleoprotein (RNP) complex comprising a class 2 CRISPR/Cas

endonuclease complexed with a corresponding CRISPR/Cas guide RNA and an AAV vector comprising a plasmid comprising a polynucleotide sequence encoding a chimeric antigen receptor (CAR); wherein the polynucleotide sequence is adjacent to one PAM and one polynucleotide sequence encoding crRNA or flanked by two PAMs and two polynucleotide sequences encoding crRNAs; and

b) introducing the polynucleotide sequence encoding the CAR polypeptide and the RNP complex into the NK cell or NK T cell; wherein the polynucleotide sequence encoding the CAR polypeptide is introduced into the cell via infection with the Adeno-associated virus (AAV) into a target cell; wherein in the ribonucleoprotein (RNP) complex hybridizes to a target sequence within the genomic DNA of the cell, and the cell's DNA repair enzymes insert the polynucleotide sequence encoding the chimeric antigen receptor (CAR) into the host genome at the target sequence, thereby creating a modified cell.

42. The method of claim 40 or 41, wherein the plasmid comprises in order a first PAM sequence and a first polynucleotide sequence encoding crRNAs, the polynucleotide sequence encoding the CAR polypeptide, and a second PAM sequence and a second polynucleotide sequence encoding crRNA.