

SYNTHETIC CYTOKINE SIGNALING SYSTEM, PREPARATION METHOD, AND USES THEREOF

BACKGROUND

5 [0001] Adoptive T-cell therapy (ACT), which utilizes a patient's own immune cells to target and eliminate tumor cells, has demonstrated remarkable success in a number of hematological malignancy, but with limited efficacy in solid tumors. Among many factors that might be responsible for such low efficacy is poor persistence of adoptively transferred T cells. Interleukin 2, i.e., IL-2 (or IL2), is a T cell growth and survival factor. Clinical administration
10 of high-dose IL-2 supports the growth and persistence of adoptively transferred T cells and potentiates anti-tumor efficacy. While necessary for T cell expansion and persistence, high dose IL-2 is often associated systemic toxicity, such as fever, chills, hypotension, etc. As such, there is a need to develop an approach that can bypass the IL2 dependence for T cell expansion and persistence.

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SUMMARY

[0002] In order to address the IL2 dependency issue associated with existing adoptive T-cell therapies and other immunotherapies, the present disclosure provides a synthetic cytokine signaling system, its preparation method, and its usage for engineering or modifying immune
20 cells such as T cells. Immune cells modified with the synthetic cytokine signaling system display a variety of improved characteristics. Compared with non-modified immune cells, the modified immune cells exhibit increased proliferation capability and viability, and in particular the capability of expansion *in vitro* in the absence of, or in the presence of only a reduced level of a cytokine supplement (such as IL-2, IL-4, IL-7, IL-9, IL-15, or IL-21), and also exhibit
25 increased interferon gamma (IFN γ) secretion and increased cytotoxicity against co-cultured target cells in the absence of IL-2 stimulation. When transferred *in vivo* into a tumor-bearing subject, the modified immune cells also exhibit more preferred migration to tumor tissues and/or lymphoid tissues within the body of the subject, and/or displays increased tumor control ability, compared to non-modified immune cells.

30 [0003] Firstly, this present disclosure provides an engineered cytokine signaling chain, which encodes a transmembrane polypeptide when expressed in an immune cell. The transmembrane polypeptide comprises an extracellular portion and an intracellular portion, which are operably connected by a transmembrane portion. The intracellular portion comprises a variant of IL9R

ICD, comprising at least one of a first sequence alteration in a first region corresponding positions 174-230, or a second sequence alteration in a second region corresponding positions 142-151, of the sequence as set forth in SEQ ID NO: 1 (i.e. wildtype IL9R ICD). Herein, the "variant of IL9R ICD" refers to a version of IL9R ICD that comprises at least one sequence alteration compared with the wildtype IL9R ICD (SEQ ID NO: 1). The wildtype IL9R ICD (SEQ ID NO: 1) as used herein corresponds to positions 292-521 of the full-length wildtype IL9R (SEQ ID NO: 2).

[0004] Herein optionally, each of the first sequence alteration or the second sequence alteration may comprise a truncation or a substitution. According to some embodiments, the first sequence alteration may comprise a truncation at positions 174-230 of the sequence as set forth in SEQ ID NO: 1. According to some embodiments, the second sequence alteration may comprise a substitution in the second region at positions 142-151 of the sequence as set forth in SEQ ID NO: 1, such as a change of the whole second region at positions 142-151 to GD (i.e. GlyAsp), but can also have other options such as substitution to GE (i.e. GlyGlu), etc. According to some embodiments of the engineered cytokine signaling chain, the intracellular portion comprises a sequence as set forth in SEQ ID NO: 7, involving both the truncation at positions 174-230 and the substitution of amino acid residues at positions 142-151 to GD, and this specific variant is termed "IL9R ICD*" hereinafter.

[0005] In the engineered cytokine signaling chain disclosed herein, the extracellular portion may comprise an extracellular domain (ECD) of a cytokine receptor chain or a functional variant thereof. Herein optionally, the cytokine receptor chain can be IL2R β (or IL2Rb), IL4R α (or IL4Ra or IL4R), IL7R α (or IL7Ra or IL17R), IL9R, or IL21R, but can also be a cytokine receptor chain other than these cytokine receptors.

[0006] According to some embodiments of the engineered cytokine signaling chain, the cytokine receptor chain is IL2R β , and the extracellular portion may comprise a sequence having at least 70% (e.g. 70%, 72%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 3 (i.e. wildtype IL2R β ECD). The wildtype IL2R β ECD (SEQ ID NO: 3) as used herein corresponds to positions 27-240 of the full-length wildtype IL2R β (SEQ ID NO: 4).

[0007] According to some other embodiments of the engineered cytokine signaling chain, the cytokine receptor chain is IL4Ra, and the extracellular portion may comprise a sequence having at least 70% (e.g. 70%, 72%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 33 (i.e. wildtype IL4Ra ECD).

[0008] According to yet some other embodiments of the engineered cytokine signaling chain, the cytokine receptor chain is IL7Ra, and the extracellular portion may comprise a sequence having at least 70% (e.g. 70%, 72%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 5 (i.e. wildtype IL7Ra ECD). The wildtype IL7Ra (i.e. IL7R α or IL7R) ECD (SEQ ID NO: 5) as used herein corresponds to positions 21-239 of the full-length wildtype IL7Ra (SEQ ID NO: 6).

[0009] According to yet some other embodiments of the engineered cytokine signaling chain, the cytokine receptor chain is IL9R, and the extracellular portion may comprise a sequence having at least 70% (e.g. 70%, 72%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 36 (i.e. wildtype IL9R ECD).

[0010] According to yet some other embodiments of the engineered cytokine signaling chain, the cytokine receptor chain is IL21R, and the extracellular portion may comprise a sequence having at least 70% (e.g. 70%, 72%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 39 (i.e. wildtype IL21R ECD).

[0011] In any of the embodiments of the engineered cytokine signaling chain described above, the transmembrane portion is functionally compatible to both the extracellular portion and the intracellular portion of the engineered cytokine signaling chain.

[0012] Herein optionally, the transmembrane (TM) domain may comprise a transmembrane domain from the same cytokine receptor chain as in the extracellular portion. According to some embodiments, the cytokine receptor chain is IL2R β , and the transmembrane domain of the transmembrane portion comprises a sequence as set forth in SEQ ID NO: 19 (i.e. wildtype IL2R β TM). According to some other embodiments, the cytokine receptor chain is IL4Ra, and the transmembrane domain of the transmembrane portion comprises a sequence as set forth in SEQ ID NO: 34 (i.e. wildtype IL4Ra TM). According to yet some other embodiments, the cytokine receptor chain is IL7Ra, and the transmembrane domain of the transmembrane portion comprises a sequence as set forth in SEQ ID NO: 24 (i.e. wildtype IL7Ra TM). According to yet some other embodiments, the cytokine receptor chain is IL9R, and the transmembrane domain of the transmembrane portion comprises a sequence as set forth in SEQ ID NO: 37 (i.e. wildtype IL9R TM). According to yet some other embodiments, the cytokine receptor chain is IL21R, and the transmembrane domain of the transmembrane portion comprises a sequence as set forth in SEQ ID NO: 40 (i.e. wildtype IL21R TM).

[0013] Further optionally, the transmembrane domain may comprise a transmembrane domain from a polypeptide other than the cytokine receptor chain. For example, the transmembrane

domain may be a TM from a known transmembrane protein (e.g. CD4, CD8, etc.).

[0014] Depending on different embodiments of the present disclosure, the engineered cytokine signaling chain may have different structures to be either in a self-activating form or in a non-self-activating form.

- 5 [0015] According to some embodiments, the engineered cytokine signaling chain takes a self-activating form. As such, the extracellular portion further comprises a cytokine ligand domain (CLD) over an N-terminus of the ECD of the cytokine receptor chain or the functional variant thereof. Herein, the CLD is configured to be functionally compatible to the ECD of the cytokine receptor chain or the functional variant thereof. As used herein, the term "functionally compatible to" means that a CLD can functionally interact with an ECD of the cytokine receptor chain or a functional variant thereof inter- or intra-molecularly, in turn causing an intracellular domain of the cytokine receptor chain, regardless of being engineered (e.g. IL9R(ICD*)) or non-engineered (e.g. wildtype cytokine receptor), to be functionally activated. Optionally, such CLD may be a cytokine ligand or a functional variant thereof that functionally corresponds to the ECD of the cytokine receptor chain of a functional variant thereof. Examples include IL2 or IL15 corresponding to IL2R β , IL4 corresponding to IL4R α , IL7 corresponding to IL7R α , IL9 corresponding to IL9R, and IL21 corresponding to IL21R, etc. Alternatively the CLD may include another peptide sequence having a cytokine ligand function (e.g. artificial peptide sequence). In these specific embodiments of the engineered cytokine signaling chain, the CLD can substantially intramolecularly activate the engineered cytokine signaling chain expressing on the plasma membrane of an immune cell, which mechanistically bind with the ECD of the cytokine receptor chain or of a functional variant thereof in the extracellular domain of the the engineered cytokine signaling chain, thereby causing the intracellular domain of the engineered cytokine receptor chain (i.e. IL9R(ICD*)) to be functionally activated.
- 25 [0016] According to some embodiments, the cytokine receptor chain is IL2R β , and the CLD comprises IL15 or a functional variant thereof; thereby these embodiments of the engineered cytokine receptor chain have a structure "IL15-IL2R β (ECD)-IL9R(ICD*)". Herein the CLD may comprise a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 9 (i.e. wildtype IL15, without signal peptide).
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[0017] According to some other embodiments, the cytokine receptor chain is IL2R β , and the CLD comprises IL2 or a functional variant thereof; thereby these embodiments of the engineered cytokine receptor chain have a structure "IL2-IL2R β (ECD)-IL9R(ICD*)". Herein

the CLD may comprise a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 44 (i.e. wildtype IL2, without signal peptide).

5 [0018] According to yet some other embodiments, the cytokine receptor chain is IL4Ra, and the CLD comprises IL4 or a functional variant thereof; thereby these embodiments of the engineered cytokine receptor chain have a structure "IL4-IL4Ra(ECD)-IL9R(ICD*)". Herein the CLD comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 49 (i.e. wildtype IL4, without signal peptide).

10 [0019] According to yet some other embodiments, the cytokine receptor chain is IL7Ra, and the CLD comprises IL7 or a functional variant thereof; thereby these embodiments of the engineered cytokine receptor chain have a structure "IL7-IL7Ra(ECD)-IL9R(ICD*)". Herein the CLD comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 13 (i.e. wildtype IL7, without signal peptide).

15 [0020] According to yet some other embodiments, the cytokine receptor chain is IL9R, and the CLD comprises IL9 or a functional variant thereof; thereby these embodiments of the engineered cytokine receptor chain have a structure "IL9-IL9R(ECD)-IL9R(ICD*)". Herein the CLD comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 46 (i.e. wildtype IL9, without signal peptide).

20 [0021] According to yet some other embodiments, the cytokine receptor chain is IL21R, and the CLD comprises IL21 or a functional variant thereof; thereby these embodiments of the engineered cytokine receptor chain have a structure "IL21-IL21R(ECD)-IL9R(ICD*)". Herein the CLD comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 47 (i.e. wildtype IL21, without signal peptide).

25 [0022] In any of these above embodiments of the engineered cytokine receptor chain, the CLD is connected to the N-terminus of the ECD of the cytokine receptor chain or the functional variant thereof via a flexible linker. Examples of the flexible linker can include a GS linker (e.g. SEQ ID NO: 16), which will be described in greater details below.

30 [0023] Several specific embodiments of the engineered cytokine receptor chain are summarized in Table 2 below, and include "IL15-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*)"

(SEQ ID NO: 79), "IL2-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*)" (SEQ ID NO: 80), "IL4-IL4Ra(ECD)-IL4Ra(TM)-IL9R(ICD*)" (SEQ ID NO: 81), "IL7-IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*)" (SEQ ID NO: 82), "IL9-IL9R(ECD)-IL9R(TM)-IL9R(ICD*)" (SEQ ID NO: 83), and "IL21-IL21R(ECD)-IL21R(TM)-IL9R(ICD*)" (SEQ ID NO: 84). In these above
5 embodiments, the CLD and the cytokine receptor chain pair are IL15-IL2R β (ECD), IL2-IL2R β (ECD), IL4-IL4Ra(ECD), IL7-IL7Ra(ECD), IL9-IL9R(ECD), and IL21-IL21R(ECD), respectively.

[0024] In some embodiments of the engineered cytokine signaling chain, when the engineered cytokine signaling chain is expressed in an immune cell, the immune cell exhibits increased
10 STAT3 signaling in the absence of IL-2 stimulation compared to when the engineered cytokine signaling chain is not expressed in the immune cell.

[0025] In some embodiments of the engineered cytokine signaling chain, when the engineered cytokine signaling chain is expressed in an immune cell, the immune cell displays increased proliferation capability when cultured *in vitro* in the absence of IL-2, compared to when the
15 engineered cytokine signaling chain is not expressed in the immune cell.

[0026] In some embodiments of the engineered cytokine signaling chain, when the engineered cytokine signaling chain is expressed in an immune cell, the immune cell displays increased viability when cultured *in vitro* in the absence of IL-2, compared to when the engineered cytokine signaling chain is not expressed in the immune cell.

[0027] In some embodiments of the engineered cytokine signaling chain, when the engineered cytokine signaling chain is expressed in an immune cell, the immune cell exhibits increased cytotoxicity against target cells of the immune cell when co-cultured with the target cells in the
20 absence of IL-2 stimulation, compared to when the engineered cytokine signaling chain is not expressed in the immune cell.

[0028] According to some embodiments, the engineered cytokine signaling chain takes a non-self-activating form. Unlike the aforementioned embodiments which take the self-activating form, the extracellular portion in these embodiments of the engineered cytokine signaling chain does not comprise a cytokine ligand domain (CLD) that is functionally compatible to the ECD of the cytokine receptor chain or the functional variant thereof. Thus while the self-activating
25 form of the engineered cytokine signaling chain expressed in an immune cell has the intracellular domain (i.e. IL9R(ICD*)) to be functionally activated through the intramolecular binding of the CLD with the ECD of the cytokine receptor chain or a functional variant thereof,
30 the non-self-activating form of the engineered cytokine signaling chain requires the presence

of additional functionally compatible cytokine ligand molecules to bind intermolecularly to the ECD of the cytokine receptor chain or a functional variant thereof of the engineered cytokine signaling chain to thereby activate the intracellular domain (i.e. IL9R(ICD*)). Thus the engineered cytokine signaling chain in the non-self-activating form can be regarded as an "engineered cytokine receptor chain" which can be activated by the presence of the functionally compatible cytokine ligand molecules on the cell membrane of immune cells expressing the engineered cytokine receptor chain. Herein the functionally compatible cytokine ligand molecules may be provided as a non-engineered form (e.g. IL2, IL15, IL4, IL7, IL9, or IL21) or more preferably as an engineered cytokine ligand chain, which may be further engineered to in a secreted form or in a membrane-bound form.

[0029] Several specific embodiments of the engineered cytokine receptor chain in the non-self-activating form are summarized in Table 1 below, including "IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*)" (SEQ ID NO: 8), "IL4Ra(ECD)-IL4Ra(TM)-IL9R(ICD*)" (SEQ ID NO: 35), "IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*)" (SEQ ID NO: 12), "IL9R(ECD)-IL9R(TM)-IL9R(ICD*)" (SEQ ID NO: 38), and "IL21R(ECD)-IL21R(TM)-IL21R(ICD*)" (SEQ ID NO: 41).

[0030] In some embodiments, when the engineered cytokine signaling chain is expressed in an immune cell along with an engineered cytokine ligand chain capable of activating the engineered cytokine receptor chain (i.e. functionally compatible engineered cytokine ligand chain), the immune cell displays increased proliferation capability when cultured *in vitro* in the absence of IL-2, compared to when the engineered cytokine receptor chain and the engineered cytokine ligand chain are not expressed in the immune cell.

[0031] In some embodiments, when the engineered cytokine receptor chain is expressed in an immune cell along with a functionally compatible engineered cytokine ligand chain, the immune cell displays increased viability when cultured *in vitro* in the absence of IL-2, compared to when the engineered cytokine receptor chain and the engineered cytokine ligand chain are not expressed in the immune cell.

[0032] In some embodiments, when the engineered cytokine receptor chain is expressed in an immune cell along with a functionally compatible engineered cytokine ligand chain, the immune cell exhibits increased STAT signaling in the absence of IL-2 stimulation compared to when the engineered cytokine receptor chain and the engineered cytokine ligand chain are not expressed in the immune cell.

[0033] In some embodiments, when the engineered cytokine receptor chain is expressed in an immune cell along with a functionally compatible engineered cytokine ligand chain, the

immune cell exhibits increased interferon gamma (IFN γ) secretion when co-cultured with target cells corresponding thereto in the absence of IL-2 stimulation, compared to when the engineered cytokine receptor chain and the engineered cytokine ligand chain are not expressed in the immune cell.

5 [0034] In some embodiments, when the engineered cytokine receptor chain is expressed in an immune cell along with a functionally compatible engineered cytokine ligand chain, the immune cell exhibits increased cytotoxicity against target cells when co-cultured with the target cells in the absence of IL-2 stimulation, compared to when the engineered cytokine receptor chain and the engineered cytokine ligand chain are not expressed in the immune cell.

10 [0035] In some embodiments, when the engineered cytokine receptor chain is expressed in an immune cell along with a functionally compatible engineered cytokine ligand chain, the immune cell exhibits more preferred migration to tumor tissues and/or lymphoid tissues when a population of the immune cell are transferred into a subject bearing a tumor that the immune cell specifically targets, compared to when the engineered cytokine receptor chain and the engineered cytokine ligand chain are not expressed in the immune cell.

15 [0036] In some embodiments, when the engineered cytokine receptor chain is expressed in an immune cell along with a functionally compatible engineered cytokine ligand chain, the immune cell displays increased tumor control ability when a population of the immune cell are transferred into a subject bearing a tumor that the immune cell specifically targets, compared to when the engineered cytokine receptor chain and the engineered cytokine ligand chain are not expressed in the immune cell.

20 [0037] In any of the above embodiments of the engineered cytokine receptor chain where the co-expression of an engineered cytokine ligand chain capable of activating the engineered cytokine receptor chain induces improved characteristics (i.e. any of "increased proliferation capability", "increased viability", "increased STAT signaling", "increased interferon gamma (IFN γ) secretion", "increased cytotoxicity against target cells", "more preferred migration to tumor tissues and/or lymphoid tissues", "increased tumor control ability") for the immune cell that co-express the engineered cytokine receptor chain and the engineered cytokine ligand chain, the engineered cytokine ligand chain may optionally encode a secreted cytokine ligand or a membrane-bound cytokine ligand when expressed in an immune cell. Herein, the secreted cytokine ligand is capable of activating the engineered cytokine receptor chain, and the membrane-bound cytokine ligand comprises a ligand portion capable of activating the engineered cytokine receptor chain.

30 [0038] Herein, the subject can be an animal such as a mouse used in a mouse tumor model, but

can also be a human subject such as a tumor patient that receives the immunotherapies such as the adoptive T cell therapy.

- [0039] Herein, depending on the different embodiments of the ECD of the cytokine receptor, there can be different embodiments of the corresponding cytokine ligand chain. When the extracellular portion of the engineered cytokine receptor chain comprises IL2R β (ECD) or a functional variant thereof, the engineered cytokine ligand chain may optionally encode a secreted IL2, a membrane-bound IL2, a secreted IL15, or a membrane-bound IL15, or a functional variant thereof. When the extracellular portion of the engineered cytokine receptor chain comprises IL4Ra(ECD) or a functional variant thereof, the engineered cytokine ligand chain may optionally encode a secreted IL4 or a membrane-bound IL4, or a functional variant thereof. When the extracellular portion of the engineered cytokine receptor chain comprises IL7Ra(ECD) or a functional variant thereof, the engineered cytokine ligand chain may optionally encode a secreted IL7 or a membrane-bound IL7, or a functional variant thereof. When the extracellular portion of the engineered cytokine receptor chain comprises IL9R(ECD) or a functional variant thereof, the engineered cytokine ligand chain may optionally encode a secreted IL9 or a membrane-bound IL9, or a functional variant thereof. When the extracellular portion of the engineered cytokine receptor chain comprises IL21R(ECD) or a functional variant thereof, the engineered cytokine ligand chain may optionally encode a secreted IL21 or a membrane-bound IL21, or a functional variant thereof.
- [0040] Secondly, a synthetic cytokine signaling system is further provided, which comprises an engineered cytokine signaling chain according to any of the embodiments as disclosed above. The synthetic cytokine signaling system can be used for engineer or modify an immune cell.
- [0041] According to some embodiments, the synthetic cytokine signaling system consists only of the engineered cytokine signaling chain that is in the self-activating form as described above.
- [0042] According to some other embodiments, the synthetic cytokine signaling system comprises, in addition to the engineered cytokine signaling chain that is in the non-self-activating form (i.e. engineered cytokine receptor chain), further comprises an engineered cytokine ligand chain, configured such that when the engineered cytokine receptor chain and the engineered cytokine ligand chain are co-expressed in the immune cell, the engineered cytokine ligand chain is capable of activating the engineered cytokine receptor chain. Herein the extracellular portion of the engineered cytokine signaling chain can comprise an extracellular domain (ECD) of one of a cytokine receptor chain (i.e. IL2R β , IL4Ra, IL7Ra, IL9R, or IL21R), or a functional variant thereof.
- [0043] According to some embodiments of the synthetic cytokine signaling system that

comprises an engineered cytokine ligand chain in addition to the engineered cytokine receptor chain, the engineered cytokine ligand chain encodes a secreted cytokine ligand, which can be any of the following scenarios: (1) when the extracellular portion of the engineered cytokine receptor chain comprises the IL2R β ECD or a functional variant thereof, the engineered cytokine ligand chain encodes at least one of: a secreted IL2, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 44; or a secreted IL15, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 9; (2) when the extracellular portion of the engineered cytokine receptor chain comprises the IL4Ra ECD or a functional variant thereof, the engineered cytokine ligand chain encodes a secreted IL4, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 49; (3) when the extracellular portion of the engineered cytokine receptor chain comprises the IL7Ra ECD or a functional variant thereof, the engineered cytokine ligand chain encodes a secreted IL7, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 13; (4) when the extracellular portion of the engineered cytokine receptor chain comprises the IL9R ECD or a functional variant thereof, the engineered cytokine ligand chain encodes a secreted IL9, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 46; or (5) when the extracellular portion of the engineered cytokine receptor chain comprises the IL21R ECD or a functional variant thereof, the engineered cytokine ligand chain encodes a secreted IL21, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 47.

[0044] According to some other embodiments of the synthetic cytokine signaling system that comprises an engineered cytokine ligand chain in addition to the engineered cytokine receptor chain, the engineered cytokine ligand chain encodes a membrane-bound cytokine ligand, which may comprise a ligand portion operably connected to a membrane-bound portion, and the ligand portion corresponds to the ECD of the cytokine receptor chain (i.e. IL2R β , IL4Ra, IL7Ra, IL9R, or IL21R) or the functional variant thereof in the extracellular portion of the engineered cytokine receptor chain. The membrane-bound cytokine ligand can be any of the following scenarios: (1) when the extracellular portion of the engineered cytokine receptor chain comprises the IL2R β ECD or a functional variant thereof, the engineered cytokine ligand chain encodes at least one of: a membrane-bound IL2, and the ligand portion thereof comprises a

sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 44; or a membrane-bound IL15, and the ligand portion thereof comprises a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 9; (2) when the extracellular portion of the engineered cytokine receptor chain comprises the IL4Ra ECD or a functional variant thereof, the engineered cytokine ligand chain encodes a membrane-bound IL4, and the ligand portion thereof comprises a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 49; (3) when the extracellular portion of the engineered cytokine receptor chain comprises the IL7Ra ECD or a functional variant thereof, the engineered cytokine ligand chain encodes a membrane-bound IL7, and the ligand portion thereof comprises a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 13; (4) when the extracellular portion of the engineered cytokine receptor chain comprises the IL9R ECD or a functional variant thereof, the engineered cytokine ligand chain encodes a membrane-bound IL9, and the ligand portion thereof comprises a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 46; (5) when the extracellular portion of the engineered cytokine receptor chain comprises the extracellular domain (ECD) of IL21R or a functional variant thereof, wherein the engineered cytokine ligand chain encodes a membrane-bound IL21, and the ligand portion thereof comprises a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 47.

[0045] In any of the above embodiments of the synthetic cytokine signaling system where the engineered cytokine ligand chain encodes a membrane-bound cytokine ligand, the membrane-bound portion of the engineered cytokine ligand chain may optionally comprise a fragment crystallizable region (Fc) or a functional variant thereof, and further optionally, the Fc comprises a IgG4Fc, and the membrane-bound portion comprises a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 14. Herein optionally, the membrane-bound portion and the ligand portion is operably connected via a hinge, which may comprise a sequence as set forth in SEQ ID NO: 21, but may comprise other sequences as well. Further optionally, in the membrane-bound portion, the Fc or a functional variant thereof is operably connected to a transmembrane region, which may comprise a sequence as set forth in SEQ ID NO: 22, but may comprise other sequences as well. According to some specific embodiments, the engineered cytokine ligand may encode one of the following membrane-bound cytokine ligands including: membrane-

bound IL2-IgG4Fc ("mbIL2-IgG4Fc", SEQ ID NO: 54), membrane-bound IL4-IgG4Fc ("mbIL4-IgG4Fc", SEQ ID NO: 55), membrane-bound IL7-IgG4Fc ("mbIL7-IgG4Fc", SEQ ID NO: 15), membrane-bound IL9-IgG4Fc ("mbIL9-IgG4Fc", SEQ ID NO: 56), membrane-bound IL15-IgG4Fc ("mbIL15-IgG4Fc", SEQ ID NO: 57), or membrane-bound IL21-IgG4Fc ("mbIL21-IgG4Fc", SEQ ID NO: 58).

[0046] There could be other ways for constructing the membrane-bound cytokine ligand. According to some embodiments where the engineered cytokine ligand chain encodes a membrane-bound IL15, the membrane-bound portion of the membrane-bound IL15 may comprise IL15R α (i.e. IL15Ra or a functional variant thereof, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 10. Herein optionally, the membrane-bound portion and the ligand portion is operably connected via a linker, which may comprise a sequence as set forth in SEQ ID NO: 16, but can have other options. According to some specific embodiment, the engineered cytokine ligand may encode a membrane-bound IL15 having a structure "IL15-IL15Ra" (exchangeable to "membrane-bound IL15-IL15Ra", "mbIL15-IL15Ra", or "Recast IL15" hereinafter, SEQ ID NO: 11).

[0047] In an additional aspect, a synthetic cytokine signaling system is further provided, which comprises an engineered cytokine receptor chain (i.e., engineered cytokine signaling chain in the non-self-activating form) and an engineered cytokine ligand chain. The engineered cytokine receptor chain encodes a transmembrane polypeptide that comprises an extracellular portion and an intracellular portion, operably connected by a transmembrane portion (TM). The intracellular portion comprises the intracellular domain (ICD) of IL9R or a functional variant thereof; and the extracellular portion comprises the extracellular domain (ECD) of one of IL2Rb or IL7Ra or a functional variant thereof. The engineered cytokine ligand chain encodes a membrane-bound cytokine ligand that comprises a ligand portion operably connected to a membrane-bound portion, and the ligand portion corresponds to the ECD of the extracellular portion of the engineered cytokine receptor chain. It is configured such that when the engineered cytokine receptor chain and the engineered cytokine ligand chain are expressed in an immune cell, the immune cell displays increased proliferation capability when cultured *in vitro* in the absence of IL-2, compared to when the engineered cytokine receptor chain and the engineered cytokine ligand chain are not expressed in the immune cell. Herein, the intracellular domain (ICD) of IL9R or a functional variant thereof in the intracellular portion of the engineered cytokine receptor chain may comprise a variant IL9R ICD as described above

(i.e. comprising at least one of a first sequence alteration in a first region corresponding positions 174-230, or a second sequence alteration in a second region corresponding positions 142-151, of the sequence as set forth in SEQ ID NO: 1), but may comprise other variants of IL9R ICD as well, as long as the expression of the synthetic cytokine signaling system cause the immune cell to have increased proliferation capability when cultured *in vitro* in the absence of IL-2 compared with otherwise (i.e. the synthetic cytokine signaling system is not expressed).

5 [0048] According to some embodiments of the synthetic cytokine signaling system, it is further configured such that when the engineered cytokine receptor chain and the engineered cytokine ligand chain are expressed in an immune cell, the immune cell further displays at least one of increased cell viability, increased STAT signaling, increased interferon gamma (IFN γ) secretion when co-cultured with target cells, or increased cytotoxicity against the target cells when cultured *in vitro* in the absence of IL-2, or exhibits more preferred migration to tumor tissues and/or lymphoid tissues, or increased tumor control ability when a population of the immune cell are transferred into a subject bearing a tumor that the immune cell specifically targets, compared with otherwise (i.e. the engineered cytokine receptor chain and the engineered cytokine ligand chain are not expressed in the immune cell).

[0049] According to some embodiments of the synthetic cytokine signaling system, the intracellular portion of the engineered cytokine receptor chain comprises a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 1.

20 [0050] According to some other embodiments, the intracellular portion comprises a variant ICD of IL9R, comprising at least one of a first sequence alteration in a first region corresponding positions 174-230, or a second sequence alteration in a second region corresponding positions 142-151, of the sequence as set forth in SEQ ID NO: 1. Herein, optionally, the variant IL9R ICD comprises a truncation in the first region corresponding positions 174-230, and further optionally, in the variant IL9R ICD, the region corresponding positions 142-151 of the sequence as set forth in SEQ ID NO: 1 is altered to GD. According to one specific embodiment, the intracellular portion of the engineered cytokine receptor chain comprises a sequence as set forth in SEQ ID NO: 7.

30 [0051] According to some embodiments of the synthetic cytokine signaling system, the extracellular portion of the engineered cytokine receptor chain comprises the extracellular domain (ECD) of IL2Rb or a functional variant thereof, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 3. Optionally, the transmembrane (TM) portion comprises the transmembrane

domain from IL2Rb, having a sequence as set forth in SEQ ID NO: 19. According to one specific embodiment, the engineered cytokine receptor chain has a structure of "IL2Rb(ECD)-IL2Rb(TM)-IL9R(ICD*)" and comprises a sequence as set forth in SEQ ID NO: 8. In correspondence, the engineered cytokine ligand chain comprises a membrane-bound IL15, wherein the ligand portion comprises IL15 or a functional variant thereof, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 9. The membrane-bound portion of the membrane-bound IL15 may optionally comprise IL15Ra or a functional variant thereof, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 10. According to one specific embodiment, the engineered cytokine ligand chain has a structure of "membrane bound IL15-IL15Ra" or "mbIL15-ILRa", and comprises a sequence as set forth in SEQ ID NO: 11.

[0052] According to some other embodiments of the synthetic cytokine signaling system, the extracellular portion of the engineered cytokine receptor chain comprises the extracellular domain (ECD) of IL7Ra or a functional variant thereof, comprising a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 5. Optionally, the transmembrane (TM) portion comprises the transmembrane domain from IL7Ra, having a sequence as set forth in SEQ ID NO: 24. According to one specific embodiment, the engineered cytokine receptor chain has a structure of IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*), and comprises a sequence as set forth in SEQ ID NO: 12. In correspondence, the engineered cytokine ligand chain comprises a membrane-bound IL7, and the ligand portion comprises IL7 or a functional variant thereof, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 13. Herein, the membrane-bound portion comprises an Fc or a functional variant thereof operably connected to a transmembrane region. Optionally, the Fc is IgG4Fc, comprising a sequence having at least 70% (e.g. 70%, 75%, 80%, 85%, 90%, 95%, 100%, etc.) identity to the sequence as set forth in SEQ ID NO: 14. According to one specific embodiment, the engineered cytokine ligand chain has a structure of "membrane bound IL7-IgG4Fc" or "mbIL7-IgG4Fc", and comprises a sequence as set forth in SEQ ID NO: 15.

[0053] Thirdly, a vector kit that can be introduced into an immune cell to thereby allow the synthetic cytokine signaling system to be expressed therein is further provided. The vector kit may comprise at least one vector, configured such that when the at least one vector is introduced in an immune cell, the immune cell expresses the synthetic cytokine signaling system according

to any of the embodiments as described above.

[0054] In situations where the synthetic cytokine signaling system consists of only the engineered cytokine signaling chain (i.e. those in the self-activating form) according to any of the related embodiments as described above, the vector kit may consist of one single vector
5 expressing the engineered cytokine signaling chain.

[0055] In situations where the synthetic cytokine signaling system comprises an engineered cytokine receptor chain (i.e. engineered cytokine signaling chain in the non-self-activating form) and an engineered cytokine ligand chain, there can be different embodiments for the vector kit.

10 [0056] Optionally the vector kit may consist of only one vector, which comprises two nucleotide sequences that respectively encode the engineered cytokine receptor chain and the engineered cytokine ligand chain in the synthetic cytokine signaling system. According to some embodiments, the two nucleotide sequences are in a common open reading frame (ORF), and the one vector comprises a separator sequence separating the two nucleotide sequences.
15 The separator sequence may comprise an F2A (SEQ ID NO: 42) or a P2A(SEQ ID NO: 43), or can have other options. According to some other embodiments, the two nucleotide sequences are in two different open reading frames (ORFs).

[0057] According to one specific embodiment, the vector kit may comprise one vector, which comprises a polynucleotide sequence as set forth in SEQ ID: 60, and the polynucleotide
20 sequence encodes a polypeptide having a sequence as set forth in SEQ ID NO: 59. The polypeptide includes an F2A sequence that separates the engineered cytokine ligand chain mbIL15-IL15Ra (i.e. Recast-IL15) and the engineered cytokine receptor chain IL2Rb(ECD)-IL2Rb(TM)-IL9R(ICD*). Here the polypeptide is termed as "IL15-IL15R-IL2Rb-IL9R" or "IL15-IL9R".

25 [0058] According to another specific embodiment, the vector kit may comprise one vector, which comprises a polynucleotide sequence as set forth in SEQ ID: 62, and the polynucleotide sequence encodes a polypeptide having a sequence as set forth in SEQ ID NO: 61. The polypeptide includes an F2A sequence that separates the engineered cytokine ligand chain mbIL7-IgG4Fc and the engineered cytokine receptor chain IL7Ra(ECD)-IL7Ra(TM)-
30 IL9R(ICD*). Here the polypeptide is termed as "IL7-IL7R-IL9R" or "IL7-IL9R".

[0059] Further optionally, the vector kit may comprise a first vector and a second vector: the first vector comprises a first nucleotide sequence encoding the engineered cytokine signaling chain; and the second vector comprises a second nucleotide sequence encoding the engineered cytokine ligand chain.

[0060] Herein, each of the at least one vector in the vector kit may be a DNA vector that can be directly transduced into the immune cells, or may be a viral vector that is indirectly introduced into the immune cells.

[0061] In addition, a method for obtaining an engineered immune cell is further provided, the method comprising: introducing into an immune cell a vector kit according to any one of the embodiments as described above. Herein the method may include direct transduction of DNA vectors into the immune cells, or may include obtaining the vehicle viruses (e.g. retroviruses, adenoviruses, etc.) that comprise the viral DNAs and subsequently infecting the immune cells with the vehicle viruses.

[0062] Fourthly, the present disclosure further provides a method for obtaining an engineered immune cell, which comprises: introducing into an immune cell a vector kit according to any one of the embodiments as described above.

[0063] Fifthly, the present disclosure further provides an immune cell, which comprises the synthetic cytokine signaling system according to any of the embodiments as described above. The immune cell may be obtained by introducing the vector kit as described above into an immune cell, but may be obtained by other means.

[0064] According to some embodiments, the immune cell may, when cultured *in vitro* in the absence of IL-2, display at least one of increased proliferation capability, increased viability, or increased STAT signaling, compared to an immune cell that does not comprise the synthetic cytokine signaling system. According to some embodiments, the immune cell may, when co-cultured with target cells in the absence of IL-2 stimulation, display at least one of increased interferon gamma (IFN γ) secretion or increased cytotoxicity against target cells, compared to an immune cell that does not comprise the synthetic cytokine signaling system. According to some embodiments, when a population of the immune cell are transferred into a subject bearing a tumor that the immune cell specifically targets, the population of the immune cell may exhibit more preferred migration to tumor tissues and/or lymphoid tissues, and/or may display increased tumor control ability, compared to an immune cell that does not comprise the synthetic cytokine signaling system.

[0065] Herein, the immune cell may be any of a T cell, a $\gamma\delta$ T cell, a natural killer (NK) cell, a natural killer T (NKT) cell, or a tumor-infiltrating lymphocyte (TIL), or an engineered immune cell derived therefrom. The immune cell may be non-engineered or unmodified, but may also be engineered or modified, for example, to express an engineered chimeric antigen receptor (CAR), an engineered T cell receptor (TCR), or an engineered Aspire-T cell receptor (as

disclosed in WO2024138181A2, whose content is incorporated in its entirety by reference) that specifically targets certain diseases (such as tumors). Optionally, the immune cell may further express a targeting polypeptide, which may comprise at least one of a CAR, a TCR, or an Aspire-TCR that can specifically and selectively recognize and bind certain target molecule/protein/marker on the surface of target cells (e.g. tumor cells) or on the surface of other entities (e.g. bacteria, fungi, viruses).

[0066] Sixthly, a culturing method for the immune cell as described above is also provided. The method comprises the steps of:

[0067] (1) obtaining an engineered immune cell from an immune cell, such that the engineered immune cell expresses the synthetic cytokine signaling system according to any embodiment as described above; and

[0068] (2) culturing the engineered immune cell in a medium containing none or reduced level of a cytokine supplement *in vitro*.

[0069] Herein, the "reduced level of a cytokine supplement" as used herein, means a level of the cytokine supplement used for *in vitro* culture of the immune cell that is at least 50% less than the level of the cytokine supplement that is conventionally used for the *in vitro* culture of an unmodified immune cell. Herein, the conventional level of IL-2 supplement that is used for the *in vitro* culture of unmodified T cells or TILs is approximately 300 IU/mL, and "a reduced level of IL2" means a level of IL2 used for the *in vitro* culture is 150 IU/mL or less (e.g. 150 IU/mL, 120 IU/mL, 100 IU/mL, 80 IU/mL, 60 IU/mL, 50 IU/mL, 25 IU/mL, 10 IU/mL, 5 IU/mL, etc.).

[0070] Herein step (1) can be realized by introducing into the immune cell the vector kit according to any embodiments as described above.

[0071] In step (2), the cytokine supplement may comprise at least one of IL-2, IL-4, IL-7, IL-9, IL-15, or IL-21. Thus according to some embodiment, the culturing medium may lack IL-2, may lack IL-15, or may lack any of the above cytokines (i.e. IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21). According to some embodiments, the cytokine supplement may only consist of IL-2.

[0072] Seventhly, the present disclosure further provides a method for treating a disease in a subject in need thereof, comprising: administering a plurality of engineered immune cells into the subject, wherein each of the plurality of engineered immune cells expresses the synthetic cytokine signaling system according to any embodiments as described above.

[0073] Herein, the treating method may comprise: administering to the subject a therapeutically effective number of the engineered immune cells to treat the disease.

[0074] The term "subject" as used herein refers to an animal such as a mammal, which can

include human, dog, cat, mouse, rat, etc.

[0075] As used herein, the term “effective amount” means an amount or dosage sufficient to effect beneficial or desired results including halting, slowing, retarding, or inhibiting progression of a disease, e.g., a cancer. An effective amount will vary depending upon, e.g., an age and a body weight of a subject to which the therapeutic agent and/or therapeutic compositions is to be administered, a severity of symptoms and a route of administration, and thus administration can be determined on an individual basis. An effective amount can be administered in one or more administrations. By way of example, an effective amount of a composition is an amount sufficient to ameliorate, stop, stabilize, reverse, inhibit, slow and/or delay progression of a cancer in a patient or is an amount sufficient to ameliorate, stop, stabilize, reverse, slow and/or delay proliferation of a cell (e.g., a biopsied cell, any of the cancer cells described herein, or cell line (e.g., a cancer cell line)) *in vitro*. As is understood in the art, an effective may vary, depending on, *inter alia*, patient history as well as other factors such as the type (and/or dosage) of compositions used. Effective amounts and schedules for administrations may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage that must be administered will vary depending on, for example, the mammal that will receive the treatment, the route of administration, the particular type of therapeutic agents and other drugs being administered to the mammal. Guidance in selecting appropriate doses can be found in the literature. In addition, a treatment does not necessarily result in the 100% or complete treatment or prevention of a disease or a condition. There are multiple treatment/prevention methods available with a varying degree of therapeutic effect which one of ordinary skill in the art recognizes as a potentially advantageous therapeutic mean. In any of the methods described herein, the engineered immune cells and, and/or at least one additional therapeutic agent can be administered to the subject at least once a week (e.g., once a week, twice a week, three times a week, four times a week, once a day, twice a day, or three times a day). In some embodiments, at least two different engineered cells (e.g., cells express different binding molecules) are administered in the same composition (e.g., a liquid composition). In some embodiments, engineered immune cells and at least one additional therapeutic agent are administered in the same composition (e.g., a liquid composition). In some embodiments, engineered immune cells and the at least one additional therapeutic agent are administered in two different compositions. In some embodiments, the at least one additional therapeutic agent is administered as a pill, tablet, or capsule. In some embodiments, the at least one additional therapeutic agent is administered in a sustained-release oral formulation.

[0076] Herein, the disease can be a cancer, an autoimmune disease (e.g. Type 1 diabetes), a viral infection (e.g. adenovirus, Epstein-Barr virus, cytomegalovirus, BK virus, JC virus, human herpes virus, or HIV, etc.).

[0077] The term "cancer" as used herein can mean any type, and exemplary cancers for treatment include a solid tumor, leukemia, and lymphoma. The therapeutic method can be characterized according to the cancer to be treated. For example, in certain embodiments, the cancer is a solid tumor. In certain other embodiments, the cancer is brain cancer, bladder cancer, breast cancer, cervical cancer, colon cancer, colorectal cancer, endometrial cancer, esophageal cancer, leukemia, lung cancer, liver cancer, melanoma, ovarian cancer, pancreatic cancer, prostate cancer, rectal cancer, renal cancer, stomach cancer, testicular cancer, or uterine cancer. In yet other embodiments, the cancer is a vascularized tumor, squamous cell carcinoma, adenocarcinoma, small cell carcinoma, melanoma, glioma, neuroblastoma, sarcoma (e.g., an angiosarcoma or chondrosarcoma), larynx cancer, parotid cancer, biliary tract cancer, thyroid cancer, acral lentiginous melanoma, actinic keratoses, acute lymphocytic leukemia, acute myeloid leukemia, adenoid cystic carcinoma, adenomas, adenosarcoma, adenosquamous carcinoma, anal canal cancer, anal cancer, anorectum cancer, astrocytic tumor, Bartholin's gland carcinoma, basal cell carcinoma, biliary cancer, bone cancer, bone marrow cancer, bronchial cancer, bronchial gland carcinoma, carcinoid, cholangiocarcinoma, chondrosarcoma, choroid plexus papilloma/carcinoma, chronic lymphocytic leukemia, chronic myeloid leukemia, clear cell carcinoma, connective tissue cancer, cystadenoma, digestive system cancer, duodenum cancer, endocrine system cancer, endodermal sinus tumor, endometrial hyperplasia, endometrial stromal sarcoma, endometrioid adenocarcinoma, endothelial cell cancer, ependymal cancer, epithelial cell cancer, Ewing's sarcoma, eye and orbit cancer, female genital cancer, focal nodular hyperplasia, gallbladder cancer, gastric antrum cancer, gastric fundus cancer, gastrinoma, glioblastoma, glucagonoma, heart cancer, hemangioblastomas, hemangioendothelioma, hemangiomas, hepatic adenoma, hepatic adenomatosis, hepatobiliary cancer, hepatocellular carcinoma, Hodgkin's disease, ileum cancer, insulinoma, intraepithelial neoplasia, interepithelial squamous cell neoplasia, intrahepatic bile duct cancer, invasive squamous cell carcinoma, jejunum cancer, joint cancer, Kaposi's sarcoma, pelvic cancer, large cell carcinoma, large intestine cancer, leiomyosarcoma, lentigo maligna melanomas, lymphoma, male genital cancer, malignant melanoma, malignant mesothelial tumors, medulloblastoma, medulloepithelioma, meningeal cancer, mesothelial cancer, metastatic carcinoma, mouth cancer, mucoepidermoid carcinoma, multiple myeloma, muscle cancer, nasal tract cancer, nervous system cancer, neuroepithelial adenocarcinoma nodular melanoma,

non-epithelial skin cancer, non-Hodgkin's lymphoma, oat cell carcinoma, oligodendroglial cancer, oral cavity cancer, osteosarcoma, papillary serous adenocarcinoma, penile cancer, pharynx cancer, pituitary tumors, plasmacytoma, pseudosarcoma, pulmonary blastoma, rectal cancer, renal cell carcinoma, respiratory system cancer, retinoblastoma, rhabdomyosarcoma, sarcoma, serous carcinoma, sinus cancer, skin cancer, small cell carcinoma, small intestine cancer, smooth muscle cancer, soft tissue cancer, somatostatin-secreting tumor, spine cancer, squamous cell carcinoma, striated muscle cancer, submesothelial cancer, superficial spreading melanoma, T cell leukemia, tongue cancer, undifferentiated carcinoma, ureter cancer, urethra cancer, urinary bladder cancer, urinary system cancer, uterine cervix cancer, uterine corpus cancer, uveal melanoma, vaginal cancer, verrucous carcinoma, VIPoma, vulva cancer, well differentiated carcinoma, or Wilms tumor.

[0078] According to some embodiments, the treatment method may further comprise, prior to the administering step:

[0079] obtaining an engineered immune cell based on an immune cell; and

[0080] culturing the engineered immune cell in a medium containing none or reduced level of a cytokine supplement *in vitro* to thereby obtain a plurality of engineered immune cells.

[0081] Herein, the engineered immune cell may further express a targeting polypeptide, wherein the targeting polypeptide comprises at least one of a chimeric antigen receptor (CAR), a T cell receptor (TCR), or an Aspire-T cell receptor, and the immune cell may be any of a T cell, a $\gamma\delta$ T cell, a natural killer (NK) cell, a natural killer T (NKT) cell, or a tumor-infiltrating lymphocyte (TIL), or an engineered immune cell derived therefrom.

[0082] Herein the targeting polypeptide may specifically recognize a particular antigen expressed or presented on the surface of a target cell. In certain embodiments where the disease is a cancer, the target cell is a cancer cell that expresses one or more of the following: ALPP, NY-ESO-1, HBV, HPV, H3.3K27M, PD-L1, BCMA, Caudin18.2, CCR4, CD10, CD123, CD147, CD171, CD19, CD20, CD22, CD276, CD319, CD33, CD38, CD70, CLL-1, DLL3, EGFR, EGFRvIII, EpCAM, FLT3, FR α , GD2, GPC3, HER2, HGFR, IL13R α 2, Mesothelin, MG7, MUC1, MUC16, Nectin4, PSCA, ROR1, ROR2, TACI, TRBC1, TSLPR, and VEGFR. In some embodiments, the cancer cell expresses one or more of the following: IL13R α 2, APN/CD13, APP, PD-L1, CD44, P32/gC1qR, E-cadherin, N-cadherin, CD21, EGFR, EphA2, EphB4, HER2, FGFR1, FGFR2, FGFR3, FGFR4, VEGFR1, VEGFR3, PSMA, GPC3, IL-10RA, IL-11R α , IL-6R α , GP130, VEGFR2, MUC18, Met, MMP9, Thomsen-Friedenreich carbohydrate antigen, NRP-1, PDGFR β , CD133, PTPRJ, HSPG, E-selectin, Tie2, VPAC1,

ActRIIB, CD163, CXCR4, Ephrin A4, Ephrin B1, Ephrin B2, Ephrin B3, Gonadotrophin releasing hormone receptor, G Protein-coupled receptor 55, Bombesin receptor 2, IL4 receptor, Low-density lipoprotein receptor, Leptin receptor, LRP1, Melanocortin 1 receptor, Melanocortin 4 receptor, CD206, Urokinase plasminogen activator receptor, Neurokinin-1
 5 receptor, VPAC2, ITGB1, CD27, ITGB5, ITGA1, CD27, LRP1, ACVR2B, COL13A1, NOTCH3, EGFR, VEGFR2, VEGFR3, PDGFR, HER2, ErbB3, ErbB4, RET, and FGFR102, etc.

[0083] In the following, definitions for some of the terms as described above and elsewhere in the disclosure are provided.

10 **[0084]** The terms "wild-type" (i.e. wildtype or "WT") or "native" relative to a protein, a polypeptide, or a polynucleotide, refers to the form in which that is typically found in nature, but it is to be noted that the terms can also be applied to a trait, a phenotype, a given cell, or a given organism. Related, the term "variant" relative to "wildtype", refers to a form in which there is at least one alteration or change compared to the wildtype form. In the context of this
 15 disclosure, a "variant" refers to a polypeptide or a polynucleotide that contains at least one sequence alteration (e.g. of point mutation(s), insertion mutation(s), deletion mutation(s), or other sequence alterations etc.) or chemical modification (e.g. glycosylation, connecting to a peptide etc.) compared to the wildtype form thereof. The term "functional variant" can be regarded as one type of a "variant" that substantially retains the functionality or the capability
 20 (i.e. more than 30%, such as 40%, 50%, 60%, 70%, 80%, 90%, or 100%, etc.) of the wildtype counterpart polypeptide or polynucleotide.

[0085] The term "intracellular domain (ICD) of IL9R thereof", "ICD of IL9R", "IL9R ICD" or alike, is referred to as the full-length ICD of IL9R having an amino acid sequence as set forth in SEQ ID NO: 1), which substantially corresponds to a polypeptide region of the IL9R with
 25 amino acid residues 292-521 of the full-length wildtype IL9R (whose sequence is set forth in SEQ ID NO: 2). As used herein, the term "functional variant" of IL9R ICD refers to a sequence variant of IL9R ICD, which contains one or more of point mutation(s), insertion mutation(s), deletion mutation(s), indels, or other sequence alterations etc. relative to the wildtype IL9R ICD and substantially retains the JAK-STAT signaling capability of the wildtype IL9R ICD.

30 **[0086]** The term "extracellular domain (ECD) of IL2R β ", "ECD of IL2R β ", "IL2R β ECD" or alike, is referred to as the full-length ECD of IL2R β having an amino acid sequence as set forth in SEQ ID NO: 3), which substantially corresponds to a polypeptide region of the IL2R β with amino acid residues 27-240 of the full-length wildtype IL2R β (whose sequence is set forth in

SED ID NO: 4).

[0087] The term "extracellular domain (ECD) of IL7R", "ECD of IL7Ra", "IL7Ra ECD" or alike, is referred to as the full-length ECD of IL7Ra having an amino acid sequence as set forth in SEQ ID NO: 5), which substantially corresponds to a polypeptide region of the IL2Rb with amino acid residues 21-239 of the full-length wildtype IL7Ra (whose sequence is set forth in SED ID NO: 6).

[0088] The term "functional variant" of IL2Rb or IL7Ra ECD refers to a sequence variant of IL2R β ECD or IL7Ra ECD (involving one or more of point mutation(s), insertion mutation(s), deletion mutation(s), or other sequence alterations etc.) that substantially retains the capability of binding both the IL2R γ/γ C (i.e. common γ chain of IL2R) and the corresponding cytokine, i.e. IL15 or membrane-bound IL15 (corresponding to IL2R β ECD), or IL7 or membrane-bound IL7 (corresponding to IL7Ra ECD).

[0089] The term "correspond to", "corresponding", "in correspondence to" or alike, means that the ligand portion of the engineered cytokine ligand chain is capable of, when expressed together with the engineered cytokine receptor chain in an immune cell, specifically binding to the ECD of the extracellular portion of the transmembrane cytokine receptor encoded by the engineered cytokine receptor chain, in turn activating the synthetic receptor complex. In one example, the ECD of the extracellular portion of the engineered cytokine receptor chain can be IL2Rb, and the corresponding ligand portion can be IL15. In another example, ECD of the extracellular portion of the engineered cytokine receptor chain can be IL7Ra, and the corresponding ligand portion can be IL7.

[0090] The term "identity" means the percentage of identical nucleotide or amino acid residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions. Identity can be readily calculated by known methods, including but not limited to those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; and Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993, etc. Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Molec. Biol. 215: 403-410 (1990) and Altschul et al. Nuc. Acids Res. 25: 3389-3402

(1997)).

[0091] Each of the terms "increased proliferation capability", "increased viability", "increased STAT signaling", "increased IFN γ secretion", "increased cytotoxicity against target cells", "more preferred migration", and "increased tumor control ability" is to be interpreted as a relative term for an immune cell of interest as compared with a reference immune cell. Herein, the immune cell of interest may be an immune cell that is modified in a certain manner, such as an immune cell modified to express the synthetic cytokine signaling system as provided in this disclosure, and the reference immune cell may be an immune cell absent said modification.

[0092] As such, the "increased proliferation capability" means that after the *in vitro* culturing of the immune cell of interest and the reference immune cell in the absence of IL-2 starts (i.e. Day 0), at any day beyond Day 5 (i.e. Day 6, Day 7, Day 8, etc.), the cell expansion ratio of the immune cell of interest is at least 10% (e.g. 10%, 20%, 40%, 50%, 70%, 90%, 150%, 300%, etc.) higher than the cell expansion ratio of the reference immune cell. The "cell expansion ratio" or "cell expansion fold" as used herein, means the total number of living cells at calculation as compared to the total number of cells at the beginning of the experiment.

[0093] The "increased viability" means that after the *in vitro* culturing of the immune cell of interest and the reference immune cell in the absence of IL-2 starts (i.e. Day 0), at any day beyond Day 5 (i.e. Day 6, Day 7, Day 8, etc.), the cell viability of the immune cell of interest is at least 5% (e.g. 5%, 10%, 25%, 40%, 50%, 70%, 90%, etc.) higher than the cell viability of the reference immune cell. The "cell viability" as used herein, means the percentage of living cells in a population of cells that include living cells and dead cells, which can be calculated by the number of living cells divided by the total number of cells in the whole population. Cell viability can be determined by live/dead dye staining, but can also be determined by other means.

[0094] The "increased STAT signaling" means that under substantially same assay condition, the level of phosphorylation at least one of STAT 1, STAT 3, and STAT5 in the immune cell of interest is at least 10% (e.g. 10%, 20%, 40%, 50%, 70%, 90%, etc.) higher than that in the reference immune cell.

[0095] The "increased IFN γ secretion" means that under substantially same assay condition, such as that that adopted in Example 3 and illustrated FIG. 10I which nonetheless does not impose any limitation to the scope of the disclosure, the level of secreted IFN γ that is detected in the immune cell of interest is at least 10% (e.g. 10%, 20%, 40%, 50%, 70%, 90%, 200%, etc.) higher than that in the reference immune cell.

[0096] The "increased cytotoxicity against target cells" means that under substantially same assay condition, such as that that adopted in Example 4 and illustrated FIGS. 12A and 12B which nonetheless does not impose any limitation to the scope of the disclosure, the percentage of killed target cells (i.e. % killing) for the immune cell of interest co-cultured with the target cells is at least 5% (e.g. 5%, 10%, 20%, 40%, 50%, etc.) higher than that for the reference immune cell co-cultured with the target cells. The term "cytotoxicity" refers to the cell killing property of an agent, such as an immune effector cell or a chemical compound, etc. Herein, there is no limitation to the specific cellular death mechanisms for cytotoxicity, which may include necrosis and apoptosis, but could include other mechanisms as well.

10 [0097] The "more preferred migration" to a tissue of interest means that under substantially same assay condition in a subject receiving *in vivo* transfer of the immune cell of interest or of the reference immune cell, such as that that adopted in Example 5 and illustrated FIG. 15 which nonetheless does not impose any limitation to the scope of the disclosure, the percentage of the donor immune cell detected in the tissue of interest in the subject receiving the immune cell of interest is at least 5% (e.g. 5%, 10%, 20%, 40%, 50%, etc.) higher than the percentage of the donor immune cell detected in the same tissue of interest in the subject receiving the reference immune cell.

20 [0098] The "increased tumor control ability" means that under substantially same assay condition in a subject receiving *in vivo* transfer of the immune cell of interest or of the reference immune cell, such as that that adopted in Example 5 and illustrated FIG. 16A which nonetheless does not impose any limitation to the scope of the disclosure, the tumor volume in the subject receiving the immune cell of interest is at least 10% (e.g. 10%, 20%, 40%, 50%, 100%, 300%, etc.) higher than the tumor volume in the subject receiving the reference immune cell.

25 [0099] The terms "operably linked" and "operatively linked," as used interchangeably herein, refer to the positioning of two or more polypeptide/polynucleotide sequences or sequence elements in a manner which permits them to function in their intended manner.

[0100] The terms "a," "an," and "the" include plural referents, unless the context clearly indicates otherwise.

30 [0101] It is further to be noted that the disclosures of all of the references cited herein are incorporated by reference in their entireties.

BRIEF DESCRIPTION OF THE DRAWINGS

[0102] FIG. 1A and FIG. 1B respectively illustrate a non-self-activated form and a self-activated form of an engineered cytokine signaling chain provided in this disclosure;

[0103] FIG. 2A and FIG. 2B respectively illustrate two embodiments of the synthetic cytokine signaling system provided in the disclosure;

[0104] FIGS. 3A-3D illustrates the characterization of the two synthetic cytokine signaling systems as illustrated in FIGS. 2A and 2B in terms of pSTAT3 signaling (FIGS. 3A and 3B), cell proliferation (FIG. 3C) and survival (FIG. 3D) in T cells modified with these two synthetic cytokine signaling systems and cultured *in vitro* in the absence of IL2;

[0105] FIG. 4A shows the construct structure of one exemplary synthetic cytokine signaling system "IL15-IL15R-IL2R β -IL9R" (or "IL15-IL9R"), and illustrates the functional cytokine signaling complexes formed on the cell membrane of an immune cell engineered to express the synthetic cytokine signaling system, as well as the corresponding downstream JAK-STAT signaling pathway thus activated; and FIG. 4B shows the construct structure of another exemplary synthetic cytokine signaling system "IL7-IL7R-IL9R" (or "IL7-IL9R"), and illustrates the functional cytokine signaling complexes formed on the cell membrane of an immune cell engineered to express the synthetic cytokine signaling system, as well as the corresponding downstream JAK-STAT signaling pathway thus activated;

[0106] FIG. 5A and FIG. 5B respectively show the transduction efficiency of the IL15-IL15Ra-IL9R construct and the IL7-IL7R-IL9R construct in Jurkat cells;

[0107] FIG. 6A shows the process for characterizing pSTAT signaling in transduced Jurkat cells; FIGS. 6B and 6C show the comparison results and the bar graphs in terms of STAT1 phosphorylation (i.e. "pSTAT1") among unmodified Jurkat cells ("UTD"), IL15-IL9R modified Jurkat cells, and IL7-IL9R modified Jurkat cells; FIGS. 6D and 6E show the comparison results and the bar graphs in terms of STAT3 phosphorylation (i.e. "pSTAT3") among unmodified Jurkat cells ("UTD"), IL15-IL9R modified Jurkat cells, and IL7-IL9R modified Jurkat cells; and FIGS. 6F and 6G show the comparison results and the bar graphs in terms of STAT5 phosphorylation (i.e. "pSTAT5") among unmodified Jurkat cells ("UTD"), IL15-IL9R modified Jurkat cells, and IL7-IL9R modified Jurkat cells;

[0108] FIG. 7A and FIG. 7B respectively show the transduction efficiency of the IL15-IL15Ra-IL9R construct and the IL7-IL7R-IL9R construct in peripheral blood mononuclear cells (PBMC);

[0109] FIG. 8A and FIG. 8B respectively show the comparison results in terms of *in vitro* cell proliferation and cell viability among unmodified T cells, IL15-IL9R modified T cells, and IL7-IL9R modified T cells when cultured *in vitro* in the presence ("With IL-2") or in the absence ("Without IL-2") of the IL-2 supplement; and FIG. 8C and FIG. 8D respectively show

the comparison results in terms of the *in vitro* cell proliferation (FIG. 8C) and the cell viability (FIG. 8D) among unmodified TILs, IL15-IL9R modified TILs, and IL7-IL9R modified TILs when cultured *in vitro* in the presence ("With IL-2") or in the absence ("Without IL-2") of the IL-2 supplement;

5 [0110] FIG. 9 shows the comparison results in terms of the phosphorylation of STAT proteins ("pSTATs") among unmodified T cells, IL15-IL9R modified T cells, IL7-IL9R modified T cells, when cultured *in vitro* without IL-2 stimulation, with the untransduced T cells stimulated by IFN α or IL-2 as positive control;

[0111] FIGS. 10A and 10B show the comparison results and the bar graphs in terms of the *in vitro* killing efficacy towards target cells (i.e. anti-CD3 Hela cells) among unmodified T cells ("UTD"), IL15-IL9R modified T cells, and IL7-IL9R modified T cells when they are co-cultured with the target cells *in vitro* without IL-2 stimulation, with the target cells alone ("anti-CD3 Hela") as negative control; FIGS. 10C and 10D further shows the comparison results and the bar graphs in terms of the killer cytokine interferon gamma (i.e. "IFN γ ") production by the
10 CD8 T cells, as indicated by the intracellular cytokine staining, during activation among unmodified T cells ("UTD"), IL15-IL9R modified T cells, and IL7-IL9R modified T cells when they are co-cultured with the target cells *in vitro* without IL-2 stimulation, with the "CD3/28 beads" as positive control; FIGS. 10E and 10F further shows the comparison results and the bar graphs in terms of the killer cytokine interferon gamma production by the CD4 T cells, as
15 indicated by the intracellular cytokine staining, during activation among unmodified T cells ("UTD"), IL15-IL9R modified T cells, and IL7-IL9R modified T cells when they are co-cultured with the target cells *in vitro* without IL-2 stimulation, with the "CD3/28 beads" as positive control; FIGS. 10G and 10H further shows the comparison results and the bar graphs in terms of the cytolytic granzyme B (i.e. "Granzyme B") production by the CD8 T cells, as
20 indicated by the intracellular cytokine staining, during activation among unmodified T cells ("UTD"), IL15-IL9R modified T cells, and IL7-IL9R modified T cells when they are co-cultured with the target cells *in vitro* without IL-2 stimulation, with the "CD3/28 beads" as positive control; FIGS. 10I, 10J and 10K respectively show the bar graph comparison results of the killer cytokine/cytolytic proteins IFN γ , Granzyme B, and Perforin that are secreted by
25 the T cells, as indicated by the secretion in the supernatant, during activation among unmodified T cells ("UTD"), IL15-IL9R modified T cells, and IL7-IL9R modified T cells when they are co-cultured with the target cells *in vitro* without IL-2 stimulation, with the "CD3/28" as positive control;
30

- [0112] FIG. 11A and FIG. 11B respectively show the comparison results in terms of *in vitro* cell proliferation and cell viability among untransduced CAR-T cells, unmodified CAR-T cells, IL15-IL9R modified CAR-T cells, and IL7-IL9R modified CAR-T cells when cultured *in vitro* in the presence ("With IL2") or in the absence ("Without IL2") of the IL-2 supplement;
- 5 [0113] FIG. 12A and 12B show the comparison results in terms of the CAR-T cytotoxicity towards target cells (i.e. Caski cells) among untransduced CAR-T cells, unmodified CAR-T cells, IL15-IL9R modified CAR-T cells, and IL7-IL9R modified CAR-T cells when cultured *in vitro* in the presence ("CAR-T with IL2", i.e. FIG. 12A) or in the absence ("CAR-T without IL2", i.e. FIG. 12B) of the IL2 supplement;
- 10 [0114] FIG. 13A and 13B show the comparison results in terms of whether the synthetic cytokine signaling modification of the CAR-T cells influences T cell activation among untransduced CAR-T cells, unmodified CAR-T cells, IL15-IL9R modified CAR-T cells, and IL7-IL9R modified CAR-T cells when co-cultured with the target Caski cells ("Target") or when cultured without the target cells ("Nontarget");
- 15 [0115] FIG. 14 shows the comparison results in terms of the *in vivo* tissue distribution of unmodified T cells and IL15-IL9R modified T cells as evaluated by means of the OT-1-LLC-OVA tumor model;
- [0116] FIG. 15A and FIG. 15B respectively show the comparison results in terms of the *in vivo* efficacy and the body weight loss among unmodified T cells and IL15-IL9R modified T cells
- 20 as evaluated by means of the OT-1-LLC-OVA tumor model, with PBS as negative control;
- [0117] FIGS. 16A-16B respectively show the comparison results in terms of the *in vivo* efficacy and the body weight loss among unmodified TILs, IL7-IL9R modified TILs, and IL15-IL9R modified TILs as evaluated by means of the TIL-A375(anti-CD3) tumor model, with PBS as negative control;
- 25 [0118] FIG. 17 shows the construct structure of one exemplary synthetic cytokine signaling system that consists of only a self-activating engineered cytokine signaling chain "IL15-IL2Rb-IL9R" (or "15R-del"), and illustrates the functional cytokine signaling complexes formed on the cell membrane of an immune cell engineered to express the synthetic cytokine signaling system, as well as the corresponding downstream JAK-STAT signaling pathway thus activated;
- 30 [0119] FIG. 18 shows the transduction efficiency of the synthetic cytokine signaling system "15R-del" as compared with the synthetic cytokine signaling system "15-9R";
- [0120] FIGS. 19A-19D respectively showed the pSTAT3 signaling (FIGS. 19A and 19B) and pSTAT3 signaling (FIGS. 19C and 19D) comparison results in the "15-9R" modified T cells and the "15R-del" modified T cells cultured *in vitro* in the absence of IL-2;

[0121] FIG. 20A and FIG. 20B respectively showed the cell proliferation (FIG. 20A) and survival (FIG. 20B) comparison results in the "15-9R" modified and the "15R-del" modified T cells cultured *in vitro* in the presence or absence of IL-2;

[0122] FIG. 21 shows the *in vivo* efficacy comparison results of the "15-9R" modified and the "15R-del" TILs in the absence of IL-2.

DETAILED DESCRIPTION

[0123] In a first aspect, the present disclosure provides an engineered cytokine signaling chain to be expressed in an immune cell (e.g. T cell, NK cell, TIL, etc.).

10 [0124] Depending on whether the engineered cytokine signaling chain can be self-activated, there can be two classes of embodiments for the engineered cytokine signaling chain: (1) engineered cytokine signaling chain in a non-self-activated form; and (2) engineered cytokine signaling chain in a self-activated form, whose schematic structures are respectively illustrated in FIG. 1A and FIG. 1B.

15 [0125] As shown in FIG. 1A, the non-self-activated form of the engineered cytokine signaling chain 100 substantially encodes a chimeric cytokine signaling polypeptides, which comprises an extracellular portion and an intracellular portion, operably connected by a transmembrane portion. The extracellular portion comprises the extracellular domain (ECD) 110 of a cytokine receptor (i.e. IL2R β , IL4Ra, IL7Ra, IL9R and IL21R) or a functional variant thereof, the
20 intracellular portion comprises the intracellular domain (ICD) 120 of IL9R or a functional variant thereof, and the transmembrane portion comprises a polypeptide segment that can form a transmembrane domain 130 for the engineered cytokine signaling chain 100. Herein, the cytokine receptor can optionally be any one of IL2R β , IL4Ra, IL7Ra, IL9R or IL21R, and thus depending on different embodiments, the extracellular portion of the engineered cytokine
25 signaling chain 100 may contain an ECD 110 from any of these above cytokine receptors or a functional variant thereof. The IL9R ICD 120 in the intracellular portion may comprise a wildtype or variant IL9R ICD, and may in particular comprise a variant IL9R ICD that comprises a first sequence alteration (e.g. truncation or substitution) at positions 174-230, and/or a second sequence alteration (e.g. truncation or substitution) at positions 142-151, of the
30 wildtype IL9R sequence (SEQ ID NO: 1). In one preferred embodiment, the intracellular portion comprises a special variant IL9R ICD 120 (i.e. IL9R (ICD*)) having a sequence as set forth in SEQ ID NO: 7, including a truncation at positions 174-230 and a substitution of amino acid residues at positions 142-151 to GD (i.e. GlyAsp).

[0126] According to some specific embodiment that is preferred, the intracellular portion 120 of the engineered cytokine receptor chain may comprise a variant IL9R ICD comprising both the truncation at positions 174-230 and substitution of positions 142-151 to GD (i.e. "IL9R ICD*" hereinafter), whose amino acid sequence is set forth in SEQ ID NO: 7. Yet it is noted that in addition to this preferred embodiment, the engineered cytokine receptor chain(s) with other sequence variations in the first region (i.e. positions 174-230) and/or in the second region (i.e. positions 142-151) of the ICD of IL9R are also deemed to be also covered in the scope of the present disclosure. Non-limiting examples include, for instance, deletion at positions 180-200, deletion at positions 142-151, or substitution of positions 142-151 to GE, etc.

[0127] Compared with the non-self-activated form of the engineered cytokine signaling chain 100 illustrated in FIG. 1A, the self-activated form of the engineered cytokine signaling chain 100' also encodes a chimeric cytokine signaling polypeptide, which similarly contains an ECD 110' of a cytokine receptor (i.e. IL2R β , IL4Ra, IL7Ra, IL9R and IL21R) or a functional variant thereof in its extracellular portion, a transmembrane domain 130' in its transmembrane portion, and an IL 9R ICD 120' or a functional variant thereof in its intracellular portion, as illustrated in FIG. 1B. However, the self-activated form of the engineered cytokine signaling chain 100' differs from the non-self-activated form of the engineered cytokine signaling chain 100 by further comprising a cytokine ligand domain (CLD) 140' in its extracellular portion, which is over an N-terminus of, and is functionally compatible to, the ECD 110' of the cytokine receptor chain or the functional variant thereof. A flexible linker is arranged between the CLD 140' and the cytokine receptor ECD 110', as illustrated by a short segment line between the two blocks representing the CLD 140' and the cytokine receptor ECD 110' in FIG. 1B. Mechanistically, when the self-activated form of the engineered cytokine signaling chain 100' is expressed in an immune cell, the flexible linker allows the CLD 140' to intramolecularly bind (illustrated by the curve with double arrowheads in FIG. 1B) with the cytokine receptor ECD 110' in the extracellular portion, which in turn causes the functional activation of the IL9R ICD 120' or its functional variant (e.g. IL9R (ICD*)) in the intracellular portion, thereby realizing the self-activation of the engineered cytokine signaling chain 100'.

[0128] Due to the lack of a functionally compatible CLD in the non-self-activating form of the engineered cytokine signaling chain 100, the activation of the IL9R ICD 120 or a functional variant thereof in the intracellular portion of the engineered cytokine signaling chain 100 cannot realize self-activation intramolecularly, but rather requires the presence of additional functionally compatible cytokine ligand molecules (e.g. by co-expression of an extra

engineered cytokine ligand chain encoding such, either in a secreted form or in a membrane-bound form), which can bind intermolecularly to the cytokine receptor ECD 110 of the engineered cytokine signaling chain 100. Thus the non-self-activating form of the engineered cytokine signaling chain 100 can be regarded as an "engineered cytokine receptor chain" in this disclosure.

[0129] In either the non-self-activating form 100 or the self-activating form 100' of the engineered cytokine signaling chain as described above, the engineered cytokine signaling chain is designed such that when expressed in an immune cell, the transmembrane polypeptide encoded by the engineered cytokine signaling chain can form a functional complex with the endogenous common gamma chain (i.e. γC or IL2R γ) and optionally with certain other cytokine co-receptor subunit(s), and such formed synthetic cytokine receptor complex can, upon binding of a corresponding cytokine ligand (i.e. the CLD 140' of the engineered cytokine signaling chain intramolecularly; or the functionally compatible cytokine ligand domain of the engineered cytokine ligand chain) to the ECD of the cytokine receptor contained in the extracellular portion of the engineered cytokine receptor chain, mediate the signaling pathway that is mediated by the IL9R ICD, i.e. IL9R signaling pathway, which may include the JAK-STAT signaling pathway including the JAK-dependent phosphorylation of one or more of the three STAT proteins including STAT1, STAT3 and STAT5.

[0130] The different embodiments of the engineered cytokine signaling chain as illustrated in FIG. 1A and FIG. 1B, and their signaling mechanisms are further detailed in the following second aspect.

[0131] In a second aspect, a synthetic cytokine signaling system to be expressed in immune cells (e.g. T cells, NK cells, TILs, etc.) is further provided, which comprises the engineered cytokine signaling chain as provided in the first aspect as described above, and is aimed to improve certain characteristics of such engineered or modified immune cells, such as the proliferation capability, viability, cytotoxicity against target cells of the immune cells, etc., in an *in vitro* culturing medium comprising no or a reduced level of a cytokine supplement (e.g. IL-2). Depending on whether the synthetic cytokine signaling system consists only the engineered cytokine signaling chain as provided in the first aspect, there can be different embodiments.

[0132] According to some embodiments, the synthetic cytokine signaling system comprises, in addition to the engineered cytokine signaling chain that is in the non-self-activating form (i.e. engineered cytokine receptor chain) as described above and illustrated in FIG. 1A, further comprises a functionally compatible engineered cytokine ligand chain, configured such that

when the engineered cytokine receptor chain and the engineered cytokine ligand chain are co-expressed in the immune cell, the engineered cytokine ligand chain is capable of activating the engineered cytokine receptor chain. Depending on whether the engineered cytokine ligand chain is in a secreted form or in a membrane-bound form, there can be different embodiments
5 for the synthetic cytokine signaling system.

[0133] In a first set of embodiments as illustrated in FIG. 2A, the synthetic cytokine signaling system 1000A comprises an engineered cytokine receptor chain 100 and a secreted form of a corresponding engineered cytokine ligand chain 200A. Herein, the engineered cytokine ligand chain 200A encodes a secreted form of a cytokine ligand that corresponds to the ECD 110 of
10 the cytokine receptor in the extracellular portion of the engineered cytokine receptor chain 100 (as illustrated by the double-headed arrow in FIG. 2A). As such, when the synthetic cytokine signaling system 1000A is expressed in an immune cell, the immune cell expresses a membrane-bound chimeric cytokine receptor encoded by the engineered cytokine receptor chain 100, and simultaneously expresses and secretes a corresponding cytokine encoded by the
15 cytokine ligand chain 200A. The secreted cytokine ligand serves as the activating ligand for the functional synthetic cytokine signaling complex formed among the membrane-bound chimeric cytokine receptor and the endogenously expressed common gamma subunit (γ_c) to thereby activate the IL9R signaling.

[0134] Herein in correspondence to the ECD 110 of the cytokine receptor in the extracellular
20 portion of the engineered cytokine receptor chain 100, the engineered cytokine ligand chain 200A may comprise a wildtype form of IL2 (SEQ ID NO: 44), IL4 (SEQ ID NO: 49), IL7 (SEQ ID NO: 13), IL9 (SEQ ID NO: 46), IL15 (SEQ ID NO: 9), or IL21 (SEQ ID NO: 47), or a functional variant thereof. According to some embodiments, the corresponding cytokine ligand chain 200A may comprise a sequence having at least 70% identity to the sequence as
25 set forth in any of SEQ ID NO: 9, 13 and 44-47. According to some embodiments, the N-terminus of the cytokine ligand chain 200A may be further connected to a signal peptide (SP), which allows the cytokine ligand to be secreted properly when expressed in the immune cell. The signal peptide can be from any secreted protein or membrane-bound protein that is known. Preferably, a signal peptide from an endogenous wildtype cytokine ligand may be used. For
30 example, the signal peptide (SEQ ID NO: 17) from an endogenous wildtype IL15 may be connected to the SEQ ID NO: 9 to thereby obtain a secreted form of the IL15 cytokine ligand chain (SEQ ID NO: 52). In another example, the signal peptide (SEQ ID NO: 20) from an endogenous wildtype IL7 may be connected to the SEQ ID NO: 13 to thereby obtain a secreted

form of the IL7 cytokine ligand chain (SEQ ID NO: 50). Optionally a linker can be arranged between the signal peptide and the cytokine ligand.

[0135] In a second set of embodiments as illustrated in FIG. 2B, the synthetic cytokine signaling system 1000B comprises an engineered cytokine receptor chain 100 and a membrane-bound form of an engineered cytokine ligand chain 200B. The membrane-bound engineered cytokine ligand chain 200B comprises a ligand portion 210 and a membrane-bound portion 220 that are operably connected to each other. The ligand portion 210 of the membrane-bound cytokine ligand chain 200B corresponds to the ECD 110 of the cytokine receptor in the extracellular portion of the engineered cytokine receptor chain 100 (as illustrated by the double-headed arrow in FIG. 2B). The membrane-bound portion 220 of the membrane-bound cytokine ligand chain 200B further comprises a transmembrane region 225, which is responsible for anchoring the membrane-bound cytokine ligand chain 200B on the plasma membrane of the immune cell where the engineered cytokine ligand chain 200B is expressed. There is no limitation to the relative location of the transmembrane region 225 which, for example, can be present in the middle of the membrane-bound portion 220 (as illustrated by FIG. 2B), or optionally can be present at the C-terminus of the membrane-bound portion 220, or at the N-terminus of the membrane-bound portion 220 (i.e. between the ligand portion 210 and the membrane-bound portion 220; these latter two options are not illustrated in the drawings). As such, when the synthetic cytokine signaling system 1000B is introduced in an immune cell, the immune cell expresses a membrane-bound chimeric cytokine receptor encoded by the engineered cytokine receptor chain 100, and simultaneously expresses a membrane-bound corresponding cytokine ligand encoded by the cytokine ligand chain 200B. On the cell membrane of the immune cell, the membrane-bound chimeric cytokine receptor and the endogenously expressed common gamma subunit (γ_c) forms a functional cytokine signaling complex, and the membrane-bound cytokine ligand further provides the activating cytokine ligand to the functional cytokine signaling complex to thereby activate the downstream IL9R ICD signaling pathway (i.e. JAK-STAT signaling, etc.) inside the immune cell, mediated by the binding between the ligand portion 210 of the membrane-bound cytokine ligand chain 200B and the ECD of the cytokine receptor within the extracellular portion 110 of the engineered cytokine receptor chain 100.

[0136] Herein in correspondence to the ECD of the cytokine receptor in the extracellular portion 110 of the engineered cytokine receptor chain 100, the ligand portion 210 of the cytokine ligand chain 200B may comprise a wildtype form of IL2 (SEQ ID NO: 44), IL4 (SEQ

ID NO: 49), IL7 (SEQ ID NO: 13), IL9 (SEQ ID NO: 46), IL15 (SEQ ID NO: 9), or IL21 (SEQ ID NO: 47), or a functional variant thereof.

[0137] There could be different embodiments for the membrane-bound portion 220 and for the transmembrane region 225. According to some embodiments, the membrane-bound portion 220 of the membrane-bound cytokine ligand chain 200B may comprise a fragment crystallizable region (Fc) or a functional variant thereof, which may be operably connected to the ligand portion 210 and to the transmembrane region 225. Herein according to some embodiments, the Fc may be from IgG4, comprising a sequence as set forth in SEQ ID NO: 14; and the transmembrane region 225 may be from a transmembrane domain (TM) from CD4, comprising a sequence as set forth in SEQ ID NO: 22. Herein, the Fc may be from other IgG subclasses, such as IgG1, IgG2, IgG3, or from other immunoglobulin classes such as IgA, IgD, IgE, IgM, etc. There is no limitation herein. In addition, besides CD4 TM, the transmembrane region may have other options such as TM from CD8, CD28, B7, IL2R β , IL4Ra, IL7Ra, IL9R, IL21R, etc. Optionally, the Fc may be operably connected to the ligand portion 210 via a first hinge, and according to some embodiments, the first hinge may have a sequence set forth in SEQ ID NO: 21. Optionally, the Fc may be operably connected to the transmembrane region 225 via a second hinge. It is noted that there can be other embodiments for the membrane-bound portion 220 (i.e. comprising a different region other than Fc) and the transmembrane region 225 (i.e. comprising a different region other than CD4 TM); there can be other embodiments for the first hinge and for the second hinge; each of the first hinge and/or the second hinge may be dispensable. Herein within these embodiments that comprise Fc as part of the membrane-bound portion 210 of the membrane-bound cytokine ligand chain 200B, specific examples may include membrane-bound IL2-IgG4Fc (SEQ ID NO: 54), membrane-bound IL4-IgG4Fc (SEQ ID NO: 55), membrane-bound IL7-IgG4Fc (SEQ ID NO: 15), membrane-bound IL9-IgG4Fc (SEQ ID NO: 56), membrane-bound IL15-IgG4Fc (SEQ ID NO: 57), and membrane-bound IL21-IgG4Fc (SEQ ID NO: 58), as shown in Table 1. According to some embodiments, the N-terminus of the cytokine ligand chain 200B may be further connected to a signal peptide (SP), which allows the cytokine ligand to be secreted properly when expressed in the immune cell. The signal peptide can be from any secreted protein or membrane-bound protein that is known, yet preferably can be from the signal peptide corresponding to the ligand portion 210 of the membrane-bound cytokine ligand chain 200B. For example, the membrane-bound IL2-IgG4Fc, membrane-bound IL4-IgG4Fc, membrane-bound IL7-IgG4Fc, membrane-bound IL9-IgG4Fc, membrane-bound IL15-IgG4Fc, and IL21-

IgG4Fc as listed in Table 1 may be connected to the signal peptide from IL2, IL4, IL7, IL9, IL15, and IL21, whose sequences are set forth in SEQ ID NO: 67, 68, 20, 69, 17, and 70, respectively.

- [0138]** According to some embodiments where the ligand portion 210 comprises IL15 (SEQ ID NO: 9) or a functional variant thereof, the membrane-bound portion 220 of the membrane-bound cytokine ligand chain 200B may optionally comprise IL15Ra or a functional variant thereof (SEQ ID NO: 10). One flexible linker (e.g. GS linker (SEQ ID NO: 16)) may optionally be arranged between the ligand portion 210 and the membrane-bound portion 220. It is noted that IL15Ra is itself a transmembrane protein whose transmembrane region 225 is inside the IL5Ra polypeptide. According to one specific embodiment, the membrane-bound cytokine ligand chain 200B comprises membrane bound IL15-IL15Ra having a sequence as set forth in SEQ ID NO: 11. According to some embodiments, the N-terminus of the membrane bound IL15-IL15Ra may be further connected to a signal peptide (SP), which may preferably be from IL15 (SP) (SEQ ID NO: 17), but can have other options.
- [0139]** The different embodiments of the synthetic cytokine signaling system as illustrated in FIGS. 2 and 3 and described above are summarized in Table 1, and further detailed in the following five scenarios.

Table 1. Embodiments of the synthetic cytokine signaling system shown in FIGS. 2 and 3.

Engineered Cytokine Receptor Chain	Engineered Cytokine Ligand Chain
IL2Rβ(ECD)-TM-IL9R(ICD): - IL2R β (SP): SEQ ID NO: 18 - IL2R β (ECD): SEQ ID NO: 3 - IL9R(ICD): SEQ ID NO: 1 - IL9R(ICD*): SEQ ID NO: 7 - IL2R β (TM): SEQ ID NO: 19 - IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*): SEQ ID NO: 8 - IL2R β (SP)-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*): SEQ ID NO: 25	IL-2 or IL15 - IL2 (w/o SP): SEQ ID NO: 44 - IL2 (w/ SP): SEQ ID NO: 48 - IL15 (w/o SP): SEQ ID NO: 9 - IL15 (w/ SP): SEQ ID NO: 52 - IgG4Fc: SEQ ID NO: 14 - IL15Ra: SEQ ID NO: 10 - membrane-bound IL2-IgG4Fc (w/o SP): SEQ ID NO: 54 - membrane-bound IL2(SP)-IL2-IgG4Fc (w/o SP): SEQ ID NO: 75 - membrane-bound IL15-IgG4Fc (w/o SP): SEQ ID NO: 57 - membrane-bound IL15R α (SP)-IL15-IgG4Fc (w/o SP): SEQ ID NO: 76 - membrane bound IL15-IL15Ra (w/o SP): SEQ ID NO: 11 - membrane bound IL15R α (SP)-IL15-IL15Ra (w/ SP): SEQ ID NO: 26
IL4Ra(ECD)- TM-IL9R(ICD): - IL4Ra(SP): SEQ ID NO: 63 - IL4Ra(ECD): SEQ ID NO: 33 - IL9R(ICD): SEQ ID NO: 1	IL-4 - IL4 (w/o SP): SEQ ID NO: 49 - IL4 (w/ SP): SEQ ID NO: 45 - IgG4Fc: SEQ ID NO: 14

- IL9R(ICD*): SEQ ID NO: 7 - IL4R(TM): SEQ ID NO: 34 - IL4Ra(ECD)-IL4Ra(TM)-IL9R(ICD*): SEQ ID NO: 35 - IL4Ra(SP)-IL4Ra(ECD)-IL4Ra(TM)-IL9R(ICD*): SEQ ID NO: 71	- membrane-bound IL4-IgG4Fc (w/o SP): SEQ ID NO: 55 - membrane-bound IL4(SP)-IL4-IgG4Fc (w/ SP): SEQ ID NO: 74
IL7Ra(ECD)-TM-IL9R(ICD): - IL7Ra(SP): SEQ ID NO: 23 - IL7Ra(ECD): SEQ ID NO: 5 - IL9R(ICD): SEQ ID NO: 1 - IL9R(ICD*): SEQ ID NO: 7 - IL7Ra(TM): SEQ ID NO: 24 - IL7Ra(ECD)-IL7R(TM)-IL9R(ICD*): SEQ ID NO: 12 - IL7Ra(SP)-IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*): SEQ ID NO: 27	IL-7 - IL7 (w/o SP): SEQ ID NO: 13 - IL7 (w/ SP): SEQ ID NO: 50 - IgG4Fc: SEQ ID NO: 14 - membrane bound IL7-IgG4Fc (w/o SP): SEQ ID NO: 15 - membrane bound IL7(SP)-IL7-IgG4Fc (w/ SP): SEQ ID NO: 28
IL9R(ECD)-TM-IL9R(ICD): - IL9R(SP): SEQ ID NO: 64 - IL9R(ECD): SEQ ID NO: 36 - IL9R(ICD): SEQ ID NO: 1 - IL9R(ICD*): SEQ ID NO: 7 - IL9R(TM): SEQ ID NO: 37 - IL9R(ECD)-IL9R(TM)-IL9R(ICD*): SEQ ID NO: 38 - IL4Ra(SP)-IL4Ra(ECD)-IL4Ra(TM)-IL9R(ICD*): SEQ ID NO: 72	IL-9 - IL9 (w/o SP): SEQ ID NO: 46 - IL9 (w/ SP): SEQ ID NO: 51 - IgG4Fc: SEQ ID NO: 14 - membrane-bound IL9-IgG4Fc (w/o SP): SEQ ID NO: 56 - membrane-bound IL9(SP)-IL9-IgG4Fc (w/o SP): SEQ ID NO: 77
IL21R(ECD)-TM-IL9R(ICD): - IL21R(SP): SEQ ID NO: 66 - IL21R(ECD): SEQ ID NO: 39 - IL9R(ICD): SEQ ID NO: 1 - IL9R(ICD*): SEQ ID NO: 7 - IL21R(TM): SEQ ID NO: 40 - IL21R(ECD)-IL21R(TM)-IL9R(ICD*): SEQ ID NO: 41 - IL4Ra(SP)-IL4Ra(ECD)-IL4Ra(TM)-IL9R(ICD*): SEQ ID NO: 73	IL-21 - IL21 (w/o SP): SEQ ID NO: 47 - IL21 (w/ SP): SEQ ID NO: 53 - IgG4Fc: SEQ ID NO: 14 - membrane-bound IL21-IgG4Fc (w/o SP): SEQ ID NO: 58 - membrane-bound IL21(SP)-IL21-IgG4Fc (w/o SP): SEQ ID NO: 78

NOTE: SP, ECD, TM, and ICD means the signal peptide, extracellular domain, transmembrane domain, and intracellular domain respectively; all sequence listings correspond to the wildtype form, except for IL9R (ICD*), which is a functional variant of IL9R ICD; "w/" means "with", and "w/o" means "without".

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[0140] Scenario 1: the cytokine receptor ECD 110 contained in the extracellular portion of the engineered cytokine receptor chain 100 is from IL2R β , and the chimeric cytokine receptor polypeptide encoded by the engineered cytokine receptor chain 100 is substantially "IL2R β (ECD)-TM-IL9R(ICD)" which, when expressed in an immune cell, can activate the

10 IL9R signaling upon stimulation of its corresponding cytokine ligand IL-2 and/or IL15, or their

functional variants. More specifically, the encoded chimeric cytokine receptor polypeptide "IL2R β (ECD)-TM-IL9R(ICD)" may form a functional complex with the common gamma chain and IL2R α subunit to thereby be responsive to the activation of IL2 or its functional variant; alternatively, the chimeric cytokine receptor polypeptide "IL2R β (ECD)-TM-IL9R(ICD)" may form a functional complex with the common gamma chain and IL2R α subunit to thereby be responsive to the activation of IL15 or its functional variant.

[0141] Scenario 2: the cytokine receptor ECD 110 contained in the extracellular portion of the engineered cytokine receptor chain 100 is from IL4Ra, and the chimeric cytokine receptor polypeptide encoded is "IL4Ra(ECD)-TM-IL9R(ICD)" which, when expressed in an immune cell, can activate the IL9R signaling upon stimulation of its corresponding cytokine ligand IL4 or its functional variants. More specifically, the chimeric cytokine receptor polypeptide "IL4Ra(ECD)-TM-IL9R(ICD)" may form a functional complex with the common gamma chain to thereby be responsive to the activation of IL4 or its functional variant.

[0142] Scenario 3: the cytokine receptor ECD contained in the extracellular portion 110 of the engineered cytokine receptor chain 100 is from IL7Ra, and the chimeric cytokine receptor polypeptide encoded is "IL7Ra(ECD)-TM-IL9R(ICD)" which, when expressed in an immune cell, can activate the IL9R signaling upon stimulation of its corresponding cytokine ligand IL7 or its functional variants. More specifically, the chimeric cytokine receptor polypeptide "IL7Ra(ECD)-TM-IL9R(ICD)" may form a functional complex with the common gamma chain to thereby be responsive to the activation of IL7 or its functional variant.

[0143] Scenario 4: the cytokine receptor ECD 110 contained in the extracellular portion of the engineered cytokine receptor chain 100 is from IL9R, and the chimeric cytokine receptor polypeptide encoded is "IL9R(ECD)-TM-IL9R(ICD)" which, when expressed in an immune cell, can activate the IL9R signaling upon stimulation of its corresponding cytokine ligand IL9 or its functional variants. More specifically, the chimeric cytokine receptor polypeptide "IL9R(ECD)-TM-IL9R(ICD)" may form a functional complex with the common gamma chain to thereby be responsive to the activation of IL9 or its functional variant.

[0144] Scenario 5: the cytokine receptor ECD 110 contained in the extracellular portion of the engineered cytokine receptor chain 100 is from IL21R, and the chimeric cytokine receptor polypeptide encoded is "IL21R(ECD)-TM-IL9R(ICD)" which, when expressed in an immune cell, can activate the IL9R signaling upon stimulation of its corresponding cytokine ligand IL21 or its functional variants.

[0145] In any of the scenarios provided above, the term "functional variant" refers to an

alteration of a parental polypeptide, such as a sequence alteration (e.g. of point mutation(s), insertion mutation(s), deletion mutation(s), or other sequence alterations etc.), a chemical modification (e.g. glycosylation, connecting to a peptide etc.) that substantially retains the capability (i.e. more than 30%, such as 40%, 50%, 60%, 70%, 80%, 90%, or 100%, etc.) of the parental polypeptide. Several examples are provided for better illustration. For example, a functional variant of the ECD of IL7Ra may include one or more point mutations yet still retaining more than 50% of the capability of the wildtype IL7Ra ECD to be able to bind, and be responsive to, the IL7 ligand. A functional variant of the of IL9R ICD may include one or more mutations yet still retaining more than 50% of the capability of wildtype IL9R ICD to, upon the stimulation of the chimeric cytokine receptor polypeptide by a corresponding cytokine ligand, activate the JAK-STAT signaling pathway. A functional variant of IL21 may include one or more mutations, and/or may be operably connected to a membrane-bound polypeptide (e.g. IL15Ra or Fc), yet still retaining more than 50% of the capability of wildtype IL21 to bind to IL21R and common gamma chain to thereby stimulate the IL21R signaling.

[0146] In any of the scenarios/embodiments provided above, the intracellular portion 120 of the engineered cytokine receptor chain may comprise a wildtype IL9R ICD or a functional variant of IL9R ICD. According to yet some preferred embodiments, the intracellular portion 120 of the engineered cytokine receptor chain may comprise a functional variant of IL9R ICD (termed "IL9R ICD'") that is characterized such that it comprises: (1) at least one sequence alteration in a first region within approximately 57 amino acid residues from the C-terminus of the wildtype IL9R ICD (i.e. corresponding to positions 174-230 of SEQ ID NO: 1); and/or (2) at least one sequence alteration in a second region corresponding positions 142-151, of the wildtype IL9R ICD (e.g. SEQ ID NO: 1). Herein optionally, a truncation/substitution may occur at the first and/or the second region. According to some specific embodiment that is preferred, the intracellular portion 120 of the engineered cytokine receptor chain may comprise a variant IL9R ICD comprising both the truncation at positions 174-230 and substitution of positions 142-151 to GD (i.e. "IL9R ICD*" hereinafter), whose amino acid sequence is set forth in SEQ ID NO: 7. Yet it is noted that in addition to this preferred embodiment, the engineered cytokine receptor chain(s) with other sequence variations in the first region (i.e. positions 174-230) and/or in the second region (i.e. positions 142-151) of the ICD of IL9R are also deemed to be also covered in the scope of the present disclosure. Non-limiting examples include, for instance, deletion at positions 180-200, deletion at positions 142-151, or substitution of positions 142-151 to GE, etc.

[0147] In any of the scenarios/embodiments provided above, the transmembrane (TM) portion

130 may comprise any sequence that can form a transmembrane helix in the cell membrane. Non-limiting examples may include the TM from any known transmembrane proteins such as CD4, CD8, CD28, B7, IL2R β , IL4Ra, IL7Ra, IL9R, IL21R, etc. Preferably, the TM portion 130 may comprise the TM sequence that corresponds to the ECD of the cytokine receptor in the extracellular portion 110. For example, the engineered cytokine receptor chain "IL2R β (ECD)-TM-IL9R(ICD)" may comprise the TM of IL2R β to thereby have a structure of "IL2R β (ECD)-IL2R β (TM)-IL9R(ICD)"; the engineered cytokine receptor chain "IL4Ra(ECD)-TM-IL9R(ICD)" may comprise the TM of IL4Ra to thereby have a structure of "IL4Ra(ECD)-IL4Ra(TM)-IL9R(ICD)"; the engineered cytokine receptor chain "IL7Ra(ECD)-TM-IL9R(ICD)" may comprise the TM of IL7Ra to thereby have a structure of "IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD)"; the engineered cytokine receptor chain "IL9R(ECD)-TM-IL9R(ICD)" may comprise the TM of IL9R to thereby have a structure of "IL9R(ECD)-IL9R(TM)-IL9R(ICD)"; and the engineered cytokine receptor chain "IL21R(ECD)-TM-IL9R(ICD)" may comprise the TM of IL21R to thereby have a structure of "IL21R(ECD)-IL21R(TM)-IL9R(ICD)". The sequences for these above embodiments are listed in Table 1.

[0148] Optionally, a flexible linker can be arranged between the extracellular portion 110 and the transmembrane portion 130, and/or between the transmembrane portion 130 and the intracellular portion 120 to thereby provide a spacer between the two functional portions to minimize the potential interference therefrom. Examples for the linker sequence may include a GS linker (e.g. SEQ ID NO: 16), but can have other options.

[0149] In order to allow the accurate and effective translocation of the engineered cytokine receptor chain when a nucleic acid encoding the engineered cytokine receptor chain is expressed in the immune cell, according to some embodiments, the engineered cytokine receptor may further comprise a signal peptide (i.e. "SP") on the N-terminus thereof (i.e. on the N-terminus of the extracellular portion 110), which may come from any known signal peptides. Preferably the signal peptide may correspond to the ECD of the cytokine receptor in the extracellular portion 110, and as such the engineered cytokine receptor chain as listed in Table 1 may have structures including: "IL2R β (SP)-IL2R β (ECD)-TM-IL9R(ICD)", "IL4Ra(SP)-IL4Ra(ECD)-TM-IL9R(ICD)", "IL7Ra(SP)-IL7Ra(ECD)-TM-IL9R(ICD)", "IL9R(SP)-IL9R(ECD)-TM-IL9R(ICD)", and "IL21R(SP)-IL21R(ECD)-TM-IL9R(ICD)". Herein, the IL2R β (SP), IL4Ra(SP), IL7Ra(SP), IL9R(SP), and IL21R(SP) may comprise a sequence as set forth in SEQ ID NOS: 18, 63, 23, 64, and 66, respectively; and the above four engineered cytokine receptor chains may comprise a sequence as set forth in SEQ ID NOS: 25, 71, 27, 72,

and 73, respectively. It is noted that there is no limitation to the signal peptide for each of the engineered cytokine receptor chain as listed in Table 1.

[0150] In any of the scenarios/embodiments of the engineered cytokine receptor chain 100 as provided above, the corresponding cytokine ligand(s) encoded by the engineered cytokine ligand chain as listed in Table 1 may be in any forms. Optionally, the cytokine ligand(s) may be endogenously expressed, or may be exogenously provided. Optionally, the cytokine ligand(s) may be in a wildtype form, or may be in an engineered form, such as containing at least one sequence variations or chemical modification that modifies the function of the cytokine ligand(s). Optionally, the cytokine ligand(s) may be in a native form, or may be in an orthogonal form. Optionally, the cytokine ligand(s) may be in a secreted form, or may be in a membrane-bound form. As used herein, the term "corresponding", "correspond", or alike, means that the cytokine ligand(s) is/are capable of binding to the ECD of the cytokine receptor in the extracellular portion 110 of the engineered cytokine receptor chain 100 to thereby activate the IL9R ICD in the intracellular portion 120 of the engineered cytokine receptor chain 100. Herein, the corresponding cytokine ligand encoded by the engineered cytokine ligand chain may be one of IL2, IL4, IL7, IL9, IL15 and IL21 or a functional variant thereof, which respectively correspond to IL2Rb ECD, IL4Ra ECD, IL7Ra ECD, IL9R ECD, IL2R β ECD and IL21R ECD that may potentially be used in the extracellular portion 110 of the engineered cytokine receptor chain 100 (the corresponding relationship is listed in Table 1).

[0151] According to some other embodiments, the synthetic cytokine signaling system consists only of the engineered cytokine signaling chain as provided in the first aspect described above.

[0152] Optionally, the synthetic cytokine signaling system may consist only of the engineered cytokine receptor chain 100 as illustrated in FIG. 1A. As such, when the synthetic cytokine signaling system is expressed in an immune cell, the chimeric cytokine receptor encoded by the engineered cytokine receptor chain 100 may form a synthetic cytokine signaling complex with the endogenously expressed common gamma subunit (γ_c) on the cell membrane of such modified or engineered immune cell. The synthetic cytokine signal complex may further, upon binding of the corresponding cytokine ligand(s), activate the IL9R signaling. Herein the corresponding cytokine ligand(s) may be cytokines that are endogenously produced and secreted by other cell types of the host, or may be cytokines that are exogenously provided as cytokine supplement(s) when cultured *in vitro* or transferred *in vivo*. Optionally, the cytokine(s) may be in wildtype form(s) or may be functional variant(s).

[0153] Further optionally, the synthetic cytokine signaling system may consist only of the

engineered cytokine signaling chain that is in the self-activating form as described above and illustrated in FIG. 1B. The different embodiments of the self-activating form of the engineered cytokine signaling chain in the synthetic cytokine signaling system provided herein are summarized in Table 2 below.

5 Table 2. Embodiments of the self-activating form of the engineered cytokine signaling chain.

Engineered Cytokine Signaling Chain	Engineered Cytokine Signaling Chain
IL2/IL15-IL2Rβ(ECD)- IL2Rβ(TM)-IL9R(ICD*): Signal peptide: - IL15R α (SP): SEQ ID NO: 85 - IL2R α (SP): SEQ ID NO: 86 Extracellular portion: (1) cytokine ligand domain: - IL2 (w/o SP): SEQ ID NO: 44 - IL15 (w/o SP): SEQ ID NO: 9 (2) flexible linker: SEQ ID NO: 16 (3) cytokine receptor domain: - IL2R β (ECD): SEQ ID NO: 3 Transmembrane domain: - IL2R β (TM): SEQ ID NO: 19 Intracellular portion: - IL9R(ICD): SEQ ID NO: 1 - IL9R(ICD*): SEQ ID NO: 7 Full-length sequence(s): - IL15-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*) (w/o SP): SEQ ID NO: 79 - IL15R α (SP)-IL15-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*) (w/ SP): SEQ ID NO: 87 - IL2-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*) (w/o SP): SEQ ID NO: 80 - IL2R α (SP)-IL2-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*) (w/ SP): SEQ ID NO: 88	IL4-IL4Ra(ECD)-IL4Ra(TM)-IL9R(ICD*): Signal peptide: - IL4R α (SP): SEQ ID NO: 63 Extracellular portion: (1) cytokine ligand domain: - IL4 (w/o SP): SEQ ID NO: 49 (2) flexible linker: SEQ ID NO: 16 (3) cytokine receptor domain: - IL4Ra(ECD): SEQ ID NO: 33 Transmembrane domain: - IL4R(TM): SEQ ID NO: 34 Intracellular portion: - IL9R(ICD): SEQ ID NO: 1 - IL9R(ICD*): SEQ ID NO: 7 Full-length sequence: - IL4-IL4Ra(ECD)-IL4Ra(TM)-IL9R(ICD*) (w/o SP): SEQ ID NO: 81 - IL4R α (SP)-IL4-IL4Ra(ECD)-IL4R α (TM)-IL9R(ICD*) (w/ SP): SEQ ID NO: 89
IL7-IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*): Signal peptide: - IL7R α (SP): SEQ ID NO: 23 Extracellular portion: (1) cytokine ligand domain: - IL7 (w/o SP): SEQ ID NO: 13 (2) flexible linker: SEQ ID NO: 16 (3) cytokine receptor domain: - IL7R α (ECD): SEQ ID NO: 5 Transmembrane domain: - IL7R α (TM): SEQ ID NO: 24	IL9-IL9R(ECD)-IL9R(TM)-IL9R(ICD*): Signal peptide: - IL9R(SP): SEQ ID NO: 64 Extracellular portion: (1) cytokine ligand domain: - IL9 (w/o SP): SEQ ID NO: 46 (2) flexible linker: SEQ ID NO: 16 (3) cytokine receptor domain: - IL9R(ECD): SEQ ID NO: 36 Transmembrane domain: - IL9R(TM): SEQ ID NO: 37

Intracellular portion: - IL9R(ICD): SEQ ID NO: 1 - IL9R(ICD*): SEQ ID NO: 7 Full-length sequence: - IL7-IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*) (w/o SP): SEQ ID NO: 82 - IL7Ra(SP)-IL7-IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*) (w/ SP): SEQ ID NO: 90	Intracellular portion: - IL9R(ICD): SEQ ID NO: 1 - IL9R(ICD*): SEQ ID NO: 7 full-length sequence: - IL9-IL9R(ECD)-IL9R(TM)-IL9R(ICD*) (w/o SP): SEQ ID NO: 83 - IL9R(SP)-IL9-IL9R(ECD)-IL9R(TM)-IL9R(ICD*) *) (w/ SP): SEQ ID NO: 91
IL21-IL21R(ECD)-IL21R(TM)-IL9R(ICD*): Signal peptide: - IL21R(SP): SEQ ID NO: 66 Extracellular portion: (1) cytokine ligand domain: - IL21 (w/o SP): SEQ ID NO: 47 (2) flexible linker: SEQ ID NO: 16 (3) cytokine receptor domain: - IL21R(ECD): SEQ ID NO: 39 Transmembrane domain: - IL21R(TM): SEQ ID NO: 40 Intracellular portion: - IL9R(ICD): SEQ ID NO: 1 - IL9R(ICD*): SEQ ID NO: 7 Full-length sequence: - IL21-IL21R(ECD)-IL21R(TM)-IL9R(ICD*)(w/o SP): SEQ ID NO: 84 - IL21R(SP)-IL21-IL21R(ECD)-IL21R(TM)-IL9R(ICD*)(w/ SP): SEQ ID NO: 92	

NOTE: SP, ECD, TM, and ICD means the signal peptide, extracellular domain, transmembrane domain, and intracellular domain respectively; all sequence listings correspond to the wildtype form, except for IL9R (ICD*), which is a functional variant of IL9R ICD; "w/" means "with", and "w/o" means "without".

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[0154] As shown in Table 2, the engineered cytokine signaling chain that is in the self-activating form may substantially comprise, from the N-terminus to the C-terminus, a structure of CLD-ECD-TM-ICD, where each of CLD, ECD, TM and ICD may take different embodiments. Herein, the ICD may optionally comprise IL9R (ICD), comprising a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 1, or may preferably comprise IL9R (ICD*), comprising a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 7. The ECD may optionally comprise any of IL2Rβ(ECD), IL4Ra(ECD), IL7Ra(ECD), IL9R(ECD), or IL21R(ECD) or a functional variant thereof. A flexible linker may be optionally arranged between the CLD and ECD, configured to allow the

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CLD to bind with ECD intramolecularly to thereby cause the ICD to be activated. Herein, the flexible linker may optionally comprise a GS linker having a sequence as set forth in SEQ ID NO: 16, but may have other sequences (e.g. SEQ ID NO: 4, 11, or 28). The TM may preferably be derived from the same cytokine receptor as the ECD, but may optionally be derived from other transmembrane proteins/polypeptides. The engineered cytokine signaling chain may further comprise a signal peptide (SP), and may comprise a structure of SP-CLD-ECD-TM-ICD from the N-terminus to the C-terminus. Herein, the SP may be derived from the same cytokine receptor as the ECD, but may optionally be derived from other proteins/polypeptides containing signal peptides.

10 **[0155]** According to some embodiments, the ECD comprises IL2R β (ECD) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 3; the CLD comprises IL15 or a functional variant thereof, having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 9; the TM may comprise IL2R β (TM), having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 19; the ICD comprises
15 IL9R (ICD*) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 7; the flexible linker between the CLD and the ECD is a GS linker (SEQ ID NO: 16). As such, some embodiments of the engineered cytokine signaling chain may comprise a structure "IL15-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*)", comprising a sequence at least 70% to the sequence as set forth in SEQ ID NO: 79. Further optionally, these embodiments of the
20 engineered cytokine signaling chain may further comprise an IL15Ra(SP) (SEQ ID NO: 85). As such, one specific embodiment of the engineered cytokine signaling chain has a structure "IL15Ra(SP)-IL15-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*)" (SEQ ID NO: 87), which can be encoded by a nucleic acid sequence SEQ ID NO: 93 in a vector used to transduce immune cells (see Example 6).

25 **[0156]** According to some other embodiments, the ECD also comprises IL2R β (ECD) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 3; the CLD comprises IL2 or a functional variant thereof, having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 44; the TM may comprise IL2R β (TM), having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 19; the ICD
30 comprises IL9R (ICD*) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 7; the flexible linker between the CLD and the ECD is a GS linker (SEQ ID NO: 16). As such, these embodiments of the engineered cytokine signaling chain may comprise a structure "IL2-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*)", comprising a sequence at

least 70% to the sequence as set forth in SEQ ID NO: 80. Further optionally, these embodiments of the engineered cytokine signaling chain may further comprise an IL2Ra(SP) (SEQ ID NO: 86).

[0157] According to some other embodiments, the ECD also comprises IL4Ra(ECD) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 33; the CLD comprises IL4 or a functional variant thereof, having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 49; the TM may comprise IL4R(TM), having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 34; the ICD comprises IL9R (ICD*) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 7; the flexible linker between the CLD and the ECD is a GS linker (SEQ ID NO: 16). As such, these embodiments of the engineered cytokine signaling chain may comprise a structure "IL4-IL4Ra(ECD)-IL4Ra(TM)-IL9R(ICD*)", comprising a sequence at least 70% to the sequence as set forth in SEQ ID NO: 81. Further optionally, these embodiments of the engineered cytokine signaling chain may further comprise a SP that is IL4Ra(SP) (SEQ ID NO: 63).

[0158] According to some other embodiments, the ECD also comprises IL7Ra(ECD) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 5; the CLD comprises IL7 or a functional variant thereof, having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 13; the TM may comprise IL7Ra(TM), having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 24; the ICD comprises IL9R (ICD*) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 7; the flexible linker between the CLD and the ECD is a GS linker (SEQ ID NO: 16). As such, these embodiments of the engineered cytokine signaling chain may comprise a structure "IL7-IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*)", comprising a sequence at least 70% to the sequence as set forth in SEQ ID NO: 82. Further optionally, these embodiments of the engineered cytokine signaling chain may further comprise an IL7Ra(SP) (SEQ ID NO: 23).

[0159] According to some other embodiments, the ECD also comprises IL9R(ECD) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 36; the CLD comprises IL9 or a functional variant thereof, having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 46; the TM may comprise IL9R(TM), having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 37; the ICD comprises IL9R (ICD*) having a sequence having at least 70% identity to the sequence as set

forth in SEQ ID NO: 7; the flexible linker between the CLD and the ECD is a GS linker (SEQ ID NO: 16). As such, these embodiments of the engineered cytokine signaling chain may comprise a structure "IL9-IL9R(ECD)-IL9R(TM)-IL9R(ICD*)", comprising a sequence at least 70% to the sequence as set forth in SEQ ID NO: 83. Further optionally, these embodiments of the engineered cytokine signaling chain may further comprise an IL9R(SP) (SEQ ID NO: 64).

[0160] According to some other embodiments, the ECD also comprises IL21R(ECD) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 39; the CLD comprises IL21 or a functional variant thereof, having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 47; the TM may comprise IL21R(TM), having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 40; the ICD comprises IL9R (ICD*) having a sequence having at least 70% identity to the sequence as set forth in SEQ ID NO: 7; the flexible linker between the CLD and the ECD is a GS linker (SEQ ID NO: 16). As such, these embodiments of the engineered cytokine signaling chain may comprise a structure "IL21-IL21R(ECD)-IL21R(TM)-IL9R(ICD*)", comprising a sequence at least 70% to the sequence as set forth in SEQ ID NO: 84. Further optionally, these embodiments of the engineered cytokine signaling chain may further comprise an IL21R(SP) (SEQ ID NO: 66).

[0161] When any of these above embodiments of the synthetic cytokine signaling system provided in the second aspect of the disclosure is expressed in an immune cell, the immune cell may exhibit one or more of the following characteristics compared to when the synthetic cytokine signaling system is not expressed in the immune cell: (1) increased STAT1/STAT3/STAT5 signaling in the absence of IL2 stimulation; (2) increased proliferation capability when cultured *in vitro* in the absence of IL2; (3) increased viability when cultured *in vitro* in the absence of IL2; (4) increased interferon gamma (IFN γ or IFN γ) secretion when co-cultured with target cells of the immune cell in the absence of IL-2 stimulation; (5) increased cytotoxicity against target cells of the immune cell when co-cultured with the target cells in the absence of IL-2 stimulation when cultured *in vitro* in the absence of IL2; (6) more preferred migration to tumor tissues and/or lymphoid tissues when a population of the immune cell are transferred into a subject bearing a tumor that the immune cell specifically targets; and/or (7) increased tumor control ability when a population of the immune cell are transferred into a subject bearing a tumor that the immune cell specifically targets.

[0162] In a third aspect, the present disclosure further provides a vector kit that is designed to

introduce the synthetic cytokine signaling system provided in the second aspect into the immune cell for expression.

[0163] The vector kit may comprise at least one expression vector (or short as "vector"), each comprising a nucleic acid sequence that encodes the engineered cytokine receptor chain and/or the engineered cytokine ligand chain of the synthetic cytokine signaling system as described above.

[0164] In a first situation where the synthetic cytokine signaling system consists only of an engineered cytokine ligand chain, the vector kit may consist of only one vector that comprises a nucleic acid sequence encoding the engineered cytokine receptor chain.

10 [0165] In second situation where the synthetic cytokine signaling system comprises both an engineered cytokine receptor chain and an engineered cytokine ligand chain, the vector kit may comprise two vectors (i.e. first vector and second vector), each comprising a nucleic acid sequence that encodes one of the engineered cytokine receptor chain or the engineered cytokine ligand chain.

15 [0166] Alternatively in the second situation, the vector kit may consist of only one vector, which comprises two nucleic acid sequence encoding the engineered cytokine receptor chain and the engineered cytokine ligand chain respectively. Optionally, the two nucleotide sequences are in two different open reading frames (ORFs); yet optionally, the two nucleotide sequences are in a common open reading frame (ORF), and the vector comprises a separator sequence separating the two nucleotide sequences, which comprises an F2A (SEQ ID NO: 42) or a P2A(SEQ ID NO: 43), or can have other options.

20 [0167] According to one specific embodiment, the vector kit may comprise one vector, which comprises a polynucleotide sequence as set forth in SEQ ID: 60, and the polynucleotide sequence encodes a polypeptide having a sequence as set forth in SEQ ID NO: 59. The polypeptide includes an F2A sequence that separates the engineered cytokine ligand chain mbIL15-IL15Ra (i.e. Recast-IL15) and the engineered cytokine receptor chain IL2Rb(ECD)-IL2Rb(TM)-IL9R(ICD*). Here the polypeptide is termed as "IL15-IL15R-IL2Rb-IL9R" or "IL15-IL9R".

25 [0168] According to another specific embodiment, the vector kit may comprise one vector, which comprises a polynucleotide sequence as set forth in SEQ ID: 62, and the polynucleotide sequence encodes a polypeptide having a sequence as set forth in SEQ ID NO: 61. The polypeptide includes an F2A sequence that separates the engineered cytokine ligand chain mbIL7-IgG4Fc and the engineered cytokine receptor chain IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*). Here the polypeptide is termed as "IL7-IL7R-IL9R" or "IL7-IL9R".

[0169] As used herein, a “nucleotide sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an RNA may optionally also include introns to the extent that the nucleotide sequence encoding the protein
5 may in some version contain an intron(s).

[0170] Herein each one vector may include an isolated polynucleotide disclosed herein (e.g., a polynucleotide that encodes a polypeptide disclosed herein), host cells into which are introduced the recombinant vectors (i.e., such that the host cells contain the polynucleotide and/or a vector comprising the polynucleotide), and the production of recombinant
10 polypeptides or fragments thereof by recombinant techniques. As used herein, a “vector” is any construct capable of delivering one or more polynucleotide(s) of interest to a host cell when the vector is introduced to the host cell. An “expression vector” is capable of delivering and expressing the one or more polynucleotide(s) of interest as an encoded polypeptide in a host cell into which the expression vector has been introduced. Thus, in an expression vector, the
15 polynucleotide of interest is positioned for expression in the vector by being operably linked with regulatory elements such as a promoter, enhancer, and/or a poly-A tail, either within the vector or in the genome of the host cell at or near or flanking the integration site of the polynucleotide of interest such that the polynucleotide of interest will be translated in the host cell introduced with the expression vector.

[0171] A vector can be introduced into the host cell by methods known in the art, e.g., electroporation, chemical transfection (e.g., DEAE-dextran), transformation, transfection, and infection and/or transduction (e.g., with recombinant virus). Thus, non-limiting examples of vectors include viral vectors (which can be used to generate recombinant virus), naked DNA or RNA, plasmids, cosmids, phage vectors, and DNA or RNA expression vectors associated
20 with cationic condensing agents.

[0172] The present disclosure provides a recombinant vector comprising a nucleic acid construct suitable for genetically modifying a cell, which can be used for treatment of pathological disease or condition.

[0173] Any vector or vector type can be used to deliver genetic material to the cell. These
30 vectors include but are not limited to plasmid vectors, viral vectors, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), and human artificial chromosomes (HACs). Viral vectors can include but are not limited to recombinant retroviral vectors, recombinant lentiviral vectors, recombinant adenoviral vectors, foamy virus vectors, recombinant adeno-associated viral (AAV) vectors, hybrid vectors, and plasmid transposons

(e.g., sleeping beauty transposon system, and PiggyBac transposon system) or integrase based vector systems. Other vectors that are known in the art can also be used in connection with the methods described herein.

5 [0174] In some embodiments, the vector is a viral vector. The viral vector can be grown in a culture medium specific for viral vector manufacturing. Any suitable growth media and/or supplements for growing viral vectors can be used in accordance with the embodiments described herein.

10 [0175] In some embodiments, the vector used is a recombinant retroviral vector. A retroviral vector is capable of directing the expression of a nucleic acid molecule of interest. A retrovirus is present in the RNA form in its viral capsule and forms a double-stranded DNA intermediate when it replicates in the host cell. Similarly, retroviral vectors are present in both RNA and double-stranded DNA forms. The retroviral vector also includes the DNA form which contains a recombinant DNA fragment and the RNA form containing a recombinant RNA fragment. The vectors can include at least one transcriptional promoter/enhancer, or other elements which
15 control gene expression. Such vectors can also include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used. Long terminal repeats (LTRs) are identical sequences of DNA that repeat many times (e.g., hundreds or thousands of times) found at either end of retrotransposons or proviral DNA formed by reverse transcription of retroviral RNA. They are
20 used by viruses to insert their genetic material into the host genomes. Optionally, the vectors can also include a signal which directs polyadenylation, selectable markers such as Ampicillin resistance, Neomycin resistance, TK, hygromycin resistance, phleomycin resistance histidinol resistance, or DHFR, as well as one or more restriction sites and a translation termination sequence. For example, such vectors can include a 5' LTR, a leading sequence, a tRNA binding
25 site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR or a portion thereof. Additionally, retroviral vector used herein can also refers to the recombinant vectors created by removal of the retroviral gag, pol, and env genes and replaced with the gene of interest.

30 [0176] In some embodiments, the vector or construct can contain a single promoter that drives the expression of one or more nucleic acid molecules. In some embodiments, such promoters can be multicistronic (bicistronic or tricistronic). For example, in some embodiments, transcription units can be engineered as a bicistronic unit containing an IRES (internal ribosome entry site), which allows coexpression of gene products (e.g. encoding an alpha chain and/or beta chain of a TCR) by a message from a single promoter. Alternatively, in some cases,

a single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three genes separated from one another by sequences encoding a self-cleavage peptide (e.g., F2A, P2A or T2A) or a protease recognition site (e.g., furin). The ORF thus encodes a single polyprotein, which, either during (in the case of 2A e.g., T2A) or after translation, is cleaved into the individual proteins. In some cases, the peptide, such as T2A, can cause the ribosome to skip (ribosome skipping) synthesis of a peptide bond at the C-terminus of a 2A element, leading to separation between the end of the 2A sequence and the next peptide downstream.

[0177] The term “linker” or “linker peptide” as used herein refer to an oligo- or polypeptide region from about 1 to 100 amino acids in length, which links together any of the domains/regions. Linkers can be composed of flexible residues like glycine and serine so that the adjacent protein domains are free to move relative to one another. Longer linkers can be used when it is desirable to ensure that two adjacent domains do not sterically interfere with one another. Linkers can be cleavable or non-cleavable. Examples of cleavable linkers include 2A linkers (e.g. F2A, P2A, T2A), 2A-like linkers or functional equivalents thereof and combinations thereof. In some embodiments, the linkers include the picornaviral 2A-like linker, CHYSEL sequences of porcine teschovirus (P2A), Thosea asigna virus (T2A) or combinations, variants and functional equivalents thereof. Other linkers will be apparent to those of skill in the art and can be used in the methods described herein.

[0178] The successful construction of a recombinant fusion protein often requires two elements: the component proteins and the linkers. The choice of the component proteins is based on the desired functions of the fusion protein product and, in most cases, is relatively straightforward. On the other hand, the selection of a suitable linker to join the protein domains together can be complicated and is often neglected in the design of fusion proteins. Direct fusion of functional domains without a linker may lead to many undesirable outcomes, including misfolding of the fusion proteins, low yield in protein production, or impaired bioactivity. Therefore, the selection or rational design of a linker to join fusion protein domains is an important, yet underexplored, area in recombinant fusion protein technology. Details of linker design, especially the selection between flexible linkers and rigid linkers, can be found, e.g., in Chen, X. et al. "Fusion protein linkers: property, design and functionality." *Advanced Drug Delivery Reviews* 65.10 (2013): 1357-1369, which is incorporated herein by reference in its entirety.

[0179] In some embodiments, the fusion proteins or fusion polypeptides described herein includes a linker that connects a first moiety and a second moiety. In some embodiments, the linker is a flexible linker, e.g., a linker with an amino acid sequence that is at least 70%, 75%,

80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identical to SEQ ID NO: 4, 11, or 28.

In some embodiments, the linker includes an amino acid sequence that is at least 80%, 85%, 90%, 95%, or 100% identical to one or more (e.g., 1, 2, 3, 4, 5, 6, 7, or 8) repeats of GGGGS (SEQ ID NO: 29) or GGGS (SEQ ID NO: 30). In some embodiments, at least 50%, 60%, 70%, 80%, or 90% of the amino acid residues in the flexible linker are glycine residues.

[0180] The present disclosure also provides a nucleic acid sequence comprising a nucleotide sequence encoding any of the chimeric cytokine signaling polypeptides (e.g., the engineered cytokine signaling chain, and/or the engineered cytokine ligand chain). “Nucleic acid” as used herein can include “polynucleotide,” “oligonucleotide,” and “nucleic acid molecule,” and generally means a polymer of DNA or RNA, which can be single-stranded or double-stranded, synthesized or obtained from natural sources, which can contain natural, non-natural or altered nucleotides. Furthermore, the nucleic acid comprises complementary DNA (cDNA). It is generally preferred that the nucleic acid does not comprise any insertions, deletions, inversions, and/or substitutions. However, it can be suitable in some instances, as discussed herein, for the nucleic acid to comprise one or more insertions, deletions, inversions, and/or substitutions.

[0181] The nucleic acids as described herein can be constructed based on chemical synthesis and/or enzymatic ligation reactions using procedures known in the art. For example, a nucleic acid can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides. In some of any such embodiments, the nucleotide sequence is codon-optimized.

[0182] The present disclosure also provides the nucleic acids comprising a nucleotide sequence complementary to the nucleotide sequence of any of the nucleic acids described herein or a nucleotide sequence which hybridizes under stringent conditions to the nucleotide sequence of any of the nucleic acids described herein. In some embodiments, the nucleic acid is synthetic. In some embodiments, the nucleic acid is cDNA.

[0183] In some of any such embodiments, the chimeric cytokine signaling polypeptides (e.g., the engineered cytokine signaling chain, and/or the engineered cytokine ligand chain) described herein are encoded by a nucleotide sequence that has been codon-optimized.

[0184] The disclosure also provides a nucleic acid sequence that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any nucleotide sequence as described herein, and an amino acid sequence that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any amino acid sequence as described herein. In some embodiments, the disclosure

relates to nucleotide sequences encoding any peptides that are described herein, or any amino acid sequences that are encoded by any nucleotide sequences as described herein.

[0185] In some embodiments, the nucleic acid sequence is at least or about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 150, 200, 250, 300, 350, 400, 500, or 600 nucleotides. In some embodiments, the amino acid sequence is at least or about 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 amino acid residues. In some embodiments, the nucleic acid sequence is less than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 150, 200, 250, 300, 350, 400, 500, or 600 nucleotides. In some embodiments, the amino acid sequence is less than 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 amino acid residues.

[0186] To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[0187] Related, a method for obtaining or manufacturing an engineered immune cell is further provided, which comprises a step of: introducing into an immune cell a vector kit according to any one of the embodiments as described above.

[0188] The immune cells for introduction of the vector kit described herein, can be isolated from a sample, such as a biological sample, e.g., one obtained from or derived from a subject. In some embodiments, the subject from which the cell is isolated is one having the disease or condition or in need of a cell therapy or to which cell therapy will be administered. The subject in some embodiments is a human in need of a particular therapeutic intervention, such as the adoptive cell therapy for which cells are being isolated, processed, and/or engineered.

[0189] Accordingly, the immune cells in some embodiments are primary cells, e.g., primary human cells. In some embodiments, the cells are cord blood-derived NK cells. The samples include tissue, fluid, and other samples taken directly from the subject, as well as samples resulting from one or more processing steps, such as separation, centrifugation, genetic

engineering (e.g., transduction with viral vector), washing, and/or incubation. The biological sample can be a sample obtained directly from a biological source or a sample that is processed. Biological samples include, but are not limited to, body fluids, such as blood, plasma, serum, cerebrospinal fluid, synovial fluid, urine and sweat, tissue and organ samples, including
5 processed samples derived therefrom.

[0190] In some aspects, the sample from which the cells are derived or isolated is blood or a blood-derived sample, or is or is derived from an apheresis or leukapheresis product. Exemplary samples include whole blood, peripheral blood mononuclear cells (PBMCs), leukocytes, bone marrow, thymus, tissue biopsy, tumor, leukemia, lymphoma, lymph node, gut
10 associated lymphoid tissue, mucosa associated lymphoid tissue, spleen, other lymphoid tissues, liver, lung, stomach, intestine, colon, kidney, pancreas, breast, bone, prostate, cervix, testes, ovaries, tonsil, or other organ, and/or cells derived therefrom. Samples include, in the context of cell therapy, e.g., adoptive cell therapy, samples from autologous and allogeneic sources.

[0191] In some embodiments, the cells are derived from cell lines, e.g., NK cell lines. The cells
15 in some embodiments are obtained from a xenogeneic source, for example, from mouse, rat, or non-human primate.

[0192] In some embodiments, the blood cells collected from the subject are washed, e.g., to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In some embodiments, the cells are washed with phosphate
20 buffered saline (PBS). In some embodiments, the wash solution lacks calcium and/or magnesium and/or many or all divalent cations. In some aspects, a washing step is accomplished a semi-automated "flow-through" centrifuge. In some aspects, a washing step is accomplished by tangential flow filtration (TFF). In some embodiments, the cells are resuspended in a variety of biocompatible buffers after washing, such as, for example, Ca²⁺/Mg²⁺
25 free PBS. In certain embodiments, components of a blood cell sample are removed and the cells directly resuspended in culture media. In some embodiments, the methods include density-based cell separation methods, such as the preparation of white blood cells from peripheral blood by lysing the red blood cells and centrifugation through a Percoll or Ficoll gradient.

[0193] In some embodiments, the method comprises one or more steps of: e.g., isolating the
30 NK cells from a patient's cord blood; transducing the population of NK cells with a viral vector including the nucleic acid construct encoding the chimeric cytokine signaling polypeptide(s) (e.g., the engineered cytokine signaling chain, and/or the engineered cytokine ligand chain); expanding the transduced cells *in vitro*; and/or infusing the expanded cells into the patient,

where the engineered NK cells will seek and destroy tumor cells expressing a target antigen or receptor. In some embodiments, the method further comprises: transfection of NK cells with the viral vector containing the nucleic acid construct.

[0194] In some embodiments, the methods involve introducing any vectors described herein into a cell *in vitro* or *ex vivo*. In some embodiments, the vector is a viral vector and the introducing is carried out by transduction. In some embodiments, the methods further involve introducing into the cell one or more agents, wherein each of the one or more agents is independently capable of inducing a genetic disruption. In some embodiments, the one or more agent is an inhibitory nucleic acid (e.g., siRNA). In some embodiments, the one or more agent is a fusion protein comprising a DNA-targeting protein and a nuclease or an RNA-guided nuclease (e.g., a clustered regularly interspaced short palindromic nucleic acid (CRISPR)-associated nuclease).

[0195] The transfection of immune cells (e.g., T cells or NK cells) may be achieved by using any standard method such as calcium phosphate, electroporation, liposomal mediated transfer, microinjection, biolistic particle delivery system, or any other known methods by skilled artisan. In some embodiments, transfection of immune cells is performed using the calcium phosphate method. Methods of preparing engineered cells and administering these engineered cells to a subject are known in the art.

[0196] In a fourth aspect, the present disclosure further provides an immune cell, which comprises and expresses the synthetic cytokine signaling system provided in the first aspect.

[0197] Herein, the immune cell may be obtained by introducing the vector kit as described above in the second aspect, or alternatively may be obtained by gene editing to the genome of the immune cell.

[0198] As used herein, and throughout other part of the present disclosure as well, the term "immune cell" or "immune cells", which is exchangeable to "immunological cell" or "immunological cells", is referred to as a type of cells in the immune system in a host that helps fight infections of or certain diseases like cancers, and can include, without limitation, any of a T cell, a $\gamma\delta$ T cell, a natural killer (NK) cell, a natural killer T (NKT) cell, a tumor-infiltrating lymphocyte (TIL), etc. Herein, the immune cell can be in a non-engineered form, or in an engineered form. In a non-engineered form, the immune cells may be the naive immune cells (e.g. T cells, TILs, or NK cells) taken directly from a subject (e.g. a mammal like a human being). The term "engineered immune cell" means an immune cell that is engineered in any manner to comprise at least one genetic, genomic, epigenomic, or proteomic alteration

compared with the non-engineered immune cell. Non-limiting examples of an engineered immune cell may include an immune cell engineered to introduce one or more alterations (e.g. substitution, addition, deletion, transposition, indel, etc.) to specific genomic sequence(s) (e.g. exon, promoter, enhancer, etc.), to express one or more exogenous RNA molecules (e.g. shRNAs, miRNAs, etc.), one or more exogenous polypeptides (e.g. secreted, transmembrane, or intracellular proteins), to exhibit one or more epigenetic changes (e.g. histone modifications such as methylation, acetylation, etc.). In one specific example, an engineered immune cell may be an engineered T cell expressing a chimeric antigen receptor (CAR) that specifically targets certain tumor cells by specifically recognizing certain cell surface markers, such as ALPP, CD19, etc., and as such the engineered immune cell may be an engineered CAR-T cell. In another example, an engineered immune cell may be an engineered T cell expressing a T cell receptor (TCR) that specifically targets certain tumor cells by specifically recognizing certain cell surface markers, such as NY-ESO-1, etc., and as such the engineered immune cell may be an engineered TCR-T cell. In yet another example, an engineered immune cell may be an engineered NK cell expressing and secreting an antibody that specifically targets certain cell surface molecules, such as CD19, PD-L1, etc. on tumor cells, thereby allowing the antibody-dependent cellular cytotoxicity (ADCC) of the engineered NK cells towards the tumor cells. According to some embodiments, the engineered immune cell may be an engineered CAR-T, an engineered TCR-T cell, an engineered CAR-NK cell, an engineered TCR-NK cell, an engineered CAR- $\gamma\delta$ T cell, or an engineered TCR- $\gamma\delta$ T cell, etc.

[0199] The term “chimeric antigen receptor (CAR)” as used herein refers to an artificial polypeptide which, when expressed in an immune effector cell (e.g. T cell, NK cell, etc.), provides the cell with specificity for target cells expressing certain antigen molecules (e.g. cancer cells that expresses a tumor associated antigen), and with intracellular signal generation. In some embodiments, a CAR comprises an intracellular activation domain, a transmembrane domain, and an extracellular domain comprising an antigen binding region that specifically recognizes and binds the antigen molecules on the target cells. For example, the antigen binding region in a CAR may typically comprise a single-chain variable fragments (scFv) derived from a monoclonal antibody that specifically recognizes the target antigen molecules. The intracellular activation domain may comprise a functional signaling domain derived from a stimulatory molecule and/or costimulatory molecule (e.g. 4-1BB, CD27 and/or CD28, etc.). CARs are typically employed to engineer immune effector cells for use in adoptive cell therapy.

[0200] The term “T-cell receptor (TCR)” as used herein refers to a protein receptor on T cells

which provides the specificity towards certain target cells expressing certain antigen molecules (e.g. cancer cells that expresses a tumor associated antigen). A TCR typically comprises a heterodimer of an alpha (α) and beta (β) chain that is responsible for the specific recognition of target antigen molecules, but may comprise gamma and delta (γ/δ) chains in some other embodiments. In mediating the specific cytotoxicity towards target cells that expressing the specific antigens, the TCR may form a functional TCR signaling complex with CD3 δ , CD3 γ , CD3 ϵ and CD3 ζ in the plasma membrane of the immune effector cells. In the use in adoptive cell therapy, TCRs may be expressed in any of a helper T cell, a cytotoxic T cell, a memory T cell, a regulatory T cell, a natural killer T cell, and a $\gamma\delta$ T cell, a tumor-infiltrating lymphocyte (TIL), or a natural killer (NK) cell, etc.

[0201] The term “Aspire-TCR” as used herein refers to a protein receptor on T cells which provides the specificity towards certain target cells expressing certain antigen molecules (e.g. cancer cells that expresses a tumor associated antigen). An Aspire-TCR is based on a regular TCR as defined above, but with certain modifications of certain subunits, and the detailed information can refer to WO2024138181A2.

[0202] Regardless of whether the cytokine ligand chain is in a secreted form (i.e. 200A) or in a membrane-bound form (i.e. 200B), when it is co-expressed in an immune cell along with the corresponding engineered cytokine receptor chain 100, the immune cell may exhibit zero or reduced dependency to certain cytokine(s) which are usually required to be supplied to the immune cells culturing *in vitro*, or transferred *in vivo*.

[0203] In various embodiments, the immune cell that is engineered can be obtained from e.g., humans and non-human animals. In various embodiments, the cell that is engineered can be obtained from bacteria, fungi, humans, rats, mice, rabbits, monkeys, pig or any other species. Preferably, the cell is from humans, rats or mice. More preferably, the cell is obtained from humans. In various embodiments, the cell that is engineered is a blood cell. Preferably, the cell is a leukocyte, lymphocyte or any other suitable blood cell type. In some embodiments, the cell is a peripheral blood cell. In some embodiments, the cell is a T cell, a tumor-infiltrating cell (TIL), or an NK cell.

[0204] Different cell types can be obtained from appropriate isolation methods. The isolation methods include the separation of different cell types based on the expression or presence in the cell of one or more specific molecules, such as surface markers, e.g., surface proteins, intracellular markers, or nucleic acid. In some embodiments, any known method for separation based on such markers can be used. In some embodiments, the separation is affinity- or

immunoaffinity-based separation. For example, the isolation in some aspects includes separation of cells and cell populations based on the cells' expression or expression level of one or more markers, typically cell surface markers, for example, by incubation with an antibody or binding partner that specifically binds to such markers, followed generally by washing steps and separation of cells having bound the antibody or binding partner, from those cells having not bound to the antibody or binding partner.

[0205] Such separation steps can be based on positive selection, in which the cells having bound the reagents are retained for further use, and/or negative selection, in which the cells having not bound to the antibody or binding partner are retained. In some examples, both fractions are retained for further use. In some aspects, negative selection can be particularly useful where no antibody is available that specifically identifies a cell type in a heterogeneous population, such that separation is best carried out based on markers expressed by cells other than the desired population.

[0206] Also provided are methods, nucleic acids, compositions, and kits, for expressing the chimeric cytokine signaling polypeptide(s) (e.g., the engineered cytokine signaling chain, and/or the engineered cytokine ligand chain) described herein, and for producing the genetically engineered immune cells (e.g., NK cells) expressing such molecules. The genetic engineering generally involves introduction of a nucleic acid encoding these molecules into the cell, such as by retroviral transduction, transfection, or transformation. In some embodiments, gene transfer is accomplished by first stimulating the cell, such as by combining it with a stimulus that induces a response such as proliferation, survival, and/or activation, e.g., as measured by expression of a cytokine or activation marker, followed by transduction of the activated cells, and expansion in culture to numbers sufficient for clinical application.

[0207] In some embodiments, recombinant nucleic acids are transferred into cells using recombinant infectious virus particles, such as, e.g., vectors derived from simian virus 40 (SV40), adenoviruses, adeno-associated virus (AAV). In some embodiments, recombinant nucleic acids are transferred into T cells using recombinant lentiviral vectors or retroviral vectors, such as gamma-retroviral vectors. In some embodiments, the retroviral vector has a long terminal repeat sequence (LTR), e.g., a retroviral vector derived from the Moloney murine leukemia virus (MoMLV), myeloproliferative sarcoma virus (MPSV), murine embryonic stem cell virus (MESV), murine stem cell virus (MSCV), or spleen focus forming virus (SFFV). Most retroviral vectors are derived from murine retroviruses. In some embodiments, the retroviruses include those derived from any avian or mammalian cell source. The retroviruses typically are amphotropic, meaning that they are capable of infecting host cells of several

species, including humans. In some embodiments, the vector is a lentivirus vector. In some embodiments, recombinant nucleic acids are transferred into T cells via electroporation. In some embodiments, recombinant nucleic acids are transferred into T cells via transposition. Other methods of introducing and expressing genetic material in immune cells include calcium phosphate transfection, protoplast fusion, cationic liposome-mediated transfection; tungsten particle-facilitated microparticle bombardment and strontium phosphate DNA co-precipitation. Many of these methods are described e.g., in WO2019195486, which is incorporated herein by reference in its entirety.

[0208] Also provided are populations of engineered immune cells, compositions containing such cells and/or enriched for such cells, such as in which cells expressing the binding molecule make up at least 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 80%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more percent of the total cells in the composition or cells of a certain type such as NK cells, T cells, or TILs.

[0209] According to some embodiments, the immune cell may, when cultured *in vitro* in the absence of IL-2, display at least one of increased proliferation capability, increased viability, or increased STAT signaling, compared to an immune cell that does not comprise the synthetic cytokine signaling system.

[0210] According to some embodiments, the immune cell may, when co-cultured with target cells in the absence of IL-2 stimulation, display at least one of increased interferon gamma (IFN γ) secretion when co-cultured with target cells of the immune cell (or in the presence of the specific antigen targeted by the immune cells), or increased cytotoxicity against the target cells, compared to an immune cell that does not comprise the synthetic cytokine signaling system.

[0211] According to some embodiments, when a population of the immune cell are transferred into a subject bearing a tumor that the immune cell specifically targets, the population of the immune cell may exhibit more preferred migration to tumor tissues and/or lymphoid tissues, and/or may display increased tumor control ability, compared to an immune cell that does not comprise the synthetic cytokine signaling system.

[0212] Herein, the immune cell may be any of a T cell, a $\gamma\delta$ T cell, a natural killer (NK) cell, a natural killer T (NKT) cell, or a tumor-infiltrating lymphocyte (TIL), or an engineered immune cell derived therefrom. The immune cell may be non-engineered or unmodified, but may also be engineered or modified, for example, to express an engineered chimeric antigen receptor (CAR) and/or an engineered T cell receptor that specifically targets certain diseases (such as

tumors). Optionally, the immune cell may further express a targeting polypeptide, which may comprise at least one of a chimeric antigen receptor (CAR) or a T cell receptor (TCR) that can specifically and selectively recognize and bind certain target molecule/protein/marker on the surface of target cells (e.g. tumor cells) or on the surface of other entities (e.g. bacteria, fungi, viruses).

[0213] A culturing method for the immune cell as described above is also provided. The method comprises the steps of:

[0214] (1) obtaining an engineered immune cell based on an immune cell, such that the engineered immune cell expresses the synthetic cytokine signaling system according to any embodiments as described above; and

[0215] (2) culturing the engineered immune cell in a medium containing none or reduced level of a cytokine supplement *in vitro*.

[0216] Herein step (1) can be realized by introducing into the immune cell the vector kit according to any embodiments as described above.

[0217] In step (2), the cytokine supplement may comprise at least one of IL-2, IL-4, IL-7, IL-9, IL-15, or IL-21. Thus according to some embodiment, the culturing medium may lack IL-2, may lack IL-15, or may lack any of the above cytokines (i.e. IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21).

[0218] The present disclosure further provides a method for treating or preventing a disease or disorder in a subject in need thereof, comprising: administering a plurality of engineered immune cells into the subject, wherein each of the plurality of engineered immune cells expresses the synthetic cytokine signaling system according to any embodiments as described above

[0219] In some embodiments, the cells, populations, and compositions, described herein are administered to a subject or patient having a particular disease or condition to be treated, e.g., via adoptive cell therapy, such as adoptive T cell therapy. In some embodiments, cells and compositions prepared by the provided methods, such as engineered compositions and end-of-production compositions following incubation and/or other processing steps, are administered to a subject, such as a subject having or at risk for the disease or condition. In some aspects, the methods thereby treat, e.g., ameliorate one or more symptom of, the disease or condition, such as by lessening tumor burden in cancer expressing an antigen recognized by the engineered T cells.

[0220] Methods for administration of cells for adoptive cell therapy are known and can be used in connection with the provided methods and compositions. For example, adoptive cell therapy

methods are described, e.g., in U.S. 2003/0170238; U.S. Pat. No. 4,690,915; Rosenberg, "Cell transfer immunotherapy for metastatic solid cancer-what clinicians need to know." *Nature reviews Clinical oncology* 8.10 (2011): 577; Themeli *et al.* "Generation of tumor-targeted human T lymphocytes from induced pluripotent stem cells for cancer therapy." *Nature biotechnology* 31.10 (2013): 928; Tsukahara *et al.* "CD19 target-engineered T-cells accumulate at tumor lesions in human B-cell lymphoma xenograft mouse models." *Biochemical and biophysical research communications* 438.1 (2013): 84-89; Davila *et al.* "CD19 CAR-targeted T cells induce long-term remission and B Cell Aplasia in an immunocompetent mouse model of B cell acute lymphoblastic leukemia." *PloS one* 8.4 (2013); each of which is incorporated
5
10 herein by reference in its entirety.

[0221] In some embodiments, the cell therapy, e.g., adoptive cell therapy, is carried out by autologous transfer, in which the T cells are isolated and/or otherwise prepared from the subject who is to receive the cell therapy, or from a sample derived from such a subject. Thus, in some aspects, the cells are derived from a subject, e.g., patient, in need of a treatment and the cells,
15 following isolation and processing are administered to the same subject.

[0222] In some embodiments, the cell therapy, e.g., adoptive cell therapy, is carried out by allogeneic transfer, in which the T cells are isolated and/or otherwise prepared from a subject other than a subject who is to receive or who ultimately receives the cell therapy, e.g., a first subject. In such embodiments, the cells then are administered to a different subject, e.g., a
20 second subject, of the same species. In some embodiments, the first and second subjects are genetically identical. In some embodiments, the first and second subjects are genetically similar. In some embodiments, the second subject expresses the same HLA class or supertype as the first subject.

[0223] In some embodiments, the HLA class or HLA supertype of the subject is identified. In
25 some embodiments, the subject is treated with a cell therapy that can recognize the antigen in the context of the HLA class or HLA supertype.

[0224] In some embodiments, the subject has been treated with a therapeutic agent targeting the disease or condition, e.g., the tumor, prior to administration of the cells or composition containing the cells. In some aspects, the subject is refractory or non-responsive to the other
30 therapeutic agent. In some embodiments, the subject has persistent or relapsed disease, e.g., following treatment with another therapeutic intervention, including chemotherapy, radiation, and/or hematopoietic stem cell transplantation (HSCT), e.g., allogenic HSCT. In some embodiments, the administration effectively treats the subject despite the subject having become resistant to another therapy.

[0225] In some embodiments, the subject is responsive to the other therapeutic agent, and treatment with the therapeutic agent reduces disease burden. In some aspects, the subject is initially responsive to the therapeutic agent, but exhibits a relapse of the disease or condition over time. In some embodiments, the subject has not relapsed. In some such embodiments, the subject is determined to be at risk for relapse, such as at high risk of relapse, and thus the cells are administered prophylactically, e.g., to reduce the likelihood of or prevent relapse. In some embodiments, the subject has not received prior treatment with another therapeutic agent.

[0226] In some embodiments, the cells are administered at a desired dosage, which in some aspects includes a desired dose or number of cells or cell type(s) and/or a desired ratio of cell types. Thus, the dosage of cells in some embodiments is based on a total number of cells (or number per kg body weight). In some embodiments, the dosage of cells is based on a desired total number (or number per kg of body weight) of cells in the individual populations or of individual cell types. In some embodiments, the dosage is based on a combination of such features, such as a desired number of total cells, desired ratio, and desired total number of cells in the individual populations.

[0227] In some embodiments, the populations of T cells are administered at or within a tolerated difference of a desired dose of total cells. In some embodiments, the desired dose is a desired number of cells or a desired number of cells per unit of body weight of the subject to whom the cells are administered, e.g., cells/kg. In some embodiments, the desired dose is at or above a minimum number of cells or minimum number of cells per unit of body weight.

[0228] In some embodiments, the cells are administered at or within a tolerated difference of a desired dose of one or more of the individual populations or sub-types of cells, such as a desired dose of T cells. In some embodiments, the desired dose is a desired number of cells of the sub-type or population, or a desired number of such cells per unit of body weight of the subject to whom the cells are administered, e.g., cells/kg. In some embodiments, the desired dose is at or above a minimum number of cells of the population or sub-type, or minimum number of cells of the population or sub-type per unit of body weight.

[0229] Thus, in some embodiments, the dosage is based on a desired fixed dose of total cells, and/or based on a desired fixed dose of one or more, e.g., each, of the individual sub-types or sub-populations. Thus, in some embodiments, the dosage is based on a desired fixed or minimum dose of NK cells, and/or is based on a desired fixed or minimum dose of T cells.

[0230] In certain embodiments, the cells or individual populations of sub-types of cells, are administered to the subject at a range of about one million to about 100 billion cells, such as, e.g., 1 million to about 50 billion cells (e.g., about 5 million cells, about 25 million cells, about

500 million cells, about 1 billion cells, about 5 billion cells, about 20 billion cells, about 30 billion cells, about 40 billion cells, or a range defined by any two of the foregoing values), such as about 10 million to about 100 billion cells (e.g., about 20 million cells, about 30 million cells, about 40 million cells, about 60 million cells, about 70 million cells, about 80 million cells, about 90 million cells, about 10 billion cells, about 25 billion cells, about 50 billion cells, about 75 billion cells, about 90 billion cells, or a range defined by any two of the foregoing values), and in some cases about 100 million cells to about 50 billion cells (e.g., about 120 million cells, about 250 million cells, about 350 million cells, about 450 million cells, about 650 million cells, about 800 million cells, about 900 million cells, about 3 billion cells, about 30 billion cells, about 45 billion cells) or any value in between these ranges.

[0231] In some embodiments, the dose of total cells and/or dose of individual sub-populations of cells is within a range of between at or about 10^4 and at or about 10^9 cells/kilograms (kg) body weight, such as between 10^5 and 10^6 cells/kg body weight, for example, at least or at least about or at or about 1×10^5 cells/kg, 1.5×10^5 cells/kg, 2×10^5 cells/kg, or 1×10^6 cells/kg body weight. For example, in some embodiments, the cells are administered at, or within a certain range of error of, between at or about 10^4 and at or about 10^9 NK cells/kilograms (kg) body weight, such as between 10^5 and 10^6 NK cells/kg body weight, for example, at least or at least about or at or about 1×10^5 NK cells/kg, 1.5×10^5 NK cells/kg, 2×10^5 NK cells/kg, or 1×10^6 NK cells/kg body weight. In some embodiments, the residual CD3+ cells are administered with less than 2×10^5 cells/kg, 1×10^5 cells/kg, 5×10^4 cells/kg, or 1×10^4 cells/kg body weight.

[0232] In some embodiments, the cells are administered at or within a certain range of error of, greater than, and/or at least about 1×10^6 , about 1×10^7 , about 1×10^9 , about 1×10^9 , or about 1×10^{10} CD56+ cells, and/or no greater than about 1×10^6 , about 1×10^7 , about 1×10^9 , about 1×10^9 , or about 1×10^{10} CD3+ cells. In some embodiments, the cells are administered at or within a certain range of error of between about 10^8 and 10^{12} or between about 10^{10} and 10^{11} NK cells, between about 10^8 and 10^{12} or between about 10^{10} and 10^{11} CD56+ cells, and/or between about 10^6 and 10^{10} or between about 10^8 and 10^9 CD3+ cells.

[0233] In some embodiments, the cells are administered at or within a tolerated range of a desired output ratio of multiple cell populations or sub-types, such as CD56+ and CD3+ cells or sub-types. In some aspects, the desired ratio can be a specific ratio or can be a range of ratios. for example, in some embodiments, the desired ratio (e.g., ratio of CD56+ to CD3+ cells) is between at or about 500: 1 and at or about 20:1, e.g., about 500: 1, about 400: 1, about 300: 1, about 200: 1, about 100: 1, about 90: 1, about 80: 1, about 70: 1, about 60: 1, about 50: 1, about

40: 1, about 30: 1, or about 20: 1. In some aspects, the tolerated difference is within about 1%, about 2%, about 3%, about 4% about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50% of the desired ratio, including any value in between these ranges.

- 5 [0234] Optimal response to therapy can depend on the ability of the engineered recombinant receptors such as the chimeric polypeptides (or the fusion polypeptide), antibodies or antigen-binding fragments thereof, and/or peptibodies described herein, to be consistently and reliably expressed on the surface of the cells and/or bind the target antigen. For example, in some cases, properties of certain chimeric polypeptides (or the fusion polypeptide), antibodies or antigen-binding fragments thereof, and/or peptibodies, can affect the expression and/or activity of the
- 10 polypeptide or protein, in some cases when expressed in a cell, such as a human NK cell, used in cell therapy. In some contexts, the level of expression of particular polypeptide or protein, can be low, and activity of the engineered cells, such as human NK cells, expressing such polypeptides or proteins, may be limited due to poor expression or poor signaling activity. In
- 15 some cases, consistency and/or efficiency of expression of the polypeptide or protein, and activity of the polypeptide or protein is limited in certain cells or certain cell populations of available therapeutic approaches. In some cases, a large number of engineered NK cells (a high effector to target (E:T) ratio) is required to exhibit functional activity. In some embodiments, the desired ratio (E:T ratio) is between at or about 1:10 and at or about 10:1 (or greater than
- 20 about 1:10 and less than about 10:1), or between at or about 1:1 and at or about 10:1 (or greater than about 1:1 and less than about 5:1), such as between at or about 2:1 and at or about 10:1. In some embodiments, the E:T ratio is greater than or about 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, or 10:1.

- [0235] For the prevention or treatment of disease, the appropriate dosage may depend on the
- 25 type of disease to be treated, the type of cells or recombinant receptors, the severity and course of the disease, whether the cells are administered for preventive or therapeutic purposes, previous therapy, the subject's clinical history and response to the cells, and the discretion of the attending physician. The compositions and cells are in some embodiments suitably administered to the subject at one time or over a series of treatments.

- 30 [0236] The cells described herein can be administered by any suitable means, for example, by bolus infusion, by injection, e.g., intravenous or subcutaneous injections, intraocular injection, periocular injection, subretinal injection, intravitreal injection, trans-septal injection, subcleral injection, intrachoroidal injection, intracameral injection, subconjunctival injection, subconjunctival injection, sub-Tenon's injection, retrobulbar injection, peribulbar injection, or

posterior juxtасcleral delivery. In some embodiments, they are administered by parenteral, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In some embodiments, a given dose is administered by a single
5 bolus administration of the cells. In some embodiments, it is administered by multiple bolus administrations of the cells, for example, over a period of no more than 3 days, or by continuous infusion administration of the cells.

[0237] In some embodiments, the cells are administered as part of a combination treatment, such as simultaneously with or sequentially with, in any order, another therapeutic intervention,
10 such as an antibody or engineered cell or receptor or agent, such as a cytotoxic or therapeutic agent. The cells in some embodiments are co-administered with one or more additional therapeutic agents or in connection with another therapeutic intervention, either simultaneously or sequentially in any order. In some contexts, the cells are co-administered with another therapy sufficiently close in time such that the cell populations enhance the effect of one or
15 more additional therapeutic agents, or *vice versa*. In some embodiments, the cells are administered prior to the one or more additional therapeutic agents. In some embodiments, the cells are administered after the one or more additional therapeutic agents. In some embodiments, the one or more additional agents includes a cytokine, such as IL-2, for example, to enhance persistence. In some embodiments, the methods comprise administration of a
20 chemotherapeutic agent.

[0238] Following administration of the cells, the biological activity of the engineered cell populations in some embodiments is measured, e.g., by any of a number of known methods. Parameters to assess include specific binding of engineered T cells to the antigen, *in vivo*, e.g., by imaging, or *ex vivo*, e.g., by ELISA or flow cytometry. In certain embodiments, the ability
25 of the engineered cells to destroy target cells can be measured using any suitable method known in the art, such as cytotoxicity assays described in, for example, Kochenderfer *et al.* "Construction and pre-clinical evaluation of an anti-CD19 chimeric antigen receptor." Journal of immunotherapy (Hagerstown, Md.: 1997) 32.7 (2009): 689 and Hermans *et al.* "The VITAL assay: a versatile fluorometric technique for assessing CTL-and NKT-mediated cytotoxicity
30 against multiple targets in vitro and in vivo." Journal of immunological methods 285.1 (2004): 25-40. In certain embodiments, the biological activity of the cells is measured by assaying expression and/or secretion of one or more cytokines, such as CD107a, IFN γ , IL-2, and TNF. In some aspects the biological activity is measured by assessing clinical outcome, such as reduction in tumor burden or load.

[0239] EXAMPLES

[0240] In order to provide a more detailed explanation for the synthetic cytokine signaling system as described above, in the following, several examples are provided. Please note that these examples are mainly for the purpose of demonstration, and shall not be interpreted to impose any limitation to the disclosure.

[0241] Example 1: Characterization of the variant form of IL9R(ICD)

[0242] A mutant form of IL9R(ICD), i.e. IL9R(ICD*) (SEQ ID NO: 7) containing a truncation at the first region (i.e. positions 174-230) and a substitution of the second region (i.e. positions 142-151) to GD, relative to the wild type IL9R(ICD) (SEQ ID NO: 1). Each of the wildtype IL9R(ICD) and the mutant IL9R(ICD*) is used to construct the synthetic cytokine signaling system "15-9R", comprising an engineered cytokine receptor chain (i.e. IL2Rb(ECD)-IL2Rb(TM)-IL9R(ICD) or IL2Rb(ECD)-IL2Rb(TM)-IL9R(ICD*)) and an engineered cytokine ligand chain (i.e. membrane-bound IL15-IL15Ra or Recast-IL15).

[0243] These above two synthetic cytokine signaling systems (i.e. "15-9R(WT)" and "15-9R(*)") are respectively expressed in T cells (e.g. PBMCs). Both synthetic cytokine signaling systems expressed well in T cells transduced with the relevant vector kit expressing the synthetic cytokine signaling system (data not shown).

[0244] In a subsequent comparison experiment where T cells transduced with indicated constructs and p-STAT3 levels were examined with p-STAT3 antibody via flow cytometry (FIG. 3A, quantification data shown in FIG. 3B), we found that both synthetic cytokine signaling systems mediated higher pSTAT3 (i.e. phosphorylated STAT3, as an indicator for STAT3 signaling) in transduced T cells compared with non-transduced control cells (i.e. "UTD"), indicating that both synthetic cytokine signaling systems can induce STAT3 signaling. Unexpectedly, however, the pSTAT3 level in the 15-9R(*)-transduced cells is significantly (~25%) higher than that in the 15-9R(WT)-transduced cells, suggesting an unexpectedly significantly stronger STAT3 signaling for 15-9R(*) than 15-9R(WT).

[0245] To further investigate whether the synthetic cytokine signaling systems can reduce IL-2 dependence of T cell expansion, T cells transduced with indicated constructs were cultured with or without 100 IU/mL IL-2, and their expansion ratio (FIG. 3C) and cell viability (FIG. 3D) were continuously monitored. As shown in FIGS. 3C and 3D (especially the "without IL-2" arm), both the 15-9R(WT)- and 15-9R(*)-transduced cells showed significantly better IL-2 independency in terms of the cell proliferation and survival compared with the non-transduced control cells (i.e. "UTD"). However, 15-9R(*) seemed to confer a surprisingly much better IL-2 independency compared with 15-9R(WT), which is nonetheless consistent with the stronger

STAT3 signaling shown in FIGS. 3A and 3B.

[0246] While the cell expansion ratio of the 15-9R(WT)-transduced cells cultured in the absence of IL-2 peaked at day 9 ("D9"), the cell expansion ratio of the 15-9R(*)-transduced cells cultured under the same condition still didn't peak even at day 11 ("D11"); and at day 9,
5 the cell expansion ratio of the 15-9R(*)-transduced cells was ~2.0 times that of the 15-9R(WT)-transduced cells (see FIG. 3C, "without IL-2" arm). Consistently, while only ~86% of the 15-9R(WT)-transduced cells survived at day 11 when cultured in the absence of IL-2, ~92% of the 15-9R(*)-transduced cells cultured under the same condition survived (see FIG. 3D, "without IL-2" arm).

10 [0247] These above results indicates a surprising or unexpected benefit of the loss-of-function mutation(s) at positions 174-230 and/or 142-151 of wild type IL9R(ICD) (SEQ ID NO: 1), especially the variant IL9R(ICD*) (SEQ ID NO: 7) that comprises both a truncation at positions 174-230 and a substitution at positions 142-151 to GD. Thus such mutant IL9R(ICD) can be used to obtain a series of engineered cytokine signaling chain (as illustrated in FIGS.
15 1A and 1B) and synthetic cytokine signaling system (as illustrated in FIGS. 2A and 2B)

[0248] In the following Examples 2-5, two specific embodiments of the synthetic cytokine signaling system are characterized, including "IL15-IL15R-IL2Rb-IL9R" (i.e. short as "IL15-IL15Ra-IL9R" or "IL15-IL9R", including an engineered cytokine receptor chain IL2Rb(ECD)-IL2Rb(TM)-IL9R(ICD*) (SEQ ID NO: 8) and an engineered cytokine ligand
20 chain membrane-bound IL15-IL15Ra/Recast-IL15 (SEQ ID NO: 11)), and "IL7-IL7R-IL9R" (short as "IL7-IL9R", including an engineered cytokine receptor chain IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*) (SEQ ID NO: 12) and an engineered cytokine ligand chain membrane-bound IL7-IgG4Fc (SEQ ID NO: 15)).

[0249] Each of these two specific synthetic cytokine signaling systems is independently
25 introduced into a variety of immune cells such as immortalized line of T cells (Jurkat cells), peripheral blood mononuclear (PMBC) cells, tumor infiltrated lymphocytes (TILs), and engineered T cells that express tumor antigen-targeting CAR (i.e. CAR-T cells), etc., and the effects of the ectopically expressed synthetic cytokine signaling system on a variety of characteristics of such modified immune cells, including: (1) proliferation capability, viability
30 and STAT signaling when cultured *in vitro* in the absence of IL2; (2) interferon gamma (IFN γ) production and cytotoxicity when co-cultured with target cells in the absence of IL2 stimulation; and (3) migration preference and tumor control ability, when transferred *in vivo* into a subject bearing a tumor; all investigated with unmodified immune cells as comparison reference.

[0250] FIG. 4A and FIG. 4B respectively illustrate the schematic construct structures of the synthetic cytokine signaling systems IL15-IL9R and IL7-IL9R, the functional cytokine signaling complexes formed thereby on the cell membrane of an engineered or modified immune cell, and the corresponding downstream JAK-STAT signaling pathways thus activated.

5 [0251] As shown in FIG. 4A, the construct of IL15-IL9R substantially comprises a polynucleotide having a nucleic acid sequence set forth in SEQ ID NO: 60, encoding a single polypeptide having an amino acid sequence set forth in SEQ ID NO: 59. The polypeptide comprises, from N-terminus to C-terminus, the engineered cytokine ligand chain "IL15-IL15Ra" (i.e. "Recast IL15" or "membrane-bound IL15-IL15Ra" or mbIL15-IL15Ra), the F2A separator sequence, and the engineered cytokine receptor chain "IL2Rb-ECD-IL9R" (i.e. "IL2Rb(SP)-IL2Rb(ECD)-IL2Rb(TM)-IL9R(ICD*) or "IL2Rb-IL9R"). When expressed in an immune cell, the engineered cytokine receptor chain IL2Rb-IL9R is anchored as a membrane-bound chimeric cytokine receptor that forms functional complex with the common gamma subunit (i.e. IL2R γ or γ C) on the cell membrane, which may then be activated by the binding of the membrane bound IL15-IL15Ra to thereby cause the downstream JAK-STAT signal pathway to be subsequently activated, resulting in the phosphorylation of STAT1, STAT3, and STAT5. In an independent manner, the membrane bound IL15-IL15Ra may also activate the functional complex formed between the endogenous IL7Ra (exchangeable to IL7R α or IL7R) and γ C, which can be in the same modified immune cell or in a neighboring immune cell, to result in the phosphorylation of STAT5 in the modified immune cell itself or in a neighboring immune cell (which could be modified or unmodified).

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[0252] Similar to IL15-IL9R shown in FIG. 4A and described above, the construct of IL7-IL9R also comprises a polynucleotide (SEQ ID NO: 61), encoding a single polypeptide having an amino acid sequence set forth in SEQ ID NO: 62. As shown in FIG. 4B, the polypeptide comprises, from N-terminus to C-terminus, the engineered cytokine ligand chain "IL7-IgG4Fc" (i.e. "membrane-bound IL7" or mbIL7), the F2A separator sequence, and the engineered cytokine receptor chain "IL7R-IL9R" (i.e. "IL7Ra(SP)-IL7Ra(ECD)-IL7Ra(TM)-IL9R(ICD*)"). When expressed in an immune cell, the engineered cytokine receptor chain IL7R-IL9R is anchored as a membrane-bound chimeric cytokine receptor that forms functional complex with γ C on the cell membrane, which may then be activated by the binding of the IL7 ligand from mbIL7 to thereby cause the downstream JAK-STAT signal pathway to be subsequently activated, resulting in the phosphorylation of STAT1, STAT3, and STAT5. In an independent manner, the mbIL7 may also activate the functional complex formed between the

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endogenous IL7Ra and γC , which can be in the same modified immune cell or in a neighboring immune cell, to result in the phosphorylation of STAT5 in the modified immune cell itself or in a neighboring immune cell (which could be modified or unmodified).

[0253] Example 2: Synthetic cytokine signaling characterization in Jurkat cells

5 **[0254]** In this example, the immortalized line of T cells (i.e. Jurkat cells) are modified with each of the IL15-IL15Ra-IL9R and IL7-IL7R-IL9R synthetic cytokine signaling system, and the effects of the modification on the JAK-STAT signaling is investigated.

[0255] Briefly, Jurkat cells were transduced with each of the IL15-IL15Ra-IL9R and IL7-IL7R-IL9R constructs to separately overexpress the engineered cytokine receptor chain and the engineered cytokine ligand chain. Expression was detected at day 7 after transduction, with
10 FIG. 5A and FIG. 5B respectively showing the transduction efficiency of the IL15-IL15Ra-IL9R construct (as indicated by the "IL15Ra" staining in the flow cytometry) and the IL7-IL7R-IL9R construct (as indicated by the "Fc γ " staining in the flow cytometry) in Jurkat cells. As shown in the figures, each of the two synthetic cytokine signaling systems is decently
15 overexpressed in Jurkat cells 7 days after transduction.

[0256] To further elucidate the effects of the synthetic cytokine signaling system modifications on JAK-STAT signaling in Jurkat cells, the pSTAT signaling characterization of the synthetic cytokine signaling in Jurkat cells were carried out. This experiment is firstly aimed to confirm that the ICD of IL9R in modified cells plays a role in their own pSTAT activation, and is
20 secondly aimed to investigate whether the membrane-bound cytokines are also able to activate the UTD cells around them.

[0257] FIG. 6A shows the process for characterizing pSTAT signaling in transduced Jurkat cells. Briefly, at day 9 after transduction, untransduced (UTD) Jurkat cells were labeled with CellTrace Violet. Labeled UTD Jurkat cells and modified Jurkat cells were starved overnight
25 separately. On the second day, according to the percentage of chimeric receptor-expressing cells, we mixed the labeled UTD cells with the modified Jurkat cells at a ratio of 1:5 and co-cultured them at 37°C for 1h. The cells were fixed and permeabilized before intracellular staining of pSTATs. Through CellTrace Violet labeling, UTD and modified cells were distinguished on flow cytometry and the activation of STAT signaling pathway was analyzed
30 separately, with STAT1 phosphorylation (i.e. "pSTAT1"), STAT3 phosphorylation (i.e. "pSTAT3") and STAT5 phosphorylation (i.e. "pSTAT5") results shown in FIGS. 6B-6C, FIGS. 6D-6E, and FIGS. 6F-6G, respectively.

[0258] Based on these results, it can be seen that each of IL15-IL9R or IL7-IL9R modified

Jurkat cells can activate STAT signaling in the neighboring cells and the modified cells themselves via membrane-bound cytokines and the ICD of IL9R, respectively.

[0259] Example 3: Synthetic cytokine signaling characterization in PBMC/TIL cells

5 **[0260]** In this example, more detailed *in vitro* characterization of the synthetic cytokine signaling was carried out in peripheral blood mononuclear cells (PBMC) and TIL cells that are separately modified to express the synthetic cytokine signaling system IL15-IL15Ra-IL9R or IL7-IL7R-IL9R.

10 **[0261]** Each of the IL15-IL15Ra-IL9R construct and the IL7-IL7R-IL9R construct was transduced into PBMC cells, and the transduction efficiency was tested. Briefly, at day 6 and day 14 after T cell transduction, 1 million cells were taken and stained with flow antibodies, including IL15R α and Fc γ , to examine the expression. As shown in FIG. 7A and FIG. 7B, both constructs can be efficiently transduced into PMBC cells, and overexpression of the transgenes can be maintained for at least two weeks during *in vitro* amplification stage. Then a variety of characterizations were performed for the modified T cells 7 days after transduction.

15 **[0262]** To investigate the effects of the synthetic cytokine signaling system modification in terms of the cell proliferation capability and of the cell viability, unmodified T cells, IL15-IL9R modified T cells, and IL7-IL9R modified T cells were cultured *in vitro* in the presence ("With IL-2") or in the absence ("Without IL-2") of the IL-2 supplement.

20 **[0263]** As shown in FIG. 8A, all three groups of cells can expand in the presence of IL-2, with total cell numbers increasing constantly over time (see the left panel labelled with "With IL-2"). In the absence of IL-2 (see the right panel labelled with "Without IL-2"), however, these three groups of cells showed dramatic differences in terms of the cell expansion ratio. Although unmodified T cells experienced a short period of initial increase in total cell number from Day 0 (i.e. Day 7 post-transduction) to Day 3, starting from Day 3 and over time, the total cell number began to constantly reduce, and became lower at Day 10 compared with Day 0. Each
25 of the IL15-IL9R modified T cells, and IL7-IL9R modified T cells, in contrast, showed relatively better proliferation capability than the unmodified T cells: At Day 6, while the cell expansion ratio of the unmodified T cells is approximately 1.6, the cell expansion ratio of the IL15-IL9R modified T cells is approximately 2.5, and the cell expansion ratio of the IL7-IL9R
30 modified T cells is approximately 2.1. Thus at Day 6, the cell expansion ratio of the IL15-IL9R modified T cells is approximately 56.3% (i.e. (2.5-1.6)/1.6) higher, and the cell expansion ratio of the IL7-IL9R modified T cells is approximately 31.3% (i.e. (2.1-1.6)/1.6) higher, than that of the unmodified T cells. At Day 8, the cell expansion ratios for the unmodified T cells, the

IL15-IL9R modified T cells, and the IL7-IL9R modified T cells are 1.2, 2.1 and 1.5 respectively, with the latter two group of cells having their cell expansion ratios approximately 75% (i.e. $(2.1-1.2)/1.2$) and 25% (i.e. $(1.5-1.2)/1.2$) and higher than the unmodified T cells. At Day 10, the cell expansion ratios for the unmodified T cells, the IL15-IL9R modified T cells, and the IL7-IL9R modified T cells are 0.9, 2.1 and 1.9 respectively, with the latter two group of cells having their cell expansion ratios approximately 133% and 111% higher than the unmodified T cells. At Day 13, the cell expansion ratios for the unmodified T cells, the IL15-IL9R modified T cells, and the IL7-IL9R modified T cells are 0.4, 3.0 and 1.8 respectively, with the latter two group of cells having their cell expansion ratios approximately 650% and 350% higher than the unmodified T cells. Therefore, both the IL15-IL9R modified T cells and the IL7-IL9R modified T cells show increased proliferation capability when cultured *in vitro* in the absence of IL-2 compared with the unmodified cells.

[0264] As shown in FIG. 8B, all three groups of cells maintain a comparable cell viability in the presence of IL-2 (see the left panel labelled with "With IL-2"). In the absence of IL-2 (see the right panel labelled with "Without IL-2"), however, these three groups of cells showed dramatic differences in terms of the cell viability. Except for the first 3 days, the cell viability of the unmodified T cells drops much more significantly than the IL15-IL9R modified T cells and the IL7-IL9R modified T cells starting at Day 6. At Day 6, the cell viability for the unmodified T cells, the IL15-IL9R modified T cells and the IL7-IL9R modified T cells are respectively 82%, 92% and 91%, with the latter two group of cells having their cell viability approximately 10% (i.e. $92\%-82\%$) and 9% (i.e. $91\%-82\%$) higher than the unmodified T cells. At Day 10, the cell viability for the unmodified T cells, the IL15-IL9R modified T cells and the IL7-IL9R modified T cells are respectively 60%, 79% and 81%, with the latter two group of cells having their cell viability approximately 19% (i.e. $79\%-60\%$) and 21% (i.e. $81\%-60\%$) higher than the unmodified T cells. At Day 13, the cell viability for the unmodified T cells, the IL15-IL9R modified T cells and the IL7-IL9R modified T cells are respectively 55%, 82% and 76%, with the latter two group of cells having their cell viability approximately 27% (i.e. $82\%-55\%$) and 21% (i.e. $76\%-55\%$) higher than the unmodified T cells. Therefore, both the IL15-IL9R modified T cells and the IL7-IL9R modified T cells show increased cell viability when cultured *in vitro* in the absence of IL-2 compared with the unmodified cells.

[0265] Similar evaluations were carried to unmodified TIL cells, IL15-IL9R modified TIL cells, and IL7-IL9R modified TIL cells, which were cultured *in vitro* in the presence ("With IL-2") or in the absence ("Without IL-2") of 300 IU/mL IL-2 supplement. As shown in FIGS. 8C and 8D, both the IL15-IL9R modified TIL cells and the IL7-IL9R modified TIL cells show

increased cell proliferation and increased cell viability when cultured *in vitro* in the absence of IL-2 compared with the unmodified TIL cells. More specifically, as shown in FIG. 8C, at Day 5, the cell expansion ratios for the unmodified TIL cells, the IL15-IL9R modified TIL cells, and the IL7-IL9R modified TIL cells are 2.0, 5.0 and 6.0 respectively, with the latter two group of cells having their cell expansion ratios approximately 150% and 200% higher than the unmodified TILs. Beyond Day 5 and through Day 10, both modified TILs can still constantly expand, whereas the unmodified TILs started dying out, thus the cell expansion ratios have even larger difference beyond Day 5. As shown in FIG. 8D, the cell viability for the unmodified TIL cells, the IL15-IL9R modified TIL cells, and the IL7-IL9R modified TIL cells are 83%, 91% and 90% respectively, with the latter two group of cells having their cell expansion ratios approximately 9.6% and 8.4% higher than the unmodified TILs. At Day 7, the cell viability for the unmodified TIL cells, the IL15-IL9R modified TIL cells, and the IL7-IL9R modified TIL cells are 58%, 90% and 91% respectively, with the latter two group of cells having their cell expansion ratios approximately 55.2% and 56.9% higher than the unmodified TILs. At Day 10, the cell viability for the unmodified TIL cells, the IL15-IL9R modified TIL cells, and the IL7-IL9R modified TIL cells are 20%, 93% and 90% respectively, with the latter two group of cells having their cell expansion ratios approximately 365% and 350% higher than the unmodified TILs. Therefore, both the IL15-IL9R and the IL7-IL9R modification can confer reduced IL-2 dependency to TIL cells.

[0266] JAK-STAT is a major signal transduction pathway downstream of cytokine receptors. To investigate the effects of the synthetic cytokine signaling system modification on the JAK-STAT signaling, the activation of STAT signaling in resting untransduced(UTD) and modified T cells without giving any stimulus, especially without IL-2 stimulation, was examined. 30 min of 10ng/mL IFN α stimulation was used as the positive control for pSTAT1 and pSTAT3, while 30 min of 100 IU/mL IL-2 stimulation as the positive control for pSTAT5. When stimulation finished, cells were then fixed by 4% PFA at room temperature and permeabilized by cold methanol before they were stained for pSTAT proteins to determine the activation of STAT signalling. Results were analyzed by flow cytometry. Antibodies used in this experiment were BD Phosflow™ PE Mouse Anti-Stat1 (pY701), BD Phosflow™ Alexa Fluor® 647 Mouse Anti-Stat3 (pY705) and BD Phosflow™ Alexa Fluor® 488 Anti-Stat5 (pY694).

[0267] FIG. 9 shows the comparison results in terms of the phosphorylation of STAT proteins ("pSTATs") among unmodified T cells, IL15-IL9R modified T cells, IL7-IL9R modified T cells, when cultured *in vitro* without IL-2 stimulation, with the untransduced T cells that are

stimulated by IFN α or IL-2 as positive control. As shown in the three panels of FIG. 9, both IL15-IL9R and IL7-IL9R modified TILs exhibit significantly increased activation of STAT signaling, as indicated by the increased phosphorylation of STAT1, STAT3 and STAT5 compared with the unmodified TILs, in the absence of IL-2 stimulation.

5 [0268] In order to further characterize the effects of the synthetic cytokine signaling system modification on T cells, the *in vitro* cytotoxicity and the secretion of killer cytokine and proteases of modified T cells upon co-culturing with target cells without IL-2 stimulation was investigated. As such, a previously reported T cell cytotoxicity study model (Judith Leitner, et al., T cell stimulator cells, an efficient and versatile cellular system to assess the role of
10 costimulatory ligands in the activation of human T cells. *J Immunol Methods*. 2010 Oct 31; 362(1-2): 131–141.) as used, with a modified HeLa cell line expressing membrane-bound anti-CD3 scFv as the target cells. Briefly, the membrane-bound anti-CD3 scFv is constructed as: VH domain and VL domain of anti-CD3 antibody are connected using GS linker followed by IgG4 hinge domain and CD4 transmembrane domain. The sequences and experimental
15 designs can be found in the above cited reference, whose disclosure is incorporated herein by reference in its entirety.

[0269] Briefly, PBMCs were transduced with indicated constructs, and the modified T cells and the target cells were mixed at a ratio of 1:1 and co-cultured them for 24 hr in 37°C without IL-2, then the proportion of tumor cells that were killed was measured by 7-AAD positive
20 percentage via flow cytometry. As shown by the flow results in FIG. 10A (flow cytometry data) and FIG. 10B (bar graph), IL15-IL9R and IL7-IL9R modified T cells killed the target cells more efficiently than UTD T cells.

[0270] In addition to the *in vitro* killing efficiency, the intracellular levels (by means of intracellular staining and flow cytometry) and secreted concentrations (by ELISA assay over
25 the collected supernatants) of the killer cytokine interferon gamma (i.e. "IFN γ ") were also investigated. Results showed that, compared with unmodified T cells ("UTD"), both the IL15-IL9R and IL7-IL9R modified T cells co-cultured with the target cells in the absence of IL2 displayed higher intracellular staining of IFN γ in both the CD8 T cell population (FIGS. 10C-10D) and the CD4 T cell population (FIGS. 10E-10F), and also displayed higher secretion level
30 of IFN γ (FIGS. 10I). Specifically, as shown in FIG. 10I, the secreted IFN γ levels of the IL7-IL9R modified T cells and of the IL15-IL9R modified T cells are about 28% and 33% higher than that of the unmodified T cells ("Blank").

[0271] Similar assays were also carried out over yet another two killer cytolytic proteins

Granzyme B and perforin. Results showed that although the intracellular staining levels of Granzyme B in the IL15-IL9R and IL7-IL9R modified T cells co-cultured with the target cells in the absence of IL2 were comparable to unmodified T cells (FIGS. 10G-10H), the IL15-IL9R modified T cells displayed notably elevated secretion of Granzyme B (FIG. 10J). Examination
5 over perforin further showed that both the IL15-IL9R and IL7-IL9R modified T cells displayed notably higher secretion levels of perforin (FIG. 10K), with the IL15-IL9R modification showing a relatively higher increase.

[0272] These results together demonstrate that both IL15-IL9R and IL7-IL9R modified T cells, when co-cultured with target cells in the absence of IL2, display increased cytotoxicity against
10 the target cells and display increased capacity to produce killer cytokine and cytolytic proteins.

[0273] Example 4: Synthetic cytokine signaling characterization in CAR-T cells

[0274] To confirm the results in Example 3 as described above, in this example, an engineered CAR-T cells, i.e. T cells engineered to express an anti-ALPP (alkaline phosphatase, placental) chimeric antigen receptor (CAR), were separately modified to express the synthetic cytokine
15 signaling system IL15-IL15Ra-IL9R or IL7-IL7R-IL9R, and the effects of such modification on multiple characteristics were investigated. The sequences for the anti-ALPP CAR construct and the engineered CAR-T cells were previously disclosed in WO2020263796A1, whose disclosure is incorporated herein by reference in its entirety.

[0275] Briefly, activated human T cells were co-transduced with ALPP CAR construct and
20 IL7-IL9R ("IL7-IL9R modified") or IL15-IL9R ("IL15-IL9R modified") constructs, or transduced with ALPP CAR construct alone ("Unmodified") in the presence of hIL2 (300 IU/mL). Five days after transduction, these T cells were split into two groups equally for culture *in vitro*: one group cultured with IL2 ("With IL2") and one group cultured without IL2 ("Without IL2"), and their cell expansion and cell viability were continuously monitored over
25 the time period of Day 0 or D0 (i.e. immediately after transduction and starting the *in vitro* culture assays) through Day 15 or D15.

[0276] In the *in vitro* cell proliferation assay, all four groups of cells could expand with little difference when cultured *in vitro* in the presence of IL2 (See FIG. 11A, left panel). Yet they displayed dramatic differences when cultured *in vitro* in the absence of IL2 (see FIG. 11B, right
30 panel): whereas both the untransduced T cells ("Untransduced") and the unmodified CAR-T cells ("Unmodified") exhibited substantially little or no cell expansion (i.e. the cell expansion ratio is no more than 2.0 during the time period of D0 through D15), both the IL15-IL9R modified CAR-T cells and the IL7-IL9R modified CAR-T cells displayed much greater cell expansion over the time period. More specifically, under the IL2-free *in vitro* culturing

condition, the IL7-IL9R modified CAR-T cells and the IL15-IL9R modified CAR-T cells expanded for about 10 fold and 20 fold respectively at Day 7 (i.e. "D7"), for about 20 fold and 62 fold respectively at Day 12 (i.e. "D12"), and for about 18 fold and 92 fold respectively at Day 15 (i.e. "D15").

- 5 [0277] In the cell viability assay, all four groups of cells were maintained similarly well when cultured *in vitro* in the presence of IL2 (See FIG. 11B, left panel). Yet they displayed dramatic differences when cultured in the absence of IL2 (see FIG. 11B, right panel): both the untransduced T cells ("Untransduced") and the unmodified CAR-T cells ("Unmodified") exhibited much reduced cell viability, with only less than 20% cells surviving starting Day 12.
- 10 In contrast, both the IL15-IL9R modified CAR-T cells and the IL7-IL9R modified CAR-T cells showed much better viability, with more than 70% such modified cells surviving even at Day 15. More specifically, at D12, the cell viability of the unmodified CAR-T cells, the IL7-IL9R modified CAR-T cells, and the IL15-IL9R modified CAR-T cells is respectively about 18%, 80%, 82%, with the cell viability of the latter two group of cells about 62% (i.e. 80%-18%, for
- 15 the IL7-IL9R modified) and 64% (i.e. 82%-18%) higher than that of the unmodified CAR-T cells. Day 15 gave a similar results, with the cell viability of the IL7-IL9R modified CAR-T cells and the IL15-IL9R modified CAR-T cells about 58% (i.e. 87%-19%) and 62% (i.e. 81%-19%) higher than the cell viability of the unmodified CAR-T cells.

- [0278] Taken these above data together, CAR-T cells modified with either IL7-IL9R or IL15-IL9R synthetic cytokine signaling system exhibit increased cell proliferation capability and increase cell viability when cultured *in vitro* in the absence of IL-2, compared with unmodified CAR-T cells.
- 20

- [0279] To determine whether the synthetic cytokine signaling modification influences the CAR-T function, an *in vitro* cytotoxicity assay was carried out. Briefly, CAR-T cells in the presence of IL2 ("CAR-T with IL2") or in the absence of IL2 ("CAR-T without IL2") were co-culture with CFSE pre-labeled Caski cells at different effector-to-target ratios (E:T ratio) for overnight, after which tumor cells were then collected, and viability was measured by 7-AAD. As shown in FIG. 12A, in the presence of IL2, all three CAR-T cells (i.e. "Unmodified", "IL7-IL9R modified" and "IL15-IL9R modified") displayed no apparent difference in their *in vitro*
- 25
- 30 killing efficiency against the target Caski cells. In the absence of IL2, however, both the IL7-IL9R modified CAR-T cells and the IL15-IL9R modified CAR-T cells exhibited significantly higher cytotoxicity against the co-cultured target Caski cells, compared with unmodified CAR-T cells (i.e. "Unmodified") at each of the three E:T ratios (i.e. "1:3", "1:1", and "3:1"), as shown in FIG. 12B.

[0280] To further determine whether the synthetic cytokine signaling modification influences T cell activation, the interferon gamma (IFN γ , or IFN γ) secretion assay was carried out. Briefly, CAR-T cells in the presence of IL2 ("CAR-T with IL2") or in the absence of IL2 ("CAR-T without IL2") were co-culture with Caski cells for 48 hrs, after which the supernatant was then collected and IFN γ ELISA was measured. As shown in FIG. 13A, in the presence of IL2, both IL7-IL9R or IL15-IL9R modification could cause significantly higher activation of CAR-T cells upon antigen stimulation (see the right panel "Target"), as compared to unmodified CAR-T cells, and IL15-IL9R modification provided an even more pronounced enhancement compared with IL7-IL9R modification. As further shown in FIG. 13B, even in the absence of IL2, both IL7-IL9R or IL15-IL9R modification could also cause activation of CAR-T cells upon antigen stimulation.

[0281] Example 5: Synthetic cytokine signaling characterization *in vivo*

[0282] To further investigate the effects of the synthetic cytokine signaling system modification on the immune cells and to confirm the *in vitro* characterization results in Examples 3 and 4, in this example, several *in vivo* characterization assays were carried out.

[0283] Firstly, the *in vivo* tissue distribution of modified T cells was examined. The OT-1-LLC-OVA tumor model (Ching-wen Chen, et al. Tumor-Specific T Cells Exacerbate Mortality and Immune Dysregulation during Sepsis. *J Immunol.* 2021 May 15;206(10):2412-2419. The disclosure of this cited reference is incorporated herein by reference in its entirety.) was used for this purpose. Briefly, at day 7 before T cell transfer, 1 million LLC-Ova tumor cells were implanted to each C57 mouse (congenic marker CD45.1) in the right flank. Then one day before T cell transfer, CTX (at 150/kg body weight) was injected for lymphodepletion, and 2 million indicated OT-1 T cells (congenic marker CD45.2) were given to each tumor-bearing C57 mouse through tail vein injection on the next day. Day 9 after T cell transfer, mice were sacrificed, and key organs/tissues were harvested to analyze T cell infiltration by flow cytometry. Mouse CD45.1 and CD45.2 immune cells were gated out first as total immune cells in each organ/tissue, the percentages of CD45.2 immune cells among total immune cells in each organ/tissue were plotted using GraphPad as indicated in FIG. 14.

[0284] As shown in the figure, the IL15-IL9R modified T cells (as indicated by "IL15-IL9R modified") have less percentage in the peripheral blood than the unmodified T cells, however, modified T cells have significant more percentages in tumor, spleen and draining lymph nodes (i.e. "TDLN-Inguinal" and "TDLN-Axillary"). In the meantime, no difference was observed for the modified T cells and the unmodified T cells in other normal tissues/organs, such as Lung, Liver, and Bone. As such, compared with unmodified T cells, the IL15-IL9R modified T cells

exhibit more preferred migration to tumor tissues and/or lymphoid tissues when transferred *in vivo* into a subject bearing a tumor that the T cells specifically targets.

[0285] Secondly, the OT-1-LLC-OVA tumor model was also adopted to investigate the *in vivo* tumor clearing ability of modified T cells. Briefly, at day 7 before T cell transfer, 1 million
5 LLC-Ova tumor cells were implanted to each C57 mouse in the right flank; 4 days before transfer, lymph nodes were collected from OT-1 mice and lymphocytes were activated by CD3/CD28 beads *in vitro* for 2 days before they were transduced with IL15-IL9R and IL7-IL9R retrovirus supernatant. The day before transfer, tumor-bearing C57 mice were intraperitoneally injected with CTX (at 150mg/kg body weight) for lymphodepletion, and 2
10 million indicated OT-1 T cells were given to each tumor-bearing C57 mouse through tail vein injection on the next day, and the tumor growth and body weight loss were examined.

[0286] As shown in FIG. 15A, while the tumors continuously grow in mice provided with unmodified OT-1 T cells, the IL15-IL9R modified OT-1 T cells displayed a remarkable *in vivo* efficacy in controlling the tumor growth. At Day 17, the tumor increased to almost 2500 mm³
15 in size for the unmodified group, whereas in sharp contrast, the tumor volume was barely increased for the IL15-IL9R modified group. As further shown in FIG. 15B, no significant weight loss was observed in all groups, indicating no toxic side effects.

[0287] To verify the above *in vivo* results, another tumor model (Judith Leitner, et al., T cell stimulator cells, an efficient and versatile cellular system to assess the role of costimulatory
20 ligands in the activation of human T cells. *J Immunol Methods*. 2010 Oct 31; 362(1-2): 131–141.) was independently adopted to confirm the capacity of the IL7-IL9R modified TILs or the IL15-IL9R modified TILs to kill tumor cells *in vivo*. Briefly, A375 tumor cells were modified to overexpress membrane-bound anti-CD3 scFv, as described above. This engineered cancer cell line is termed as A375-anti-CD3. 5 million A375-anti-CD3 tumor cells were implanted to
25 each NOG (hIL2) mouse in the right flank at day 6 before TIL transfer. At the transfer day, each tumor-bearing mouse received 15 million indicated TILs via tail vein injection. As demonstrated by the tumor volume change of each individual mouse in each group (see FIG. 16A), both the IL7-IL9R modified TILs and the IL15-IL9R modified TILs exhibited higher tumor controlling efficacy *in vivo* compared with unmodified TILs, and the body weight data
30 (see FIG. 16B) indicated no toxic side effects.

[0288] Taken together, these above data demonstrated that the modification of cancer-targeting effector immune cells (e.g. T cells or TILs) with the synthetic cytokine signaling system such as IL7-IL9R and in particular IL15-IL9R can enhance their migration to lymphoid and tumor tissues, and further result in an elevated *in vivo* tumor controlling capacity.

[0289] Example 6: Characterization of the synthetic cytokine signaling system that consists only of a self-activating engineered cytokine signaling chain "15R-del"

[0290] In this example, one specific embodiment of a synthetic cytokine signaling system that consists only of a self-activating engineered cytokine signaling chain is characterized. The self-activating engineered cytokine signaling chain, i.e. IL15-IL2R β (ECD)-IL2R β (TM)-IL9R(ICD*) (short as "15R-del" in this example; structure illustrated in FIG. 17; SEQ ID NO: 79) is compared with the "15-9R" in this example (i.e. consisting of the non-self-activating engineered cytokine receptor chain IL2Rb(ECD)-IL2Rb(TM)-IL9R(ICD*) (SEQ ID NO: 8) and an engineered cytokine ligand chain membrane-bound IL15-IL15Ra/Recast-IL15 (SEQ ID NO: 11)).

[0291] Each of "15R-del" and "15-9R" is independently introduced into immune cells such as immortalized line of T cells (Jurkat cells), PMBC cells, TILs, or engineered T cells that express tumor antigen-targeting CARs, etc., and the effects of the ectopically expressed synthetic cytokine signaling system on a variety of characteristics, with untransduced cells (i.e. "UT" or "UTD") as control.

[0292] Expression of "15R-del" and "15-9R" was first examined. Briefly, at day 7 after T cell transduction, 0.2 million cells were taken and stained with G4S flow antibody to examine the expression of the cytokine-chimeric receptor complex by flow cytometry. As shown in FIG. 18, after transduction of the activated T cells with the "15-9R" or the "15R-del" retroviral vectors, both synthetic cytokine signaling systems could be well expressed in T cells.

[0293] The activation of STAT signaling in resting untransduced (UT), 15-9R-modified and 15R-del-modified TIL cells without giving any stimulus (especially without IL-2 stimulation) was further examined. Briefly, 1 million cells per group were starved overnight, and UT cells stimulated with IL-2 (100 IU/mL) or IFN α (10 ng/mL) for 30 min served as a positive control ("Pos ctr"). After fixation and permeabilization by paraformaldehyde and methanol, cells were stained by AF647-pSTAT3 and AF488-pSTAT5 for 1 hour on ice, results were analyzed by flow cytometry. Both the 15-9R modified and 15R-del modified T cells displayed strong pSTAT3 activation, with the 15R-del modified T cells showing even stronger pSTAT3 signaling than the 15-9R modified (FIGS. 19A and 19B). Surprisingly and unexpectedly, however, while the 15-9R modified T cells displayed strong pSTAT5 signaling than the UT cells, the pSTAT5 signaling in the 15R-del modified T cells were roughly comparable to that in the UT cells (FIGS. 19C and 19D). Thus the results demonstrate somewhat difference for the two synthetic cytokine signaling systems in terms of the pSTAT3 signaling and of the pSTAT5 signaling.

[0294] To investigate whether the modifications can reduce IL-2 dependence of such modified T cells in terms of proliferation or survival, TILs transduced with indicated constructs were cultured *in vitro* with or without 300 IU/mL IL-2, and their expansion ratio (FIG. 20A) and cell viability (FIG. 20B) were continuously monitored. As shown in FIG. 20A, in the absence of IL-2, while unmodified TILs stopped proliferating at roughly day 2 or day 4, both the 15-9R modified TILs and the 15R-del-modified TILs continued proliferating till day 11, with the former showing higher proliferation capability than the latter. As shown in FIG. 20B, in the absence of IL-2, both the 15-9R modified TILs and the 15-del modified TIL cells exhibit better viability (or survival capability, or sustainability) compared with the unmodified TILs, with the former showing higher viability than the latter. Thus the results suggest that in the *in vitro* culturing condition in the absence of IL2, the 15-del showed a somewhat weaker effect compared with the 15-9R in terms of proliferation capability and viability.

[0295] To investigate the effects of 15-9R and 15R-del modifications on the *in vivo* efficacy of T cells against target tumor cells in the absence IL-2, a paired TIL and tumor model in immunodeficient mice (NKG mice) was used. First, tumor tissue from a lung cancer patients were cut into small 1x1 mm³ pieces and divided into two roughly equal parts. One part was used to culture TILs and the other was used to inoculate NKG mice and generate PDX model. From established PDX model, we further established patient-derived tumor cell line. 4 million patient primary tumor cells were implanted to each NKG mouse in the right flank on day 6 prior to TIL infusion. Patient TILs at the meantime were modified with indicated constructs. At the transfer day, each tumor-bearing mouse received 15 million ("15e6") or 5 million ("5e6") indicated TILs via tail vein injection, without any IL-2 injection. *In vivo* efficacy was demonstrated by tumor volume change of each group. As shown in FIG. 21, while mice injected with PBS continued to have the tumor volume increasing over time ("Vehicle control"), both the 15R-del modified TILs and the 15-9R modified TILs exhibited robust and dose-dependent *in vivo* efficacy against the target tumor cells. Noteworthy, it was further observed that although the 15-del showed a somewhat weaker effect compared with 15-9R in terms of proliferation capability and viability as illustrated in FIGS. 20A and 20B, the 15R-del modified T cells surprisingly and unexpectedly displayed a better tumor growth capability compared with the 15-9R modified T cells. In one example, at day 21, the tumor volume was ~400 mm³ for the "15-9R/5e6" mice and ~100 mm³ for the "15R-del/5e6" mice. In another example, at day 25, the tumor volume was ~550 mm³ for the "15-9R/15e6" mice and almost 0 mm³ for the "15R-del/15e6" mice. Thus these results indicate that compared with the 15-9R modification, the 15-del modification causes a much better *in vivo* efficacy for such modified T cells against

target tumor cells.

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 - 30 NKT-mediated cytotoxicity against multiple targets in vitro and in vivo." Journal of immunological methods 285.1 (2004): 25-40.

CLAIMS

1. An engineered cytokine signaling chain, encoding a transmembrane polypeptide when expressed in an immune cell, wherein the transmembrane polypeptide comprises an extracellular portion and an intracellular portion, operably connected by a transmembrane portion, wherein:
- the intracellular portion comprises a variant intracellular domain (ICD) of interleukin-9 receptor (IL9R), comprising at least one of a first sequence alteration in a first region corresponding positions 174-230, or a second sequence alteration in a second region corresponding positions 142-151, of the sequence as set forth in SEQ ID NO: 1.
2. The engineered cytokine signaling chain of claim 1, wherein each of the first sequence alteration or the second sequence alteration comprises a truncation or a substitution.
3. The engineered cytokine signaling chain of claim 2, wherein the first sequence alteration comprises a truncation at positions 174-230 of the sequence as set forth in SEQ ID NO: 1, and the second sequence alteration comprises a substitution in the second region at positions 142-151 of the sequence as set forth in SEQ ID NO: 1.
4. The engineered cytokine signaling chain of claim 3, wherein the variant ICD of IL9R in the intracellular portion comprises a sequence as set forth in SEQ ID NO: 7.
5. The engineered cytokine signaling chain of any one of claims 1-4, wherein the extracellular portion comprises an extracellular domain (ECD) of a cytokine receptor chain or a functional variant thereof.
6. The engineered cytokine signaling chain of claim 5, wherein the cytokine receptor chain is IL2R β , and the ECD of the cytokine receptor chain or the functional variant thereof in the extracellular portion comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 3.
7. The engineered cytokine signaling chain of claim 5, wherein the cytokine receptor chain is IL4R α , and the ECD of the cytokine receptor chain or the functional variant thereof in the

extracellular portion comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 33.

8. The engineered cytokine signaling chain of claim 5, wherein the cytokine receptor chain is IL7R α , and the ECD of the cytokine receptor chain or the functional variant thereof in the extracellular portion comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 5.

9. The engineered cytokine signaling chain of claim 5, wherein the cytokine receptor chain is IL9R, and the ECD of the cytokine receptor chain or the functional variant thereof in the extracellular portion comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 36.

10. The engineered cytokine signaling chain of claim 5, wherein the cytokine receptor chain is IL21R, and the ECD of the cytokine receptor chain or the functional variant thereof in the extracellular portion comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 39.

11. The engineered cytokine signaling chain of any one of claims 5-10, wherein the transmembrane portion comprises a transmembrane domain from the cytokine receptor chain.

12. The engineered cytokine signaling chain of claim 11, wherein one of the following is met:
the cytokine receptor chain is IL2R β , and the transmembrane domain of the transmembrane portion comprises a sequence as set forth in SEQ ID NO: 19;
the cytokine receptor chain is IL4R α , and the transmembrane domain of the transmembrane portion comprises a sequence as set forth in SEQ ID NO: 34;
the cytokine receptor chain is IL7R α , and the transmembrane domain of the transmembrane portion comprises a sequence as set forth in SEQ ID NO: 24;
the cytokine receptor chain is IL9R, and the transmembrane domain of the transmembrane portion comprises a sequence as set forth in SEQ ID NO: 37; or
the cytokine receptor chain is IL21R, and the transmembrane domain of the transmembrane portion comprises a sequence as set forth in SEQ ID NO: 40.

13. The engineered cytokine signaling chain of any one of claims 5-10, wherein the transmembrane portion comprises a transmembrane domain from a polypeptide other than the cytokine receptor chain.

- 5 14. The engineered cytokine signaling chain of any one of claims 5-13, wherein the extracellular portion further comprises a cytokine ligand domain (CLD) over an N-terminus of the ECD of the cytokine receptor chain or the functional variant thereof, wherein the CLD is functionally compatible to the ECD of the cytokine receptor chain or the functional variant thereof.

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15. The engineered cytokine signaling chain of claim 14, wherein the cytokine receptor chain is IL2R β , and the CLD comprises IL15 or a functional variant thereof, wherein the CLD comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 9.

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16. The engineered cytokine signaling chain of claim 14, wherein the cytokine receptor chain is IL2R β , and the CLD comprises IL2 or a functional variant thereof, wherein the CLD comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 44.

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17. The engineered cytokine signaling chain of claim 14, wherein the cytokine receptor chain is IL4R α , and the CLD comprises IL4 or a functional variant thereof, wherein the CLD comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 49.

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18. The engineered cytokine signaling chain of claim 14, wherein the cytokine receptor chain is IL7R α , and the CLD comprises IL7 or a functional variant thereof, wherein the CLD comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 13.

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19. The engineered cytokine signaling chain of claim 14, wherein the cytokine receptor chain is IL9R, and the CLD comprises IL9 or a functional variant thereof, wherein the CLD

comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 46.

20. The engineered cytokine signaling chain of claim 14, wherein the cytokine receptor chain is IL21R, and the CLD comprises IL21 or a functional variant thereof, wherein the CLD comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 47.

21. The engineered cytokine signaling chain of any one of claims 14-20, wherein the CLD is connected to the N-terminus of the ECD of the cytokine receptor chain or the functional variant thereof via a flexible linker.

22. The engineered cytokine signaling chain of claim 26, wherein one of the following is met:
the cytokine receptor chain is IL2R β , and the engineered cytokine signaling chain comprises a sequence as set forth in SEQ ID NO: 79 or SEQ ID NO: 80;
the cytokine receptor chain is IL4R α , and the engineered cytokine signaling chain comprises a sequence as set forth in SEQ ID NO: 81;
the cytokine receptor chain is IL7R α , and the engineered cytokine signaling chain comprises a sequence as set forth in SEQ ID NO: 82;
the cytokine receptor chain is IL9R, and the engineered cytokine signaling chain comprises a sequence as set forth in SEQ ID NO: 83; or
the cytokine receptor chain is IL21R, and the engineered cytokine signaling chain comprises a sequence as set forth in SEQ ID NO: 84.

23. The engineered cytokine signaling chain of any one of claims 14-22, wherein when the engineered cytokine signaling chain is expressed in an immune cell, the immune cell exhibits increased STAT3 signaling in the absence of IL-2 stimulation compared to when the engineered cytokine signaling chain is not expressed in the immune cell.

24. The engineered cytokine signaling chain of any one of claims 14-22, wherein when the engineered cytokine signaling chain is expressed in an immune cell, the immune cell displays increased proliferation capability when cultured *in vitro* in the absence of IL-2, compared to when the engineered cytokine signaling chain is not expressed in the immune cell.

25. The engineered cytokine signaling chain of any one of claims 14-22, wherein when the engineered cytokine signaling chain is expressed in an immune cell, the immune cell displays increased viability when cultured *in vitro* in the absence of IL-2, compared to when the
5 engineered cytokine signaling chain is not expressed in the immune cell.

26. The engineered cytokine signaling chain of any one of claims 14-22, wherein when the engineered cytokine signaling chain is expressed in an immune cell, the immune cell exhibits increased cytotoxicity against target cells of the immune cell when co-cultured with the target
10 cells in the absence of IL-2 stimulation, compared to when the engineered cytokine signaling chain is not expressed in the immune cell.

27. The engineered cytokine signaling chain of any one of claims 5-13, wherein the extracellular portion does not comprise a cytokine ligand domain (CLD) that is functionally
15 compatible to the ECD of the cytokine receptor chain or the functional variant thereof.

28. The engineered cytokine signaling chain of claim 27, wherein one of the following is met:
the cytokine receptor chain is IL2R β , and the engineered cytokine signaling chain
comprises a sequence as set forth in SEQ ID NO: 8;
20 the cytokine receptor chain is IL4R α , and the engineered cytokine signaling chain
comprises a sequence as set forth in SEQ ID NO: 35;
the cytokine receptor chain is IL7R α , and the engineered cytokine signaling chain
comprises a sequence as set forth in SEQ ID NO: 12;
the cytokine receptor chain is IL9R, and the engineered cytokine signaling chain
25 comprises a sequence as set forth in SEQ ID NO: 38; or
the cytokine receptor chain is IL21R, and the engineered cytokine signaling chain
comprises a sequence as set forth in SEQ ID NO: 41.

29. The engineered cytokine signaling chain of claim 27 or claim 28, wherein when the
30 engineered cytokine signaling chain is expressed in an immune cell along with an engineered
cytokine ligand chain capable of activating the engineered cytokine signaling chain, the
immune cell displays increased proliferation capability when cultured *in vitro* in the absence

of IL-2, compared to when the engineered cytokine signaling chain and the engineered cytokine ligand chain are not expressed in the immune cell.

30. The engineered cytokine signaling chain of claim 27 or claim 28, wherein when the engineered cytokine signaling chain is expressed in an immune cell along with an engineered cytokine ligand chain capable of activating the engineered cytokine signaling chain, the immune cell displays increased viability when cultured *in vitro* in the absence of IL-2, compared to when the engineered cytokine signaling chain and the engineered cytokine ligand chain are not expressed in the immune cell.

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31. The engineered cytokine signaling chain of claim 27 or claim 28, wherein when the engineered cytokine signaling chain is expressed in an immune cell along with an engineered cytokine ligand chain capable of activating the engineered cytokine signaling chain, the immune cell exhibits increased STAT signaling in the absence of IL-2 stimulation compared to when the engineered cytokine signaling chain and the engineered cytokine ligand chain are not expressed in the immune cell.

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32. The engineered cytokine signaling chain of claim 27 or claim 28, wherein when the engineered cytokine signaling chain is expressed in an immune cell along with an engineered cytokine ligand chain capable of activating the engineered cytokine signaling chain, the immune cell exhibits increased interferon gamma (IFN γ) secretion when co-cultured with target cells corresponding thereto in the absence of IL-2 stimulation, compared to when the engineered cytokine signaling chain and the engineered cytokine ligand chain are not expressed in the immune cell.

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33. The engineered cytokine signaling chain of claim 27 or claim 28, wherein when the engineered cytokine signaling chain is expressed in an immune cell along with an engineered cytokine ligand chain capable of activating the engineered cytokine signaling chain, the immune cell exhibits increased cytotoxicity against target cells of the immune cell when co-cultured with the target cells in the absence of IL-2 stimulation, compared to when the engineered cytokine signaling chain and the engineered cytokine ligand chain are not expressed in the immune cell.

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34. The engineered cytokine signaling chain of claim 27 or claim 28, wherein when the engineered cytokine signaling chain is expressed in an immune cell along with an engineered cytokine ligand chain capable of activating the engineered cytokine signaling chain, the immune cell exhibits more preferred migration to tumor tissues and/or lymphoid tissues when a population of the immune cell are transferred into a subject bearing a tumor that the immune cell specifically targets, compared to when the engineered cytokine signaling chain and the engineered cytokine ligand chain are not expressed in the immune cell.

35. The engineered cytokine signaling chain of claim 27 or claim 28, wherein when the engineered cytokine signaling chain is expressed in an immune cell along with an engineered cytokine ligand chain capable of activating the engineered cytokine signaling chain, the immune cell displays increased tumor control ability when a population of the immune cell are transferred into a subject bearing a tumor that the immune cell specifically targets, compared to when the engineered cytokine signaling chain and the engineered cytokine ligand chain are not expressed in the immune cell.

36. The engineered cytokine signaling chain of any one of claims 29-35, wherein the engineered cytokine ligand chain encodes a secreted cytokine ligand when expressed in the immune cell.

37. The engineered cytokine signaling chain of any one of claims 29-35, wherein the engineered cytokine ligand chain encodes a membrane-bound cytokine ligand when expressed in the immune cell.

38. A synthetic cytokine signaling system for expression in an immune cell, comprising an engineered cytokine signaling chain according to any one of claims 1-37.

39. The synthetic cytokine signaling system of claim 38, comprising an engineered cytokine signaling chain according to any one of claims 27-37, and further comprising an engineered cytokine ligand chain, wherein:

the extracellular portion of the engineered cytokine signaling chain comprises an extracellular domain (ECD) of a cytokine receptor chain or a functional variant thereof,

wherein the cytokine receptor chain is selected from a group consisting of IL2R β , IL4R α , IL7R α , IL9R, and IL21R; and

when the engineered cytokine signaling chain and the engineered cytokine ligand chain are co-expressed in the immune cell, the engineered cytokine ligand chain is capable of activating the engineered cytokine signaling chain.

40. The synthetic cytokine signaling system of claim 39, wherein the engineered cytokine ligand chain encodes a secreted cytokine ligand.

41. The synthetic cytokine signaling system of claim 40, wherein the extracellular portion of the engineered cytokine signaling chain comprises the extracellular domain (ECD) of IL2R β or a functional variant thereof, wherein the engineered cytokine ligand chain encodes at least one of:

a secreted IL2, comprising a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 44; or

a secreted IL15, comprising a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 9.

42. The synthetic cytokine signaling system of claim 40, wherein the extracellular portion of the engineered cytokine signaling chain comprises the extracellular domain (ECD) of IL4R α or a functional variant thereof, wherein the engineered cytokine ligand chain encodes a secreted IL4, comprising a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 49.

43. The synthetic cytokine signaling system of claim 40, wherein the extracellular portion of the engineered cytokine signaling chain comprises the extracellular domain (ECD) of IL7R α or a functional variant thereof, wherein the engineered cytokine ligand chain encodes a secreted IL7, comprising a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 13.

44. The synthetic cytokine signaling system of claim 40, wherein the extracellular portion of the engineered cytokine signaling chain comprises the extracellular domain (ECD) of IL9R or a functional variant thereof, wherein the engineered cytokine ligand chain encodes a secreted

IL9, comprising a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 46.

45. The synthetic cytokine signaling system of claim 40, wherein the extracellular portion of the engineered cytokine signaling chain comprises the extracellular domain (ECD) of IL21R or a functional variant thereof, wherein the engineered cytokine ligand chain encodes a secreted IL21, comprising a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 47.

46. The synthetic cytokine signaling system of claim 39, wherein the engineered cytokine ligand chain encodes a membrane-bound cytokine ligand, comprising a ligand portion operably connected to a membrane-bound portion, wherein the ligand portion is functionally compatible to the ECD of the cytokine receptor chain or the functional variant thereof in the extracellular portion of the engineered cytokine signaling chain.

47. The synthetic cytokine signaling system of claim 46, wherein the extracellular portion of the engineered cytokine signaling chain comprises the extracellular domain (ECD) of IL2R β or a functional variant thereof, wherein the engineered cytokine ligand chain encodes at least one of:

a membrane-bound IL2, wherein the ligand portion thereof comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 44; or

a membrane-bound IL15, wherein the ligand portion thereof comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 9.

48. The synthetic cytokine signaling system of claim 46, wherein the extracellular portion of the engineered cytokine signaling chain comprises the extracellular domain (ECD) of IL4R α or a functional variant thereof, wherein the engineered cytokine ligand chain encodes a membrane-bound IL4, wherein the ligand portion thereof comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 49.

49. The synthetic cytokine signaling system of claim 46, wherein the extracellular portion of the engineered cytokine signaling chain comprises the extracellular domain (ECD) of IL7R α or a functional variant thereof, wherein the engineered cytokine ligand chain encodes a membrane-bound IL7, wherein the ligand portion thereof comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 13.

50. The synthetic cytokine signaling system of claim 46, wherein the extracellular portion of the engineered cytokine signaling chain comprises the extracellular domain (ECD) of IL9R or a functional variant thereof, wherein the engineered cytokine ligand chain encodes a membrane-bound IL9, wherein the ligand portion thereof comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 46.

51. The synthetic cytokine signaling system of claim 46, wherein the extracellular portion of the engineered cytokine signaling chain comprises the extracellular domain (ECD) of IL21R or a functional variant thereof, wherein the engineered cytokine ligand chain encodes a membrane-bound IL21, wherein the ligand portion thereof comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 47.

52. The synthetic cytokine signaling system of any one of claims 46-51, wherein the membrane-bound portion of the engineered cytokine ligand chain comprises a fragment crystallizable region (Fc) or a functional variant thereof.

53. The synthetic cytokine signaling system of claim 52, wherein the Fc comprises a IgG4Fc, wherein the membrane-bound portion comprises a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 14.

54. The synthetic cytokine signaling system of claim 52 or claim 53, wherein the membrane-bound portion and the ligand portion is operably connected via a hinge.

55. The synthetic cytokine signaling system of claim 54, wherein the hinge comprises a sequence as set forth in SEQ ID NO: 21.

56. The synthetic cytokine signaling system of claim 52 or claim 53, wherein in the membrane-bound portion, the Fc or a functional variant thereof is operably connected to a transmembrane region.

57. The synthetic cytokine signaling system of claim 56, wherein the transmembrane region comprises a sequence as set forth in SEQ ID NO: 22.

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58. The synthetic cytokine signaling system of any one of claims 52-57, wherein the engineered cytokine ligand chain comprises a sequence as set forth in any one of SEQ ID NO: 15 and 54-58.

59. The synthetic cytokine signaling system of claim 47, wherein the engineered cytokine ligand chain encodes a membrane-bound IL15, wherein the membrane-bound portion of the membrane-bound IL15 comprises IL15R α or a functional variant thereof, comprising a sequence having at least 70%, at least 80%, at least 90%, at least 95%, or 100% identity to the sequence as set forth in SEQ ID NO: 10.

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60. The synthetic cytokine signaling system of claim 59, wherein the membrane-bound portion comprises the sequence as set forth in SEQ ID NO: 10.

61. The synthetic cytokine signaling system of claim 59 or claim 60, wherein the membrane-bound portion and the ligand portion is operably connected via a linker.

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62. The synthetic cytokine signaling system of claim 61, wherein the linker comprises a sequence as set forth in SEQ ID NO: 16.

63. The synthetic cytokine signaling system of any one of claims 59-62, wherein the engineered cytokine ligand chain comprises a sequence as set forth in SEQ ID NO: 11.

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64. The synthetic cytokine signaling system of claim 38, consisting of an engineered cytokine signaling chain according to any one of claims 14-26.

65. A vector kit, comprising at least one vector, wherein when the at least one vector is introduced in an immune cell, the immune cell expresses the synthetic cytokine signaling system according to any one of claims 38-64.

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66. The vector kit of claim 65, wherein the synthetic cytokine signaling system comprises an engineered cytokine signaling chain and an engineered cytokine ligand chain, wherein the vector kit consists of one vector, wherein the one vector comprises two nucleotide sequences respectively encoding the engineered cytokine signaling chain and the engineered cytokine
10 ligand chain in the synthetic cytokine signaling system.

67. The vector kit of claim 66, wherein the two nucleotide sequences are in a common open reading frame (ORF), and the one vector comprises a separator sequence separating the two nucleotide sequences, wherein the separator sequence comprises F2A or P2A, wherein the F2A
15 comprises a sequence as set forth in SEQ ID NO: 42, and the P2A comprises a sequence as set forth in SEQ ID NO: 43.

68. The vector kit of claim 66, wherein the two nucleotide sequences are in two different open reading frames (ORFs).

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69. The vector kit of claim 65, wherein the synthetic cytokine signaling system comprises an engineered cytokine signaling chain and an engineered cytokine ligand chain, wherein the vector kit comprises a first vector and a second vector, wherein:

the first vector comprises a first nucleotide sequence encoding the engineered cytokine
25 signaling chain; and

the second vector comprises a second nucleotide sequence encoding the engineered cytokine ligand chain.

70. The vector kit of claim 65, wherein the synthetic cytokine signaling system consists of an
30 engineered cytokine signaling chain, wherein the vector kit consists of one single vector encoding the engineered cytokine signaling chain.

71. A method for obtaining an engineered immune cell, comprising introducing into an immune cell a vector kit according to any one of claims 65-70.

72. An immune cell, comprising the synthetic cytokine signaling system according to any one of claims 38-64.
- 5 73. The immune cell of claim 72, wherein when cultured in *vitro* in the absence of IL-2, the immune cell displays at least one of increased proliferation capability, increased viability, or increased STAT signaling, compared to an immune cell that does not comprise the synthetic cytokine signaling system.
- 10 74. The immune cell of claim 72, wherein when co-cultured with target cells of the immune cell in the absence of IL-2 stimulation, the immune cell displays at least one of increased interferon gamma (IFN γ) secretion or increased cytotoxicity against the target cells, compared to an immune cell that does not comprise the synthetic cytokine signaling system.
- 15 75. The immune cell of claim 72, wherein when a population of the immune cell are transferred into a subject bearing a tumor that the immune cell specifically targets, the population of the immune cell exhibit more preferred migration to tumor tissues and/or lymphoid tissues, and/or displays increased tumor control ability, compared to an immune cell that does not comprise the synthetic cytokine signaling system.
- 20 76. The immune cell of any one of claims 72-75, wherein the immune cell further expresses a targeting polypeptide, wherein the targeting polypeptide comprises at least one of a chimeric antigen receptor (CAR), a T cell receptor (TCR), or an Aspire-T cell receptor.
- 25 77. The immune cell of any one of claims 72-77, wherein the immune cell is any of a T cell, a $\gamma\delta$ T cell, a natural killer (NK) cell, a natural killer T (NKT) cell, or a tumor-infiltrating lymphocyte (TIL), or an engineered immune cell derived therefrom.
78. A method, comprising:
- 30 obtaining an engineered immune cell based on an immune cell, such that the engineered immune cell expresses the synthetic cytokine signaling system according to any one of claims 38-64; and

culturing the engineered immune cell in a medium containing none or a reduced level of a cytokine supplement *in vitro*, wherein the cytokine supplement comprises at least one of IL-2, IL-4, IL-7, IL-9, IL-15, or IL-21.

5 79. The method of claim 78, wherein the cytokine supplement consists of IL-2.

80. A method for treating a disease in a subject in need thereof, comprising:

administering a population of engineered immune cells into the subject, wherein each of the population of the engineered immune cells expresses the synthetic cytokine signaling
10 system according to any one of claims 38-64.

81. The method of claim 80, further comprising, prior to the administering step:

obtaining an engineered immune cell from an immune cell; and
culturing the engineered immune cell in a medium containing none or a reduced level
15 of a cytokine supplement *in vitro* to thereby obtain the population of the engineered immune cells.

82. The method of any one of claims 80-81, wherein the engineered immune cell further expresses a targeting polypeptide, wherein the targeting polypeptide comprises at least one of
20 a chimeric antigen receptor (CAR), a T cell receptor (TCR), or an Aspire-T cell receptor.

83. The method of any one of claims 81-82, wherein the immune cell is any of a T cell, a $\gamma\delta$ T cell, a natural killer (NK) cell, a natural killer T (NKT) cell, or a tumor-infiltrating lymphocyte (TIL), or an engineered immune cell derived therefrom.

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84. The method of any of claims 80-83, wherein the disease is a cancer, an autoimmune disease, or a viral infection.