

## CONTROLLED EXPRESSION OF IMMUNE REGULATORY CYTOKINES IN GENETICALLY ENGINEERED IMMUNE CELLS

### CROSS REFERENCE TO RELATED APPLICATIONS

5           This application claims the benefit of the filing date of U.S. Provisional Application No. 63/520,756, filed August 21, 2023, the entire contents of which is incorporated by reference herein.

### SEQUENCE LISTING

10           The instant application contains a Sequence Listing which has been filed electronically in XML format and is hereby incorporated by reference in its entirety. Said XML copy, created on August 20, 2024, is named 20240820\_063604-507001WO\_Sequence\_Listing\_ST26.xml and is 36,730 bytes in size.

### BACKGROUND OF THE INVENTION

15           Currently, there are many studies involving expression of recombinant proteins in various therapeutic cells to achieve desired physiological effects in clinical applications. Some of these studies have focused on exogenous expression of cytokines, such as IL-2 and IL-15, as well as IL-6 and GM-CSF antagonists in immune cells. More recent studies have focused on  
20   tumor infiltrating T cells expressing IL-12 and chimeric antigen receptor-T (CAR-T) cells expressing IL-18 for therapeutic uses. However, given the pleiotropic effects of cytokines and other immune regulatory agents in immune cells, clinical applications involving exogenous introduction and expression of immune regulatory agents such as cytokines in immune cells have been hampered by severe toxicities in patients.

25           It is of great interest to develop approaches to develop effective and safe immune cell therapies.

### SUMMARY OF THE INVENTION

30           The present disclosure is based, at least in part, on the discovery that inclusion of a coding sequence of one or more self-cleavable peptide linkers (*e.g.*, 2A peptides) between a nucleotide sequence encoding a signal peptide and a coding sequence for a polypeptide of interest significantly reduced the production or secretion of the polypeptide of interest, thereby reducing undesired features, for example, toxicity, associated with over expression of such polypeptides. This approach would be expected to be specifically useful in controlling

expression of immune regulatory agents such as cytokines (*e.g.*, such as IL-2, IL-10, IL-15, and IL-18) in immune cells in order to improve therapeutic efficacy of the immune cells by minimizing or eliminating toxicities associated with inappropriately high levels of expression in the immune cells.

5 Accordingly, provided herein is an expression system for controlling the expression level of a polypeptide of interest. In some embodiments, the expression system provided herein is a self-cleavable 2A peptide-mediated expression system (*i.e.*, SP-2A expression system) utilizing nucleic acids encoding intervening self-cleavable peptides (*e.g.*, 2A peptides) to control the expression level of polypeptides of interest such as immune regulatory agents (*e.g.*,  
10 cytokines such as IL-2, IL-10, IL-15, or IL-18) in mature form, genetically engineered immune cells (*e.g.*, T cells such as TCR-T cells, CAR-T cells, or tumor infiltrating lymphocytes or TILs) carrying such nucleic acids, and therapeutic uses of the genetically engineered immune cells.

In one aspect, the present disclosure provides a nucleic acid, comprising from 5' to 3':  
15 (1) a first nucleotide sequence encoding a signal peptide; (2) a second nucleotide sequence encoding one or more self-cleaving peptides (*e.g.*, 2A peptides) and (3) a third nucleotide sequence encoding a polypeptide of interest. The polypeptide of interest encoded by the third nucleotide sequence is free of a signal peptide at its N-terminus (*i.e.*, in its mature form). When the nucleic acid is expressed in a cell, the polypeptide is produced or secreted at a low  
20 level as compared with a counterpart cell comprising a nucleic acid encoding a counterpart polypeptide having an N-terminus signal peptide.

In some embodiments, the polypeptide encoded by the third nucleotide sequence is a blood factor, a chimeric antigen receptor (CAR), an antibody, a hormone, an immune modulating polypeptide, or a combination thereof. In other embodiments, the third  
25 nucleotide sequence encodes an immune modulating polypeptide. In some instances, the immune modulating polypeptide can be a cytokine, for example, IL-2, IL-7, IL-9, IL-10, IL-15, IL-12, IL-17, IL-18, IL-21, IL-23, IL-24, IL-27, IL-33, or CCL19. In other instances, the immune modulating polypeptide can be an inhibitor of a checkpoint molecule, examples of which include PD1, PDL1, LAG3, TIGIT, TIM3, and CTLA4. In some examples, the  
30 checkpoint inhibitor is an anti-PD1/PDL1 antibody, an anti-LAG3 antibody, an anti-TIGIT antibody, an anti-TIM3 antibody, or an anti-CTLA4 antibody. In yet other instances, the immune modulating polypeptide can be an immune enhancer, for example, 41BBL, an anti-CD3 antibody, an anti-FcR antibody, an anti-41BB antibody, an anti-OX40 antibody, an anti-CD28 antibody, or an anti-CD40 antibody. In still other instances, the immune modulating

polypeptide can be a cytokine antagonist. Examples include an anti-IL6 antibody, an anti-GM-CSF antibody, an anti-IFN- $\gamma$  antibody, an anti-TNF antibody, an anti-IL-1 antibody, and IL-1RA.

In specific examples, the immune modulating polypeptide can be an anti-CD28 antibody, which may be derived from an agonistic anti-CD28 antibody, for example, TGN1412 or TGN1112. See **Table 1** below.

An antibody (interchangeably used in plural form) as used herein is an immunoglobulin molecule capable of specific binding to a target protein, *e.g.*, IL-6 or IL-6R, through at least one antigen recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term “antibody” encompasses not only intact (*e.g.*, full-length) antibodies and heavy chain antibodies (*e.g.*, an Alpaca heavy chain IgG antibody), but also antigen-binding fragments thereof (such as Fab, Fab', F(ab')<sub>2</sub>, Fv), single chain (scFv), single-domain antibody (sdAb; VHH), also known as a nanobody, mutants thereof, fusion proteins comprising an antibody portion, humanized antibodies, chimeric antibodies, diabodies, linear antibodies, single chain antibodies, multispecific antibodies (*e.g.*, bispecific antibodies) and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity, including glycosylation variants of antibodies, amino acid sequence variants of antibodies, and covalently modified antibodies.

In some embodiments, the polypeptide encoded by the third nucleotide sequence is a cytoplasmic signaling domain of an immune cell receptor polypeptide. For example, the third nucleotide sequence may encode the CD3 $\zeta$  signaling domain, the DAP12 signaling domain, the FcR signaling domain, the CD79 signaling domain, or the CNAIP signaling domain. In other examples, the third nucleotide sequence may encode a co-stimulatory signaling domain of a co-stimulation receptor such as CD28 or 4-1BB. In yet other examples, the third nucleotide sequence may encode a co-inhibitory signaling domain of a co-inhibitory receptor such as CTLA4, PD1, LAG3, TIGIT, or TIM3. In some examples, the third nucleotide sequence may encode a signaling domain of a cytokine receptor. Any of the cytoplasmic signaling domains provided herein may be part of a chimeric antigen receptor polypeptide (CAR).

In one specific example, a coding sequence of a 2A peptide can be placed within a coding sequence for a CAR polypeptide to control the expression level of the CAR fragment upstream to the 2A peptide (*e.g.*, the N-terminal fragment comprising the extracellular domain and the transmembrane domain) and the CAR fragment downstream to the 2A peptide (*e.g.*,

the C-terminal fragment comprising a cytoplasmic signaling domain).

In some embodiments, the polypeptide encoded by the third nucleotide sequence is a hormone. In certain embodiments, the hormone is insulin or growth hormone.

In some embodiments, the second nucleotide sequence encodes a 2A self-cleaving peptide of P2A, T2A, E2A, F2A, or a combination thereof. In one embodiment, the second nucleotide sequence encodes a combination of P2A and T2A. In specific examples, the nucleotide sequence encoding the 2A self-cleaving peptide is set forth in any one of SEQ ID NOs: 1-11.

In some embodiments, the first nucleotide sequence encoding the signal peptide is directly linked to the second nucleotide sequence encoding the one or more self-cleaving peptides (*e.g.*, 2A peptides).

In some embodiments, the genetically engineered cells further express one or more proteins of interest, which optionally are different from the polypeptide encoded by the third nucleotide sequence. In some embodiments, the proteins of interest comprise a blood factor, a chimeric antigen receptor (CAR), an immune modulating polypeptide, an antibody, a cytokine, a hormone, a cell surface marker, or a combination thereof. In certain embodiments, the immune modulating polypeptide is a cytokine antagonist, preferably an antibody that binds IL-6, IL-1, GM-CSF, TNF, IFN- $\gamma$ , or a combination thereof. In certain embodiments, the cell surface marker is CD20, a truncated epidermal growth factor receptor (tEGFR), or receptor tyrosine kinase-like orphan receptor 1 (ROR1). In some embodiments, the cell surface marker is CD20, a truncated epidermal growth factor receptor (tEGFR), or receptor tyrosine kinase-like orphan receptor 1 (ROR1). The cell surface surface markers may be used in the expression system to identify transduced cells by *e.g.*, flow cytometry and/or eliminate the genetically engineered cells when severe toxicity is observed by injecting antibodies targeting one or more of CD20, tEGFR, and/or ROR1.

In some embodiments, the first exogenous nucleic acid comprises a fourth nucleotide sequences encoding the one or more proteins of interest disclosed herein. In some embodiments, the fourth nucleotide sequence is located between the first nucleotide sequence encoding the signal peptide and the second nucleotide sequence encoding the one or more self-cleaving peptides (*e.g.*, 2A peptides). In other embodiments, the fourth nucleotide sequence is located 5' to the first nucleotide sequence encoding the signal peptide, and/or 3' to the third nucleotide sequence encoding the one or more proteins of interest. In some embodiments, the fourth nucleotide sequence comprises a self-cleaving peptide coding sequence, a promoter sequence, a coding sequence for a protease cleavage site, an mRNA splicing site, or an internal

ribosomal entry sequence.

In some embodiments, the fourth nucleotide sequence encodes a blood factor, a chimeric antigen receptor (CAR), an antibody, an immune modulating polypeptide, a cytokine, a hormone, or a combination thereof. In other embodiments, the fourth nucleotide sequence  
5 encodes a cell surface marker.

In some examples, the fourth nucleotide sequence encodes a chimeric antigen receptor (CAR). In some instances, the CAR may comprise an extracellular domain, a hinge and a transmembrane domain. In other instances, the CAR may comprise an extracellular domain, a hinge domain, a transmembrane domain and one or more signaling domains. In some  
10 examples, the coding sequence for the CAR, without or with a signaling domain(s), may be placed between the first nucleotide encoding a signal peptide and the second nucleotide encoding one or more self-cleaving peptides (*e.g.*, 2A peptide). In some instances, the third nucleotide may encode a cytoplasmic signaling domain(s).

In some embodiments, the second nucleotide sequence encoding the one or more self-cleaving peptides (*e.g.*, 2A peptides) may be directly linked to the third nucleotide sequence  
15 encoding the polypeptide of interest. Alternatively, the second nucleotide sequence and the third nucleotide sequence may be connected via an intervening sequence, which is free of any in-frame coding sequence for a signal peptide.

In some embodiments, the nucleic acid is present in an expression cassette, comprising  
20 gene expression control sequences, for example, promoter, enhancer, and/or polyA signaling sequence. In some examples, the nucleic acid (*e.g.*, the expression cassette) is located in a vector such as an expression vector or as an RNA. In some examples, the vector (*e.g.*, the expression vector) is a plasmid or a viral vector. Exemplary viral vectors include, but are not limited to, a retroviral vector, a lentiviral vector, an adeno-associated viral vector, or an  
25 adenoviral vector.

In another aspect, the present disclosure provides a population of genetically engineered cells comprising the expression system provided herein and any of the nucleic acids described herein, which are exogenous to the parent cells for making the genetically engineered cells. In some embodiments, the population of genetically engineered cells  
30 comprise immune cells, tumor cells, islet cells, or stem cells. In some examples, the population of genetically engineered cells comprise genetically engineered immune cells, which may comprise T-cells, tumor infiltrating lymphocytes (TILs), NK cells, dendritic cells, macrophages, B cells, neutrophils, eosinophils, basophils, mast cells, myeloid- derived suppressor cells, or a combination thereof.

In some embodiments, the population of genetically engineered cells may comprise immune cells expressing a chimeric antigen receptor (CAR) and/or a cytokine antagonist, in addition to the polypeptide of interest, the production/secretion of which is reduced by the self-cleavable peptide (*e.g.*, 2A peptide)-mediated approach provided herein. In some examples, the cytokine antagonist may target IL-6, IL-1, GM-CSF, TNF, IFN- $\gamma$ , or a combination thereof. In some instances, the CAR and/or the cytokine antagonist may be encoded by the same exogenous nucleic acid disclosed above. Alternatively, the CAR and/or the cytokine antagonist may be encoded by one or more additional nucleic acids different from the exogenous nucleic acid. Any of the polypeptides or proteins of interest, including the cytokine antagonists, may be expressed as a fusion polypeptide comprising an immunoglobulin Fc domain or an albumin domain.

In another aspect, the present disclosure provides a pharmaceutical composition comprising any of the population of genetically engineered cells disclosed herein and a pharmaceutically acceptable carrier.

In another aspect, the present disclosure provides a method for treating a disease in a subject. The method comprises administering to a subject in need thereof an effective amount of the population of genetically engineered cells disclosed herein or pharmaceutical composition thereof. In some embodiments, the disease is an autoimmune disorder, a cancer, an infectious disease, an immune disorder, a fibrosis disease, or a senescence disease, such as liver fibrosis, atherosclerosis, or natural aging.

Also within the scope of the present disclosure is the population of genetically engineered cells (*e.g.*, immune cells) for use in treating any of the target diseases disclosed herein, as well as use of such genetically engineered cells for manufacturing a medicament for the intended therapeutic uses.

In another aspect, the present disclosure provides a method for preparing the genetically engineered cells disclosed herein. The method comprises delivering the nucleic acid comprising the signal peptide coding sequence, the self-cleavable peptide linker (*e.g.*, 2A peptide) coding sequence as disclosed herein, and the coding sequence for the polypeptide of interest, and optionally second and/or third nucleic acids to a population of cells to produce the genetically engineered cells that produce or secrete a low level of the polypeptide of interest, and optionally one or more of the additional polypeptides, such as a CAR and/or a cytokine antagonist as also disclosed herein.

The details of one or more embodiments of the invention are set forth in the description

below. Other features or advantages of the present invention will be apparent from the following drawings and detailed description of several embodiments, and also from the appended claims.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, which can be better understood by reference to the drawing in combination with the detailed description of specific embodiments presented herein.

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**FIG. 1** shows the secretion levels of human IL-2 expressed from expression cassettes with or without (WO) a coding sequence for a P2A- or P2A-T2A linker between the coding sequence of a signal peptide and coding sequence of the human IL-2 in mature form (with no N-terminal signal peptide).

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**FIG. 2** shows secretion levels of human IL-2 expressed from a lentiviral transfer vector comprising an expression cassette encoding, from N terminus to C-terminus, an anti-IFN- $\gamma$  scFv that has an N-terminal signal peptide and the human IL-2 in mature form having no N-terminal signal peptide (WO) or from a lentivirus transfer vector comprising an expression cassette encoding, from N terminus to C-terminus, the anti-IFN- $\gamma$  scFv that has an N-terminal signal peptide, a P2A- or P2A-T2A linker, and the human IL-2 in mature form (with no N-terminal signal peptide).

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**FIG. 3** shows secretion levels of human IL-10 expressed from a lentiviral transfer vector comprising an expression cassette encoding, from N terminus to C-terminus, an anti-IL-6 scFv that has an N-terminal signal peptide, a T2A peptide, a truncated EGFR fragment with an N-terminal signal peptide, an F2A peptide, an anti-IFN- $\gamma$  scFv that carries an N-terminal signal peptide, a P2A peptide and the human IL-10 in mature form (with no N terminal signal peptide) (designated as P2A), or expressed from a lentiviral transfer vector comprising a conventional expression cassette comprising a coding sequence for an anti-CD19 CAR with an N-terminal signal peptide, a T2A peptide linker, and a coding sequence of the human IL-10 with an N terminal signal peptide(designated as WO).

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**FIGS. 4A-4C** show the concentrations of cytokines in CAR T cells co-cultured with target cells. **FIGS. 4A-4C:** human T cells were activated and transduced with an anti-CD19-CAR-encoding lentivector construct of one of 1-9 described in **Example 3** below.

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**FIG. 5** shows the killing activity of CART cells with a different 2A linker between the transmembrane domain and CD3 $\zeta$  signaling domain.

FIGS.6A-6B show the change of body weight and the concentrations of IL10 in the mice post infusion with human T cells activated and transduced with a lentivector expressing certain cytokine elements. FIG. 6A: change of body weight. FIG. 6B: comparison of concentrations of IL10.

FIGS.7A-7B show the change of body weight and the concentrations of IL18 in the mice post infusion with human T cells activated and transduced with a lentivector expressing certain cytokine elements. FIG. 7A: change of body weight. FIG. 7B: comparison of concentrations of IL18.

FIGS.8A-8B show the change of body weight and the concentrations of IL15 in mice post infusion with human T cells activated and transduced with a lentivector expressing certain cytokine elements. FIG. 8A: change of body weight. FIG. 8B: comparison of concentrations of IL15.

FIG. 9 shows the concentrations of cytokines IL-18 (1) and IL-10 (2) in CART cells expressing IL18 and IL10 with insertion of a 2A peptide cocultured with target cells.

FIG. 10 shows the concentrations of IL18 (1) and IL10 (2) in mice infused with human T cells activated and transduced with a lentivector expressing IL-18 with a 2A linker between SP and IL18 followed by a 2A linker and IL-10.

## DETAILED DESCRIPTION OF THE INVENTION

Immune cells such as CAR-T cells, TCR-T cells or tumor infiltrated T cells (TILs) have shown promising therapeutic efficacy in disease treatment (*e.g.*, in cancer treatment). Such therapeutic immune cells may be modified by transgenes coding for suitable immune regulatory agents (*e.g.*, cytokines) to enhance the therapeutic potency of the immune cells. However, the therapeutic immune cells thus modified have been found to elicit severe toxicities, hampering their clinical applications. The present disclosure aims at developing potent and safe genetically engineered cells such as immune cells to solve this problem. More specifically, the present disclosure involves the placement of one or more self-cleavable peptide linkers (*e.g.*, 2A peptides) between a signal peptide and a polypeptide of interest, such as an immune regulatory agent, to reduce the production or secretion of the polypeptide of interest in cells (*e.g.*, immune cells) engineered to express such.

Secretion of a polypeptide from a cell or insertion of a polypeptide into the plasma membrane of a cell typically requires an N-terminal signal peptide for binding to a signal recognition particle (SRP), which directs transport of the nascent polypeptide into a secretory

pathway. Without the signal peptide, a polypeptide will not enter the secretory pathway and will not be processed properly for expression and/or secretion.

Self-cleaving peptides, such as P2A or T2A harboring the conserved sequence Asp-Val/Ile-Glu-X-Asn-Pro-Gly-Pro constitute a class of peptides capable of inducing ribosomal skipping effect during translation of a protein in host cells. The apparent cleavage is triggered by ribosomal skipping of the peptide bond between the proline (P) and glycine (G) at the C-terminal end of the 2A self-cleaving peptide. As such, 2A self-cleaving peptides are often used in expressing two separate polypeptides from one nucleic acid comprising two coding sequences, each encoding one polypeptide, linked by an in-frame coding sequence for a 2A self-cleaving peptide. Such a nucleic acid can generate a bicistronic mRNA transcript, which, *via* the self-cleaving activity of the encoded 2A self-cleaving peptide, produces two separate polypeptides in cells. However, the self-cleaving mediated by a 2A self-cleaving peptide is usually not complete, leading to the production of a low amount of fusion polypeptide comprising the two polypeptides connected by the 2A self-cleaving peptide.

Internal ribosome entry sites are sequences found in certain eukaryotic mRNAs that enable translation initiation by directly serving as a ribosome-binding target. It allows for producing multiple polypeptides from a single mRNA molecule.

The present disclosure is based, at least in part, on the discovery that inclusion of the coding sequence of one or more cleavable 2A peptide linkers between the coding sequence for a signal peptide and a coding sequence for a polypeptide of interest, which lacks an N-terminal signal peptide (in mature form), significantly reduces the production or secretion of the polypeptide of interest by host cells engineered to express such. This 2A self-cleaving peptide-mediated approach to control the production level of a polypeptide of interest by host cells can be used in producing potent and safe genetically engineered cells such as immune cells for therapeutic uses.

Accordingly, provided herein is an expression system, such as a self-cleavable 2A peptide-mediated expression system (*i.e.*, SP-2A expression system) for reducing the expression level of a polypeptide of interest in host cells (*e.g.*, genetically engineered immune cells), genetically engineered cells (*e.g.*, immune cells) carrying the system for reduced expression, and therapeutic uses of such genetically engineered cells.

## I. Expression Systems

In one aspect, the present disclosure provides an expression system that modulates the expression level of a polypeptide of interest (*e.g.*, a polypeptide targeted to the secretory

pathway).

In some embodiments, the expression system provided herein is an SP-2A expression system comprising a nucleic acid encoding a 2A self-cleaving peptide between a signal peptide (SP) and a polypeptide targeted to the secretory pathway. More particularly, the SP-2A expression system comprises a first nucleic acid, which comprises from 5' to 3': (1) a first nucleotide sequence encoding a signal peptide; (2) a second nucleotide sequence encoding one or more 2A self-cleaving peptides; and (3) a third nucleotide sequence encoding a polypeptide of interest. The polypeptide encoded by the third nucleotide sequence is free of an N-terminus signal peptide.

When the polypeptide of interest is expressed from the expression system provided herein, such as the SP-2A expression system in a population of cells, the polypeptide is secreted at a low level as compared with a population of counterpart cells of the same type comprising a nucleic acid encoding a counterpart polypeptide having a N-terminus signal peptide without the intervening 2A self-cleaving peptide. As used herein, the counterpart cells refer to cells that are otherwise identical to the cells comprising the SP-2A expression system except that the counterpart cells carry a regular expression system for the polypeptide of interest, *i.e.*, does not contain the intervening 2A peptide-coding sequence and encodes the precursor form of the polypeptide that carries a signal peptide at the N-terminus (the counterpart polypeptide).

#### A. 2A Self-Cleaving Peptide

In some instances, the second nucleotide sequence in the nucleic acid encodes one or more 2A self-cleaving peptides, also referred to herein as 2A elements or 2A peptides. A 2A self-cleaving peptide is an 18-22 amino acid peptide, which can induce the cleaving of a polypeptide in a cell during translation. When expressed from an mRNA, a 2A peptide induces peptide cleavage or ribosomal skipping effect during translation of the mRNA in a cell. Inclusion of a 2A element in the nucleic acid allows multiple polypeptide coding regions to be produced from a single mRNA molecule. Exemplary 2A elements include T2A elements, P2A elements, E2A elements, and F2A elements.

2A peptides share a core sequence motif of D-V/I-E-X-N-P-G-P (SEQ ID NO: 1) at the C-terminal end and are found in a wide range of viral families. Exemplary 2A member family amino acid sequences include the core sequence motif of SEQ ID NO: 1; a P2A peptide derived from Porcine teschovirus-1 (ATNFSLLKQAGDVEENPGP, SEQ ID NO:2); a T2A peptide derived from Thoseaasigna virus (EGRGSLLTCGDVEENPGP, SEQ ID NO: 3); an

F2A peptide derived from foot-and-mouth disease virus 18, (VKQTLNFDLLKLAGDVESNPGP; SEQ ID NO: 4); and an E2A peptide derived from Equine rhinitis A virus (QCTNYALLKLAGDVESNPGP, SEQ ID NO: 5) (Kim et al., *PLOS ONE* 6(4): e18556). 2A peptides have been characterized by different cleavage efficiencies.

5 Thus, nucleic acid sequences encoding the 2A peptides described herein may be selected based on a desired cleavage efficiency.

In the controlled expression system described herein, the greater the cleavage efficiency, the greater the reduction in production or secretion of the polypeptide. Conversely, the lower the cleavage efficiency, the greater the production or secretion of the polypeptide.

10 In some embodiments, the nucleic acid encoding a 2A peptide additionally encodes Gly-Ser-Gly at its 5' terminal end, which has been reported to improve cleavage efficiency (Szymczak, A. et al., (2004) *Nat. Biotechnol.* 22:589-594). Thus, in some embodiments the nucleic acid encodes a P2A peptide comprising the amino acid sequence, GSGATNFSLLKQAGDVEENPGP, SEQ ID NO:6); a T2A peptide comprising the amino acid  
15 sequence, GSGEGRGSLTTCGDVEENPGP (SEQ ID NO: 7); an F2A peptide comprising the amino acid sequence, GSGVKQTLNFDLLKLAGDVESNPGP (SEQ ID NO: 8); an E2A peptide comprising the amino acid sequence, GSGQCTNYALLKLAGDVESNPGP, (SEQ ID NO: 9); or a combined P2A-T2A peptide comprising e.g., the amino acid sequence, GSGATNFSLLKQAGDVEENPGPGGSGEGRGSLTTCGDVEENPGP (SEQ ID NO: 10).

20 In some embodiments, the 2A-encoded nucleic acid may be modified to alter a 2A amino acid sequence to provide improved expression or altered skipping/self-cleavage efficiencies (Liu et al. *Sci Rep.* 2017; 7:2193; Sharma et al., (2012) *Nucl. Acids Res.*, 40(7):3143-3151). In some embodiments, the nucleotide sequence encoding a 2A peptide may be codon-optimized without changing the encoded peptide sequence.

25 In some embodiments, the nucleic acid may encode a functional variant of a 2A peptide having a substantially similar structure and ability to facilitate skipping/self-cleavage activity. For example, the functional variant may comprise an amino acid sequence at least 85% (e.g., at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or more) identical to that of the native counterpart. The "percent identity" of two amino acid sequences is determined  
30 using the algorithm of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 87:2264-68, 1990, modified as in Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-77, 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. *J. Mol. Biol.* 215:403-10, 1990. BLAST protein searches can be performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to

the protein molecules of interest. Where gaps exist between two sequences, Gapped BLAST can be utilized as described in Altschul et al., *Nucleic Acids Res.* 25(17):3389-3402, 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used.

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### C. Polypeptides of Interest

The third polynucleotide sequence in the nucleic acid encodes a polypeptide of interest, optionally which can be a polypeptide intended for entry into the secretory pathway. Polypeptides for entry into the secretory pathway generally require a signal peptide. However, in accordance with the present disclosure, when the coding sequence of the signal peptide is separated from the polypeptide by a coding sequence of a 2A self-cleaving peptide, entry of the polypeptide (lacking a signal peptide) into the secretory pathway and the subsequent secretion has been significantly reduced.

As used herein, the term “polypeptide”, including the specific polypeptides described herein, encompass both naturally occurring molecules (including intact molecules and subunits thereof) and functional variants thereof. In some embodiments, the polypeptide encoded by the third nucleotide sequence of the SP-2A expression system and targeted for reduced secretion or membrane insertion is a blood factor, a hormone, or an immune regulatory agent. In some embodiments, the immune regulatory agent is a cytokine, soluble receptor, or chimeric antigen receptor (CAR). In some embodiments, the cytokine or soluble receptor may be immunostimulatory for treating tumors or microbial infections. In other embodiments, the cytokine or soluble receptor may antagonize the activity of certain immune regulatory agents in disease states where excessive inflammation is harmful (*e.g.*, autoimmune diseases).

In certain embodiments, the polypeptide encoded by the third nucleotide sequence is a polypeptide known or predicted to exhibit *in vivo* toxicity. Multiple cytokines have been revealed to exhibit significantly increased expression during CAR-T therapy (*e.g.*, IFN- $\gamma$ , IL-2, IL-6, IL-10, and IL-15). Therefore, in some embodiments, the nucleic acid of the SP-2A expression system encodes a similar cytokine for reduced secretion to promote or enhance T cell function, such as, interleukin 2 (IL-2), interleukin 7 (IL-7), interleukin 9 (IL-9), interleukin 10 (IL-10), interleukin 15 (IL-15), interleukin 12 (IL-12), interleukin 17 (IL-17), interleukin 18 (IL-18), interleukin 21 (IL-21), interleukin 23 (IL-23), interleukin 24 (IL-24), interleukin 27 (IL-27), interleukin 33 (IL-33), or CCL19.

Other exemplary cytokines or soluble receptors for inclusion in the SP-2A expression system include, but are not limited to interleukin 1 alpha (IL-1 $\alpha$ ), interleukin 1 beta (IL-1 $\beta$ ),

interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 13 (IL-13), interleukin 33 (IL-33), interleukin 35 (IL-35), interleukin 37 (IL-37), soluble interleukin-1 receptor type I (sIL-1RI), soluble interleukin 2 receptor alpha (sIL-2R $\alpha$ ), soluble IL-6 receptor (sIL6R), interferon  $\alpha$  (IFN- $\alpha$ ), interferon  $\beta$  (IFN- $\beta$ ), interferon  $\gamma$  (IFN- $\gamma$ ), Macrophage inflammatory proteins (e.g., MIP- $\alpha$  and MIP- $\beta$ ), Macrophage colony-stimulating factor 1 (CSF-1), leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), C-X-C motif chemokine ligand 10 (CXCL10), chemokine (C-C motif) ligand 5 (CCL5), eotaxin, monocyte chemoattractant protein 1 (MCP-1), monokine induced by gamma interferon (MIG), receptor for advanced glycation end-products (RAGE), c-reactive protein (CRP), angiopoietin-2, and von Willebrand factor (VWF); inflammatory growth factors (e.g., transforming growth factor alpha (TGF- $\alpha$ ), transforming growth factor beta (TGF- $\beta$ ), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), human growth factor (HGF), and fibroblast growth factor (FGF)); and cytotoxic molecules (e.g., perforin, granzyme, and ferritin).

In some embodiments, the polypeptide for reduced expression is an antagonist of a cytokine listed above. In certain embodiments, the antagonist of a cytokine is selected from the group consisting of anti-IL6, anti-GM-CSF, anti-IFN- $\gamma$ , anti-TNF, anti-IL-1, or IL-1RA.

In some embodiments, the polypeptide of interest is an immune regulatory agent for reduced expression, including naturally-occurring molecules or variants, and functional variants thereof. A functional variant has substantially similar structure and bioactivity as the native counterpart. For example, the functional variant may comprise an amino acid sequence at least 85% (e.g., at least 90%, at least 95%, at least 97%, at least 98% or above) identical to that of the native counterpart (as determined above). Alternatively, or in addition, the functional variant may contain one or more conservative amino acid residue substitutions relative to the native counterpart as described above.

In some examples, a functional variant may comprise one or more amino acid substitutions such as conservative amino acid substitutions. As used herein, a “conservative amino acid substitution” refers to an amino acid substitution that does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g., Molecular Cloning: A Laboratory Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or Current Protocols in Molecular Biology, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York.

Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D. Non-conservative substitutions may include amino acid pairs that are not contained within these groups.

5           In some instances, the cytokine provided herein is a variant of a native IL18, which can be engineered to reduce the binding to the IL18 binding protein. In other instances, the cytokine can be a variant of a native IL10, which can bind to the IL10 receptors as a monomer.

          In some embodiments the coding sequence of a polypeptide of interest as disclosed herein may be codon-optimized.

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#### C. Signal Peptide

          The signal peptide is encoded by the first nucleotide sequence in the nucleic acid. The first nucleotide sequence may encode a signal peptide corresponding to any secreted protein or membrane protein in eukaryotes, particularly humans. As such, the signal peptide may be  
15   native or heterologous to a polypeptide for reduced secretion. The signal peptide may be derived from a variety of proteins, including albumin, immunoglobulins, growth factors, hormones, enzymes, and coagulation factors. Exemplary signal peptides may be derived from the coding sequences of CD8, growth hormone, IL-2, albumin, antibody kappa light chain, and *Gausia* luciferase (from *e.g.*, *Gausiaprincipis*), the sequences of which are available in public  
20   sequence databases (*e.g.*, GenBank).

#### D. Nucleic Acid of the Expression Systems

          The expression system provided herein, *e.g.*, the SP-2A expression system comprises a nucleic acid, which comprises, from 5' to 3', a first nucleotide sequence encoding a signal  
25   peptide, a second nucleotide sequence encoding one or more 2A peptides, and a third nucleotide sequence encoding a polypeptide of interest as disclosed herein, which does not have its own N-terminal signal peptide.

          Nucleic acids encoding the polypeptides or proteins of interest described herein for expression in cells may be derived from any suitable species, *e.g.*, from a mammal such as  
30   mouse, rat, rabbit, pig, a non-human primate, or human. Naturally occurring sequences from various species are well known in the art and their sequences can be retrieved from a public gene database, such as GenBank.

          In some instances, the nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system) can be an expression cassette, which further comprises regulatory elements

controlling the expression of the polypeptide of interest. Exemplary regulatory elements include promoter sequences and terminator sequences (*e.g.*, polyA, GU-rich sequences), as well as enhancer sequences and binding sites for transcriptional factors.

A promoter, as used herein, refers to a nucleotide sequence (site) on a nucleic acid to which RNA polymerase can bind to initiate the transcription of the coding DNA (*e.g.*, for a cytokine antagonist) into mRNA, which will then be translated into the corresponding protein (i.e., expression of a gene). A promoter is considered to be “operably linked” to a coding sequence when it is in a correct functional location and orientation relative to the coding sequence to control (“drive”) transcriptional initiation and expression of that coding sequence (to produce the corresponding protein molecules). In some instances, the promoter described herein can be constitutive, which initiates transcription independent other regulatory factors. In some instances, the promoter described herein can be inducible, which is dependent on regulatory factors for transcription. Exemplary promoters include, but are not limited to ubiquitin, RSV, CMV, EF1 $\alpha$  and PGK1.

In certain embodiments, the nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system) is contained in a vector (*e.g.*, an expression vector) or an RNA for delivery into cells. In certain embodiments, the nucleic acid comprises an SP-2A expression cassette. In some examples, the vector may be a viral vector, for example, a lentiviral vector, a retroviral vector, an adeno-associated vector, or an adenoviral vector.

In some embodiments, the nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system) may further encode one or more additional polypeptides whose expression or secretion is directly linked to a signal peptide for “normal” expression or secretion without an intervening 2A element for expression of the additional polypeptide. In some embodiments, the nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system) may include multiple 2A elements and/or internal ribosome entry sequences (IRES) to facilitate expression of multiple polypeptides from the same nucleic acid or another nucleic acid independently delivered transiently or stably to the cells. In some embodiments, the additional nucleotides encode other therapeutic polypeptides and/or marker polypeptides for tracking polypeptide expression. In some embodiments, the nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system) may include additional signal peptides for secreting other polypeptides with N-terminal signal peptides without intervening 2A elements.

IRES is an RNA element that allows for translation initiation in a cap-independent manner. Translation initiation in eukaryotic cells typically is dependent on the 5' cap of an mRNA molecule, wherein the translation initiation complex forms and ribosomes engage the

mRNA. An IRES element, typically located between two open read frames (ORFs), allows ribosomes to engage the mRNA and begin translation independently of the 5' cap. IRES elements are often used in molecule biology to co-express multiple genes under the control of the same promoter, thereby mimicking a polycistronic mRNA.

5 Any naturally occurring IRES elements, such as IRES elements of viral origins and IRES elements in cellular mRNAs can be used in the IRES-mediated expression system provided herein. Exemplary viral IRES elements include, but are not limited to, piconavirus IRES, aphthovirus IRES, Kaposi's carcoma-associated herpesvirus (KSHV) IRES, hepatitis A IRES, hepatitis C IRES, pestivirus IRES, cricavirus IRES, rhopalosiphumpadi virus  
10 IRES, and Marek's disease virus IRES. Exemplary IRES elements in cellular mRNAs include, but are not limited to, IRES elements from growth factor mRNAs, for example, fibroblast growth factor IRES (*e.g.*, FGF-1 IRES or FGF-2 IRES), platelet-derived growth factor B IRES(PDGF/c-sis IRES), vascular endothelial growth factor IRES(VEGF IRES), and insulin-like growth factor 2 IRES(IGF-II IRES), from transcription factor mRNAs, for example, from  
15 antennapedia, ultrabithorax, MYT-2, NF- $\kappa$  B repressing factor NRF, AML1/RUNX1, or Gtx homeodomain protein, from translation factor mRNAs, for example, from eukaryotic initiation factor 4G (eIF4G)a, eukaryotic initiation factor 4G1 (eIF4G1)a, or eukaryotic translation initiation factor 4 gamma 2 (EIF4G2,DAP5), from oncogenes, for example, c-myc, L-myc, Pim-1, Protein kinase p58PITSLRE, p53, from transporter and receptor mRNAs, for example,  
20 cationic amino acid transporter (SLC7A1,Cat-1), nuclear form of Notch 2, voltage-gated potassium channel, or muscarinic M2 receptor Muscarinic acetylcholine receptor M2, from apoptosis activators or inhibitors such as apoptotic protease activating factor (Apaf-1), X-linked inhibitor of apoptosis (XIAP), HIAP2, Bcl-xL, or Bcl-2, from neuronal dendrite-located protein mRNAs such as activity-regulated cytoskeletal protein (ARC),  $\alpha$ -subunit of calcium  
25 calmodulin dependent kinase II dendrin, microtubule-associated protein 2 (MAP2), neurogranin (RC3), or amyloid precursor protein. Additional examples include IRES elements from immunoglobulin heavy chain binding protein (BiP), Heat shock protein 70, $\beta$ -subunit of mitochondrial H<sup>+</sup>-ATP synthase, Ornithine decarboxylase, connexins 32 and 43, HIF-1 $\alpha$ , or APC. See, *e.g.*, Hellen CU, Sarnow P (July 2001). "Internal ribosome entry sites in eukaryotic  
30 mRNA molecules". *Genes & Development*. 15 (13): 1593–1612, the relevant disclosures of which are incorporated by reference for the subject matter and purpose referenced herein.

In some instances, a functional variant of a native IRES may be used in the expression system provided herein, the sequence of which is derived from encephalomyocarditis virus

(EMCV).

Sequences of the exemplary IRES elements are available in public sequence databases (*e.g.*, GenBank).

In some embodiments, the nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system) may include a fourth nucleotide sequence encoding one or more polypeptides, optionally which are different from the polypeptide expressed from the engineered cells. In some embodiments, the fourth nucleotide sequence is located between the first nucleotide sequence encoding the signal peptide and the second nucleotide sequence encoding one or more 2A self-cleaving peptides. In other embodiments, the fourth fifth nucleotide sequence is located 5' to the first nucleotide sequence encoding the signal peptide and/or 3' to the third nucleotide encoding the polypeptide, and wherein optionally the fourth nucleotide sequence comprises a ribosomal skipping site, a promoter sequence, a coding sequence for a protease cleavage site, an mRNA splicing unit, or an internal ribosomal entry site. In some examples, the ribosomal skipping site encodes a 2A self-cleaving peptide.

Additionally, any of the vectors comprising exogenous nucleic acids described herein may further contain, for example, some or all of the following: a selectable marker gene, such as the neomycin gene for selection of stable or transient transfectants in mammalian cells; enhancer/promoter sequences from the immediate early gene of human CMV for high levels of transcription; transcription termination and RNA processing signals from SV40 for mRNA stability; SV40 polyoma origins of replication and ColE1 for proper episomal replication; versatile multiple cloning sites; and T7 and SP6 RNA promoters for in vitro transcription of sense and antisense RNA. Suitable methods for producing vectors containing transgenes are well known and available in the art. Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory Press.

A nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system) according to the present disclosure may encode a plurality of signal peptides and/or a plurality of 2A elements separating a plurality of polypeptide coding regions, including non-secreted proteins (*e.g.*, marker proteins) and secreted proteins with or without N-terminal signal peptides. A polypeptide downstream of an N-signal peptide adjoined to an upstream polypeptide with a 2A element positioned between the two polypeptides will be largely expressed intracellularly due to 2A peptide cleavage or ribosome skipping but may be secreted at low levels as a fusion protein with the other polypeptide.

In one embodiment, the nucleic acid encodes a fusion protein comprising, from N- to C-terminal: (1) an N-terminal signal peptide (SP); (2) a 2A linker peptide; and (3) a

polypeptide for intracellular expression and reduced expression or secretion from the N-terminal SP.

In another embodiment, the nucleic acid encodes a fusion protein comprising, from N- to C-terminal: (1) an N-terminal SP; (2) a first polypeptide for normal expression or secretion  
5 from the N-terminal SP; (3) a 2A linker peptide; and (4) a second polypeptide for intracellular expression and reduced expression or secretion from the N-terminal SP as a fusion protein with the first polypeptide.

In another embodiment, the nucleic acid encodes a fusion protein comprising, from N- to C-terminal: (1) an N-terminal SP; (2) a first 2A linker peptide; (3) a first polypeptide, the  
10 expression and/or secretion guided by the N-terminal SP would be reduced; (4) a second 2A linker peptide; and (5) a second polypeptide for intracellular expression and reduced expression or secretion from the N-terminal SP as a fusion protein with the first polypeptide.

In another embodiment, the nucleic acid encodes a fusion protein comprising, from N- to C-terminal: (1) a first N-terminal SP; (2) a first polypeptide for normal expression or  
15 secretion from the first N-terminal SP; (3) a first 2A linker peptide; (4) a second N-terminal SP; (5) a second polypeptide for normal expression from the second N-terminal SP; (6) a second 2A linker peptide; and (7) a third polypeptide for intracellular expression and reduced expression or secretion from the second N-terminal SP as a fusion protein with the second polypeptide.

In another embodiment, the nucleic acid encodes a fusion protein comprising, from N- to C-terminal: (1) a first N-terminal SP; (2) a first polypeptide for normal expression or  
20 secretion from the first N-terminal SP; (3) a first 2A linker peptide; (4) a second polypeptide for intracellular expression and reduced expression or secretion from the first N-terminal SP as a fusion protein with the first polypeptide; (5) a second 2A linker peptide; (6) a second N-terminal SP; (7) a third polypeptide for normal expression from the second N-terminal SP; (8)  
25 a third 2A linker peptide; and (9) a fourth polypeptide for intracellular expression and reduced expression or secretion from the second N-terminal SP as a fusion protein with the third polypeptide.

In some embodiments, the expression system provided herein comprise a nucleic acid  
30 encoding a chimeric antigen receptor polypeptide, which comprises an extracellular antigen binding domain, a hinge/transmembrane domain, and optionally one or more cytoplasmic signaling domains. In some instances, a coding sequence of a 2A peptide can be placed within a coding sequence for a CAR polypeptide to control the expression level of the intact CAR fragment upstream to the 2A peptide and the CAR fragment downstream to the 2A peptide. In

some instances, the CAR polypeptide may contain only one cytoplasmic signaling domain and the 2A peptide can be placed between the transmembrane domain and the cytoplasmic signaling domains. In other instances, the CAR polypeptide may contain two or more cytoplasmic signaling domains and the 2A peptide may be placed between the transmembrane domain and the immediate downstream cytoplasmic signaling domain. Alternatively, the 2A peptide can be placed between two adjacent cytoplasmic signaling domains. More efficient cleavage will result in less amount of the cytoplasmic signaling domain(s) after the 2A linker retained with the whole CAR, therefore driving less downstream signaling elicited by the cytoplasmic signaling domain(s) after the 2A linker to avoid over-signaling in the engineered cells.

## II. Genetically Engineered Cells

One aspect of the present disclosure provides genetically engineered cells (*e.g.*, genetically engineered immune cells, stem cells, tumor cells, islet cells, etc.) comprising a nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system). In some embodiments, the genetically engineered cells comprise immune cells.

### A. Immune Cells

Any immune cells may be used to engineer the cells described herein. In some embodiments, an immune cell can be derived, for example without limitation, from a stem cell. The stem cells can be adult stem cells, non-human embryonic stem cells, more particularly non-human stem cells, cord blood stem cells, progenitor cells, bone marrow stem cells, induced pluripotent stem cells, totipotent stem cells or hematopoietic stem cells. In other embodiments, the immune cell is derived from the differentiation of a population of induced pluripotent cells (iPSCs).

Useful immune cells for making the engineer the cells disclosed herein may be T-cells, NK cells, tumor infiltrating lymphocytes, dendritic cells, macrophages, B cells, neutrophils, eosinophils, basophils, mast cells, myeloid-derived suppressor cells, mesenchymal stem cells, precursors thereof, or combinations thereof. The T-cells may be selected from the group consisting of inflammatory T-lymphocytes, cytotoxic T-lymphocytes, regulatory T-lymphocytes or helper T-lymphocytes. In some embodiments, the T-cells can be derived from the group consisting of CD4+ T-lymphocytes and CD8+ T-lymphocytes. In one example, the immune cell is a human immune cell. In some embodiments, the human immune cells are CD34+ or CD45+ cells.

In some embodiments, the immune cells may be harvested directly from a subject, e.g., a human subject. The cells are genetically modified as described herein and the genetically engineered immune cells are infused back into the same subject, for example, in a CAR-T cell therapy. In this case, the genetically engineered immune cells are autologous to the subject receiving the CAR-T cell therapy. In another embodiment, the immune cells may be harvested directly from a donor subject, modified, and the genetically engineered immune cells are infused into a recipient subject in need of therapy, e.g., a CAR-T cell therapy. The donor immune cells optionally are HLA-matched with the recipient subject, i.e., the cells are allogeneic to the recipient subject. In some embodiments, the immune cells are harvested from the peripheral blood of the subject, and expanded *in vitro* with genetical modification(s) as disclosed herein.

#### B. Reduced Expression or Secretion of Polypeptides of Interest

The engineered immune cells express or secrete the polypeptide of interest (e.g., immune regulatory agent) from the nucleic acid of the expression system provided herein (e.g., the SP-2A system), which may be delivered transiently or stably into the immune cells. For example, in some instances, the nucleic acid of the SP-2A expression system or expression cassette thereof may be stably incorporated into the genome of an immune cell. Alternatively, an expression vector comprising the expression cassette may exist extrachromosomally (e.g., episomally).

In some embodiments, the 2A element(s) are configured to reduce of the polypeptide (e.g., immune regulatory agent) by more than 20%, more than 50%, more than 80%, more than 85%, more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99%, more than 99.5%, more than 99.9%, more than 99.99%, or more compared to the level of secretion obtained from a control nucleic acid encoding the polypeptide with a signal peptide at the 5' terminus without a 2A element therebetween.

#### C. Expression of Additional Exogenous Proteins

In some embodiments, an immune cell comprising the expression system provided herein (e.g., the SP-2A system) may further encode at least one additional exogenous nucleic acid encoding one or more additional proteins whose expression or secretion is directly linked to a signal peptide for "normal" expression or secretion without an intervening 2A element for expression of the additional polypeptide. In some embodiments, the additional proteins are

expressed or secreted from the nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system). In other embodiments, the additional protein is expressed or secreted from a second and/or third exogenous nucleic acid introduced into the immune cell. As used herein, “an exogenous nucleic acid” refers to a nucleic acid that is not native to the host cells (*e.g.*, the immune cells for producing the genetically engineered immune cells). The exogenous nucleic acid carries one or more transgene(s) encoding the polypeptide or protein of interest disclosed herein and can be delivered into the host cells via a conventional method to produce genetically engineered immune cells comprising the exogenous nucleic acid and capable of expressing the immune regulatory agents encoded by the transgene(s). In some instances, the transgene carried by the exogenous nucleic acid may have an endogenous counterpart, *i.e.*, an endogenous gene encoding the same polypeptide. In other instances, the transgene may be distinct from any nucleic acid naturally present in the cell.

In some embodiments, the first or second exogenous nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system) is transiently or stably delivered into immune cells for expression or secretion of a polypeptide selected from the group consisting of immune modulating agents (*e.g.*, antibodies, immune ligands or receptors, or cytokines), chimeric antigen receptors (CARs), blood factors, hormones, or a combination thereof. In some embodiments, the additional polypeptide is encoded by the first exogenous nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system) for reduced expression. In some embodiments, the additional polypeptide is encoded by the first exogenous nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system) for normal or reduced expression without or with an intervening 2A element. In other embodiments, an additional polypeptide is encoded by a second or third exogenous nucleic acid for normal or reduced expression or secretion without or with an intervening 2A element.

In some embodiments, the additional polypeptide is a polypeptide or cytokine described in section I. In other embodiments, the additional polypeptide encodes a cytokine antagonist. In certain embodiments, the cytokine antagonist targets an immune regulatory agent associated with excessive inflammation, including those associated with cytokine release syndrome (CRS), such as IL-1, IL-6, IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$ , IL-8 (CXCL8), IL-10, GM-CSF, MIP-1 $\alpha/\beta$ , MCP-1 (CCL2), CXCL9, and CXCL10. In some embodiments, the cytokine antagonist is an scFv. In certain embodiments, the scFv is an anti-IL-6 scFv, an anti-IFN- $\gamma$  scFv, an anti-GM-CSFscFv, an anti-TNF $\alpha$  scFv, an anti-IL-17scFv, an anti-IL-10scFv, or an anti-IL-1 scFv. In certain embodiments, the anti-IFN- $\gamma$  scFv comprises the amino acid sequence of SEQ ID NO: 16.

In some embodiments, the additional polypeptide is a checkpoint inhibitor, including but not limited to anti-PD1/PDL1, anti-LAG3, anti-TIGIT, anti-TIM3, and anti-CTLA.

In some embodiments, the additional polypeptide is a hormone, including but not limited to insulin, growth hormone, including naturally-occurring molecules and functional variants thereof. In some embodiments, the additional polypeptide is human insulin comprising an amino acid sequence of SEQ ID NOs: 11 and 12. In certain embodiments, the human insulin polypeptide is encoded by a single chain insulin comprising the amino acid sequence of SEQ ID NO: 13 or a single chain insulin comprising the amino acid sequences of SEQ ID NOs: 11 and 12 joined to one another via a suitable linker, such as a linker comprising the amino acid sequence of SEQ ID NO: 14. In some embodiments the additional polypeptide is human growth hormone comprising the amino acid sequence of SEQ ID NOs: 15. *See*

**Table 1.**

In some embodiments, the additional polypeptide is a chimeric artificial receptor (CAR) with an N-terminal signal peptide and transmembrane domain for insertion into the cell membrane of an immune cell, such as a T cell or natural killer (NK) cell. In certain embodiments, the CAR is encoded by the first exogenous nucleic acid for reduced expression or normal expression. In other embodiments, the CAR is encoded by the second exogenous nucleic acid. In yet other embodiments, the CAR is expressed by a different population of immune cells, such as T cells or natural killer (NK) cells (e.g., CAR-T or CAR-NK cells).

A chimeric antigen receptor (CAR) is engineered to recognize and bind to an antigen expressed by undesired cells, for example, disease cells such as cancer cells. A T cell that expresses a CAR polypeptide is referred to as a CAR-T cell. CARs are able to redirect T-cell specificity and reactivity toward a selected target in a non-MHC-restricted manner. The non-MHC-restricted antigen recognition gives CAR-T cells the ability to recognize an antigen independent of antigen processing, thus bypassing a major mechanism of tumor escape. Moreover, when expressed on T-cells, CARs advantageously do not dimerize with endogenous T-cell receptor (TCR) alpha and beta chains.

There are various generations of CARs, each of which contains different components. First generation CARs join an antibody-derived scFv to the CD3 zeta ( $\zeta$  or  $z$ ) intracellular signaling domain of the T-cell receptor through hinge and transmembrane domains. Second generation CARs incorporate an additional co-stimulatory domain, e.g., CD28, 4-1BB (41BB), or ICOS, to supply a costimulatory signal. Third-generation CARs contain two costimulatory domains (e.g., a combination of CD27, CD28, 4-1BB, ICOS, or OX40) fused with the

TCRCD3 $\zeta$  chain. Maude *et al.*, *Blood*. 2015; 125(26):4017-4023; Kakarla and Gottschalk, *Cancer J.* 2014; 20(2):151-155). Any of the various generations of CAR constructs is within the scope of the present disclosure.

In some instances, a CAR can be a fusion polypeptide comprising an extracellular  
 5 antigen binding domain that recognizes a target antigen (*e.g.*, a single chain variable fragment (scFv) of an antibody or other antibody fragment) and an intracellular domain comprising a signaling domain of the T-cell receptor (TCR) complex (*e.g.*, CD3 $\zeta$ ) and, in most cases, a co-stimulatory domain. (Enblad *et al.*, *Human Gene Therapy*. 2015; 26(8):498-505). A CAR construct may further comprise a hinge and transmembrane domain between the extracellular  
 10 domain and the intracellular domain.

The CAR disclosed herein comprises an antigen binding extracellular domain, which may be a single-chain variable fragment (scFv) or a single domain antibody (*e.g.*, VHH). The antigen-binding extracellular domain may be specific to a target antigen of interest, for example, a pathologic antigen such as a tumor-associated antigen. Exemplary tumor-  
 15 associated antigens include, but are not limited to, AFP, ALK, BAFF-R, BAGE proteins,  $\beta$ -catenin, BCMA, bcr-abl, BRCA1, BORIS, CA9, carbonic anhydrase IX, caspase-8, CCR5, CD2, CD3, CD5, CD7, CD19, CD20, CD22, CD30, CD 33, CD38, CD70, CD123, CD133, CD171, CDK4, CEA, CEACAM5, CEACAM6, CS1, Claudin 18.2, cyclin-B1, CYP1B1, desmoglein (Dsg3), EGFR, EGFRvIII, ErbB2/Her2, ErbB3, ErbB4, ETV6-AML, EpCAM,  
 20 EphA2, FAP, 5T4, FOLR1, Fra-1, FSHR, GAGE proteins (*e.g.*, GAGE-1, -2), GD2, GD3, GloboH, glypican-3, GM3, gp100, GPC3, HER-2, HLA/B-raf, HLA/k-ras, HLA/MAGE-A3, hTERT, Lewis Y, LMP2, MAGE proteins (*e.g.*, MAGE-1, -2, -3, -4, -6, and -12), MART-1, mesothelin, ML-IAP, Muc1, Muc2, Muc3, Muc4, Muc5, Muc16 (CA-125), MUM1, NA17, NKG2D, NY-BR1, NY-BR62, NY-BR85, NY-ESO1, OX40, p15, p53, PAP, PAX3, PAX5,  
 25 PCTA-1, PLAC1, PRLR, PRAME, PSCA, PSMA (FOLH1), RAGE proteins, Ras, RGS5, Rho, ROR1, SART-1, SART-3, Steap-1, Steap-2, STn, survivin, TAG-72, TGF- $\beta$ , TMPRSS2, Tn, TRP-1, TRP-2, tyrosinase, VEGF-RII, and uroplakin-3.

The CAR polypeptide disclosed herein further contains a transmembrane domain, which can be a hydrophobic alpha helix that spans the membrane. As used herein, a  
 30 “transmembrane domain” refers to any protein structure that is thermodynamically stable in a cell membrane, preferably a eukaryotic cell membrane. The transmembrane domain can provide stability of the CAR containing such. In some embodiments, the transmembrane domain of a CAR as provided herein can be a CD8 transmembrane domain. In other

embodiments, the transmembrane domain can be a CD28 transmembrane domain.

Further, the CAR polypeptide may comprise a hinge domain located between an extracellular domain (comprising the antigen binding domain) and a transmembrane domain of a CAR, or between a cytoplasmic domain and a transmembrane domain of the CAR. A hinge domain can be any oligopeptide or polypeptide that functions to link the transmembrane domain to the extracellular domain and/or the cytoplasmic domain in the polypeptide chain. A hinge domain may function to provide flexibility to the CAR, or domains thereof, or to prevent steric hindrance of the CAR, or domains thereof.

CAR constructs contain one or more intracellular signaling domains (*e.g.*, CD3 $\zeta$ , and optionally one or more co-stimulatory domains), which are the functional end of the receptor. Following antigen recognition, receptors cluster and a signal is transmitted to the cell. CD3 $\zeta$  is the cytoplasmic signaling domain of the T cell receptor complex. CD3 $\zeta$  contains three (3) immunoreceptor tyrosine-based activation motifs (ITAMs), which transmit an activation signal to the T cell after the T cell is engaged with a cognate antigen. In many cases, CD3 $\zeta$  provides a primary T cell activation signal but not a fully competent activation signal, which requires a co-stimulatory signal. In other embodiments, the CAR polypeptides disclosed herein may comprise one or more signaling domains from other immune receptors, such as DAP10, DAP12, immunoglobulins, and FcR.

In some embodiments, the CAR polypeptides disclosed herein may further comprise one or more co-stimulatory signaling domains. For example, the co-stimulatory domains of CD28 and/or 4-1BB may be used to transmit a full proliferative/survival signal, together with the primary CD3 $\zeta$ -mediated signaling via its three ITAM motifs ( $\zeta$ a,  $\zeta$ b and  $\zeta$ c). In some examples, the CAR disclosed herein comprises a CD28 co-stimulatory molecule. In other examples, the CAR disclosed herein comprises a 4-1BB co-stimulatory molecule.

#### D. Method of Producing Genetically Engineered Immune Cells

The genetically engineered immune cells disclosed herein may be prepared by conventional methods (*e.g.*, recombinant technology) or disclosures provided herein. For example, the exogenous nucleic acids encoding the immune regulatory agents or other polypeptide disclosed herein can be delivered into parent immune cells via routine methods to produce the genetically engineered immune cells expressing the immune regulatory agents and optionally other polypeptide disclosed herein. The genetically engineered immune cells comprising a nucleic acid of the expression system provided herein (*e.g.*, the SP-2A system)

may be engineered to transiently or stably express or secrete a polypeptide of interest at a reduced level or normal level. In some instances, the coding sequence of the polypeptide or immune regulatory agent is integrated into the genome of the cell. In some instances, the coding sequence of one or more polypeptides is not integrated into the genome of the cell.

5       The exogenous nucleic acid(s) encoding one or more polypeptide(s) or interest (or immune regulatory agents) as described herein may be introduced into immune cells by viral transduction, non-viral transduction, or by gene editing into suitable target sites of interest. In certain embodiments, genetically engineered immune cells expressing immune regulatory agents, such as TNF- $\alpha$  may be administered to subjects with solid tumors in view of systemic  
10       toxicities known to be associated with their use.

          In some instances, the nucleic acids are delivered to the immune cells by viral transduction using a viral expression vector or recombinant viral particle. A “vector”, as used comprises any nucleic acid (DNA or RNA) or expression cassette capable of facilitating the transfer of a nucleic acid molecule into B (and T) cells. In general, vectors include, but are not  
15       limited to, plasmids, phagemids, viral vectors, viral particles, and other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of a target nucleotide sequence. A vector for use herein includes a nucleic acid molecule (*e.g.*, a DNA or RNA molecule) comprising a nucleotide sequence encoding a protein described as herein, which may optionally include one or more suitable regulatory elements operably linked  
20       to provide constitutive or tissue-specific (*e.g.*, B cell specific) expression, including but not limited to promoters, enhancers, 5' and 3' untranslated regions (UTRs), insulators, polyadenylation signals, as well as internal ribosome entry sites (IRES) and/or 2A peptide for expressing more than one gene from a single promoter. In certain embodiments, a vector may include nucleic acid(s) encoding one or more immune regulatory agents, wherein the  
25       recombinant vector is designed for site specific chromosomal integration (and/or gene replacement/disruption) by gene editing (*e.g.*, CRISPR). In some embodiments, the recombinant vector includes exogenous regulatory elements for driving expression in target cells. In other embodiments, the recombinant vector does not contain exogenous regulatory elements, but is designed for site-specific integration (and/or gene replacement/disruption) into  
30       chromosomal sites so that expression is driven by native regulatory agents present in the chromosome.

          A vector for viral transduction contains elements derived from a viral genome (naturally occurring or modified) and can be used to deliver genetic materials (*e.g.*, a

transgene) into suitable host cells. Viral vectors may be based on non-cytopathic eukaryotic viruses in which nonessential genes have been replaced with a target nucleotide sequence. In some embodiments, a non-cytopathic virus, such as a retrovirus (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Non-limiting examples of viral vectors include, but are not limited to, retroviral vectors (e.g., lentiviral vectors or gammaretroviral vectors), adeno-associated viral vectors (AAV), adenoviral vectors, and hybrid vectors (containing components from different viral genomes). Additional examples of viral vectors are provided in US Patent No. 5,698,443, US Patent No. 5,650,309, and US Patent No. 5,827,703, the relevant disclosures of each of which are herein incorporated by reference for the purpose and subject matter referenced herein.

In some embodiments, a retroviral vector (e.g., lentivirus-based vector) can be used to introduce the exogenous nucleic acid(s) into the immune cells disclosed herein. Preferably, the retroviruses (RVs) are replication-defective (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Retrovirus expression systems are well established and known to those skilled in the art for high-efficiency transduction of genes *in vitro*, *ex vivo*, and *in vivo*. Standard protocols for producing replication-defective RVs (including the steps of incorporating exogenous genetic material into retroviral plasmid vectors, transfecting and encapsidating retroviral plasmid vectors using retroviral packaging cell lines to produce recombinant RV particles, collecting recombinant RV particles from tissue culture media, and infecting target cells with the recombinant RV particles) are known in the art. Lentivirus vectors are particularly useful for producing engineered immune cells in view of the natural tropism of lentiviruses for immune cells. In some embodiments, a lentivirus vector is used for delivering immune regulatory agent-encoded nucleic acids into immune cells (and/or CAR-encoded nucleic acids into T cells).

In other embodiments, an AAV vector may be used to introduce the exogenous nucleic acid(s) into the immune cells disclosed herein. Adeno-associated viruses (AAVs) are small viruses which can, in certain instances, integrate site-specifically into the host genome. Inverted terminal repeats (ITRs) are present flanking the AAV genome and/or a transgene of interest and serve as origins of replication. Preferably, the AAVs are replication-defective (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). AAV expression systems are well established and known to those skilled in the art for high-efficiency transduction of genes *in vitro*, *ex vivo*, and *in vivo*. Standard protocols for producing replication-defective AAVs (including the steps of incorporating

exogenous genetic material into AAV plasmid vectors, transfecting and encapsidating AAV plasmid vectors using AAV packaging cell lines (expressing rep and cap) to produce recombinant AAV particles, collecting recombinant AAV particles from tissue culture media, and infecting target cells with the recombinant AAV particles) are known in the art.

- 5 Depending on the packaging line used, surface receptors on capsids in the recombinant AAV particles define various AAV serotypes that can determine the target organs to which the capsids will primarily bind to and most efficiently infect. There are twelve currently known human AAV serotypes. In some embodiments, an AAV serotype 6 (AAV) vector is used for delivering immune regulatory agent-encoded nucleic acids into immune cells (and/or CAR-
- 10 encoded nucleic acids into T cells). In some embodiments, the cells are transduced with a replication-defective AAV.

- In some embodiments, the non-viral plasmid vector may be complexed with an agent, such as a liposome or poloxamer, for transfection into immune cells (and/or T cells) using standard methods known in the art, such as lipofection. In other embodiments, the non-viral
- 15 plasmid vector may be introduced into cells via electroporation. In some embodiments the immune cells are activated prior to and after transfection with a plasmid vector or expression vector, or infection with a recombinant virus particle. In some embodiments, the cells are transfected or infected in the presence of an apoptosis inhibitor.

- In some embodiments, the nucleic acid or vector is delivered into immune cells via
- 20 encapsulation into lipid nanoparticles. As used herein, the term “lipid nanoparticle” or “LNP” refers to a particle comprising one or more lipids. Lipid nanoparticles include, but are not limited to, liposomes and micelles. Any of a number of lipids may be present, including cationic lipids, ionizable lipids, anionic lipids, neutral lipids, amphipathic lipids, conjugated lipids (*e.g.*, PEGylated lipids), and/or structural lipids (*e.g.*, sterols). Such lipids can be used
- 25 alone or in combination. In some embodiments, the nucleic acids encoding the immune regulatory agents may be mRNAs delivered into immune cells by electroporation or encapsulation into lipid nanoparticles according to methods known to those skilled in the art.

- A vector may also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to
- 30 be transfected or infected through viral vectors. In some embodiments, the selectable marker may be carried on a separate piece of DNA and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate transcriptional control sequences to enable expression in the host cells. Examples of selective markers include a dihydrofolate reductase gene and a neomycin resistance gene for eukaryotic cell culture, and a

tetracycline resistance gene and an ampicillin resistance gene for culture of *E. coli* and other bacteria. By use of such selection markers, it can be confirmed whether the polynucleotide encoding the polypeptide(s) of the present invention has been transferred into the host cells and then expressed without fail.

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### III. Therapeutic Applications

In some embodiments, immune cell populations comprising the SP-2A expression cassettes described herein may be used in an adoptive immune cell therapy for treating a target disease, such as cancer, an immune disorder, an infectious disease, a fibrosis disease, or a senescence disease, optionally wherein the senescence disease is liver fibrosis, atherosclerosis, or natural aging. In some embodiments, the method of adoptive immune cell therapy may include co-introduction of these immune cell populations with a second population of CAR-expressing immune cells. In other embodiments, vectors comprising an SP-2A expression cassette, including recombinant virus vectors (*e.g.*, replication defective retrovirus or AAV particles) may be used in a gene therapy for preventing or treating a disease, such as cancer, an immune disorder, an infectious disease, a fibrosis disease, or a senescence disease. Due to the expression in immune cells of the polypeptide(s) from the SP-2A expression cassette and/or second exogenous nucleic acid, the therapeutic uses of such would be expected to provide reduced toxicities associated with gene delivery or conventional adoptive immune cell therapy while achieving the similar or better therapeutic effects. In some embodiments, the reduced toxicities would be attributed to reduced inflammatory cytokine production and/or signaling by both the immune cells used in adoptive immune cell therapy and endogenous immune cells of the recipient, which can be activated by the infused immune cells.

To practice the therapeutic methods described herein, an effective amount of the any of the modified immune cell population(s) or vectors comprising the expression system provided herein (*e.g.*, the SP-2A system) may be administered to a subject in need of treatment via a suitable route (*e.g.*, intravenous infusion). The immune cell population may be mixed with a pharmaceutically acceptable carrier to form a pharmaceutical composition prior to administration, which is also within the scope of the present disclosure. The immune cells may be autologous to the subject in need of the treatment and modified to reduce expression or secretion of one or more target proteins, cytokines, cytokine antagonists and/or exogenous CAR constructs for administration to the same subject. Administration of autologous cells to a subject may result in reduced rejection of the immune cells as compared to administration of non-autologous cells. Alternatively, the immune cells can be allogenic cells, *i.e.*, the cells are

obtained from a subject and modified as described herein and then administered to a different subject of the same species. For example, allogenic immune cells may be derived from a human donor and administered to a human recipient who is different from the donor.

The subject to be treated may be a mammal (*e.g.*, human, mouse, pig, cow, rat, dog, guinea pig, rabbit, hamster, cat, goat, sheep, or monkey). The subject may be suffering from cancer, an infectious disease, an immune disorder, a fibrosis disease, or a senescence disease. Exemplary cancers include but are not limited to hematologic malignancies (*e.g.*, B-cell acute lymphoblastic leukemia, chronic lymphocytic leukemia, and multiple myeloma) and solid tumor cancers. Exemplary hematological malignancies include, but are not limited to, leukemia, lymphoma, or multiple myeloma. Exemplary leukemias include chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), and chronic myelogenous leukemia (CML). Exemplary lymphomas include mantle cell lymphoma, non-Hodgkin's lymphoma, and Hodgkin's lymphoma. Exemplary solid tumor cancers include but are not limited to breast cancer, lung cancer, pancreatic cancer, liver cancer, glioblastoma (GBM), prostate cancer, ovarian cancer, mesothelioma, colon cancer, and stomach cancer.

Exemplary infectious diseases include but are not to human immunodeficiency virus (HIV)-1 and -2 infections, severe acute respiratory syndrome coronavirus (SARS-CoV)-1 and -2 infections, Epstein-Barr virus (EBV) infection, human papillomavirus (HPV) infection, dengue virus infection, malaria, sepsis, and *E. coli* infection. Exemplary immune disorders include but are not limited to, autoimmune diseases, such as rheumatoid arthritis, type I diabetes, systemic lupus erythematosus, inflammatory bowel disease, multiple sclerosis, Guillain-Barre syndrome, chronic inflammatory demyelinating polyneuropathy, psoriasis, Graves' disease, Hashimoto's thyroiditis, myasthenia gravis, and vasculitis.

The term "an effective amount" as used herein refers to the amount of each active agent required to confer therapeutic effect on the subject, either alone or in combination with one or more active agents. Effective amounts vary, as recognized by those skilled in the art, depending on the particular condition being treated, the severity of the condition, individual patient parameters including age, physical condition, size, gender and weight, the duration of treatment, route of administration, excipient usage, co-usage (if any) with other active agents and like factors within the knowledge and expertise of the health practitioner. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner.

However, suitable dosage ranges are readily determinable by one skilled in the art.

The term “treating” as used herein refers to the application or administration of a composition including one or more active agents to a subject, who has a target disease, a symptom of the target disease, or a predisposition toward the target disease, with the purpose to  
5 cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of the disease, or the predisposition toward the disease.

In some embodiments, the immune cell populations comprising the modified immune cells as described herein may be utilized in conjunction with other types of therapy for cancer, such as chemotherapy, surgery, radiation, gene therapy, and so forth. Such therapies can be  
10 administered simultaneously or sequentially (in any order) with the immunotherapy described herein. When co-administered with an additional therapeutic agent, suitable therapeutically effective dosages for each agent may be lowered due to the additive action or synergy.

Non-limiting examples of anti-cancer therapeutic agents useful for combination with the modified immune cells described herein include, but are not limited to, immune checkpoint  
15 inhibitors (e.g., PDL1, PD1, and CTLA4 inhibitors), anti-angiogenic agents (e.g., TNP-470, platelet factor 4, thrombospondin-1, tissue inhibitors of metalloproteases, prolactin, angiostatin, endostatin, bFGF soluble receptor, transforming growth factor beta, interferon alpha, soluble KDR and FLT-1 receptors, and placental proliferin-related protein); a VEGF antagonist (e.g., anti-VEGF antibodies, VEGF variants, soluble VEGF receptor fragments);  
20 chemotherapeutic compounds. Exemplary chemotherapeutic compounds include pyrimidine analogs (e.g., 5-fluorouracil, floxuridine, capecitabine, gemcitabine and cytarabine); purine analogs (e.g., fludarabine); folate antagonists (e.g., mercaptopurine and thioguanine); antiproliferative or antimitotic agents, for example, vinca alkaloids; microtubule disruptors such as taxane (e.g., paclitaxel, docetaxel), vincristin, vinblastin, nocodazole, epothilones and  
25 navelbine, and epididodophyllotoxins; DNA damaging agents (e.g., actinomycin, amsacrine, anthracyclines, bleomycin, busulfan, camptothecin, carboplatin, chlorambucil, cisplatin, cyclophosphamide, cytoxan, dactinomycin, daunorubicin, doxorubicin, epirubicin, hexamethylene bisacetamide, hexamethylene amiloride, oxaliplatin, ifosfamide, melphalan, mechlorethamine, mitomycin, mitoxantrone, nitrosourea, plicamycin, procarbazine, taxol,  
30 taxotere, teniposide, triethylenethiophosphoramidate and etoposide).

In some embodiments, radiation or radiation and chemotherapy are used in combination with the cell populations comprising modified immune cells described herein. Additional useful agents and therapies can be found in Physician's Desk Reference, 59<sup>th</sup> edition, (2005), Thomson P D R, Montvale N.J.; Gennaro et al., Eds. Remington's The Science and Practice of

Pharmacy 20<sup>th</sup> edition, (2000), Lippincott Williams and Wilkins, Baltimore Md.; Braunwald et al., Eds. Harrison's Principles of Internal Medicine, 15<sup>th</sup> edition, (2001), McGraw Hill, NY; Berkow et al., Eds. The Merck Manual of Diagnosis and Therapy, (1992), Merck Research Laboratories, Rahway N.J.

5

#### **IV. Kits for Therapeutic Uses**

The present disclosure also provides kits for use in treating any of the target diseases described herein involving the genetically engineered immune cells or recombinant vectors described herein and kits for use in making the engineered immune cells or recombinant  
10 vectors as described herein.

In one embodiment, the kit for therapeutic use includes one or more containers comprising one or more populations of the genetically engineered immune cells, each of which may be formulated as a pharmaceutical cell therapy composition. In another embodiment, the kit for therapeutic use includes one or more containers comprising anucleic acid or vector  
15 comprising the expression system provided herein (*e.g.*, the SP-2A system) and other components for making recombinant vectors (*e.g.*, recombinant viral particles), including packaging cells and the like for pharmaceutical gene therapy applications. In some embodiments, the kit may further include suitable diagnostic agents (including antibodies and detection reagents) for monitoring the course and efficacy of the treatment.

20 In some embodiments, the kit can additionally comprise instructions for use of the immune cell population(s) or recombinant gene therapy vectors in any of the methods described herein. The kit may further include instructions for administering the pharmaceutical immune cells or recombinant vectors to a subject to achieve the intended activity in a subject. The kit may further comprise a description of selecting a subject suitable for treatment based  
25 on identifying whether the subject is in need of the treatment.

The instructions relating to the use of the genetically engineered immune cells, nucleic acid or recombinant vectors, or the pharmaceutical compositions comprising such as described herein generally include information as to dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose  
30 packages) or sub-unit doses. Instructions supplied in the kits of the disclosure are typically written instructions on a label or package insert. The label or package insert indicates that the pharmaceutical compositions are used for treating, delaying the onset, and/or alleviating a disease or disorder in a subject.

The kits provided herein are in suitable packaging. Suitable packaging includes, but is

not limited to, vials, bottles, jars, flexible packaging, and the like. Also contemplated are packages for use in combination with a specific device, such as an inhaler, nasal administration device, or an infusion device. A kit may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The container may also have a sterile access port.

Kits optionally may provide additional components such as buffers and interpretive information. Normally, the kit comprises a container and a label or package insert(s) on or associated with the container. In some embodiment, the disclosure provides articles of manufacture comprising contents of the kits described above.

Also provided here are kits for use in making the modified immune cells described herein. Such a kit may include one or more containers each containing reagents for use in introducing the SP-2A nucleic acid into immune cells. Alternatively, or in addition, the kit may further comprise one or more other exogenous nucleic acids for expressing any of the additional polypeptides described herein and reagents for delivering the exogenous nucleic acids into host immune cells.

### ***General techniques***

The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook, et al., 1989) Cold Spring Harbor Press; *Oligonucleotide Synthesis* (M. J. Gait, ed. 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J. E. Cellis, ed., 1989) Academic Press; *Animal Cell Culture* (R. I. Freshney, ed. 1987); *Introduction to Cell and Tissue Culture* (J. P. Mather and P. E. Roberts, 1998) Plenum Press; *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J. B. Griffiths, and D. G. Newell, eds. 1993-8) J. Wiley and Sons; *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D. M. Weir and C. C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J. M. Miller and M. P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F. M. Ausubel, et al. eds. 1987); *PCR: The Polymerase Chain Reaction*, (Mullis, et al., eds. 1994); *Current Protocols in Immunology* (J. E. Coligan et al., eds., 1991); *Short Protocols in Molecular Biology* (Wiley and Sons, 1999); *Immunobiology* (C. A. Janeway and P. Travers, 1997); *Antibodies* (P. Finch, 1997); *Antibodies: a practice approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal*

antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J. D. Capra, eds. Harwood Academic Publishers, 1995); *DNA Cloning: A practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. (1985»); *Transcription and Translation* (B.D. Hames & S.J. Higgins, eds. (1984»); *Animal Cell Culture* (R.I. Freshney, ed. (1986»); *Immobilized Cells and Enzymes* (IRL Press, (1986»); and B. Perbal, *A practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.).

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference for the purposes or subject matter referenced herein.

#### EXAMPLES

While the present disclosure has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the disclosure. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit, and scope of the present disclosure. All such modifications are intended to be within the scope of the disclosure.

##### **Example 1: Reduction of IL-2 Expression in Host Cells Using 2A Peptide Linkers**

A lentiviral transfer vector construct was used to express IL-2 from an SP-2A expression cassette containing an intervening P2A or P2A-T2A linker element between the coding sequence of a signal peptide and the coding sequence of human IL-2 (mature form without an N-terminal signal peptide). In the presence of the P2A or P2A-T2A element cleavage, the IL-2 coding region does not encode an N-terminal signal peptide. In the absence of the P2A or P2A-T2A linker element (as in the control), the IL-2 coding region is directly linked to coding regions for an N-terminal signal peptide. The resulting vectors were transfected into 293T cells for expression of IL-2, and a standard enzyme linked immunosorbent assay (ELISA) was performed to measure levels of IL-2 secreted into the cell media. As shown in **FIG. 1**, expression of IL-2 was significantly reduced from the expression

cassette containing a P2A linker and was further reduced by the addition of another T2A linker in the combined P2A-T2A linker. These results suggest that incorporation of one of more 2A linkers between a signal peptide and coding sequence is highly effective in reducing secretion of a polypeptide.

5           Another experiment evaluated the ability of 2A linker elements positioned between two polypeptides to express or secrete one of the polypeptides in reduced amounts where the upstream polypeptide has an N-terminal signal peptide. Briefly, a lentiviral transfer vector was constructed to express an anti-IFN- $\gamma$  scFv fused with human IL-2, with or without an intervening P2A linker or linker comprising a combination of P2A and T2A coding sequences  
10 between the anti-IFN- $\gamma$  scFv and human IL-2. The resulting vectors were transfected into 293T cells for expression of anti-IFN- $\gamma$  scFv and IL-2 using the signal peptide from the anti-IFN- $\gamma$  scFv. IL-2 expression was evaluated by ELISA. The results showed that the expression of IL-2 was significantly reduced by incorporation of a P2A linker, which could be further reduced by the addition of another T2A linker designated as P2A-T2A (**FIG. 2**). These results further  
15 confirm that incorporation of one of more 2A linkers between two fused recombinant proteins is highly effective in reducing secretion of the downstream polypeptide.

#### **Example 2: Reduction of IL-10 Expression in Host Cells Using 2A Peptide Linkers**

Another experiment was conducted to evaluate the ability of an SP-2A expression  
20 cassette to facilitate expression of multiple (i.e., four) proteins using multiple 2A linkers, including one 2A linker between two polypeptides, where reduced expression of a downstream polypeptide is driven by a N-terminal signal peptide from an upstream polypeptide coding region. In this experiment, a lentiviral transfer vector was constructed to express an anti-IFN- $\gamma$  scFv fused with human IL-10, with or without (control) a P2A linker between the coding  
25 sequences of anti-IFN- $\gamma$  scFv and human IL-10. From N to C terminus, the expression cassette encodes: (1) a first signal peptide (SP); (2) anti-IL-6 scFv; (3) a first T2A; (3) a second signal peptide and truncated epidermal growth factor receptor EGFR; (4) a second F2A; (5) a third signal peptide; (6) anti-IFN- $\gamma$  scFv (SEQ ID NO: 16); (7) a third P2A; and (7) IL-10. The control lentivirus transfer vector was constructed to secrete IL-10 without an intervening P2A  
30 between a signal peptide and the IL-10 coding region. The resulting vectors were transfected to 293T cells for expression of IL-10, which was evaluated by ELISA. As shown in **FIG. 3**, the results showed that secretion of IL-10 was significantly reduced by an intervening P2A in between, further confirming that incorporation of 2A linkers between two recombinant proteins can be highly effective for reducing secretion of a protein downstream.

**Example 3: Reduction of Cytokine Expression in Human T Cells Using 2A Peptide Linkers**

Human T cells were activated and transduced with a lentivector comprising a coding sequence for a signal peptide (*e.g.*, SEQ ID NO: 22) and an anti-CD19 CAR (SEQ ID NO: 17) and one of the following elements:

1. a coding sequence for T2A, a coding sequence for a signal peptide (SP) and a nucleotide sequence encoding mature IL18 (IL18; SEQ ID NO: 20);
2. a coding sequence for T2A, a coding sequence for SP, a coding sequence for P2A, and the nucleotide sequence encoding mature IL18;
3. an IRES, a coding sequence for the SP, and the nucleotide sequence encoding the mature-IL18;
4. a coding sequence for T2A, a coding sequence for SP and a coding sequence for mature IL15 (IL15; SEQ ID NO: 21);
5. a coding sequence for T2A, a coding sequence for SP, a coding sequence for P2A, and a coding sequence for mature IL15;
6. an IRES element, a coding sequence for SP, and a coding sequence for mature IL15;
7. a coding sequence for T2A, a coding sequence for SP and a coding sequence for mature IL10 (IL10; SEQ ID NO: 19);
8. a coding sequence for T2A, a coding sequence for SP, a coding sequence for P2A, and a coding sequence for mature IL10; and
9. an IRES, a coding sequence for SP, and a coding sequence for mature IL10.

The resulting T cells were cocultured with Nalm6 tumor cells at the ratio of 1:1.5, and the coculture supernatant was collected for ELISA to evaluate the concentrations of cytokines. As shown in **FIGS. 4A-4C**, the results suggest that using the P2A linker between the SP coding sequence and the cytokine coding sequence or an IRES upstream to the SP coding sequence can effectively reduce the expression levels of the cytokines.

**Example 4: Expression of Signaling Domain of CAR in Human T Cells Using 2A Peptide Linkers**

Human T cells were activated and transduced with a lentivector comprising a coding sequence for a signal peptide (*e.g.*, SEQ ID NO: 22) and an anti-CD19 CAR (SEQ ID NO: 18) and one of the following features:

1. no linker between the transmembrane domain and the CD3 $\zeta$  signaling domain in the

anti-CD19CAR;

2. a P2A linker between transmembrane domain and CD3 $\zeta$  signaling domain;
3. a T2A linker between transmembrane domain and CD3 $\zeta$  signaling domain;
4. a F2A linker between transmembrane domain and CD3 $\zeta$  signaling domain;
5. a E2A linker between transmembrane domain and CD3 $\zeta$  signaling domain.

The resulting T cells were cocultured with GFP expressing Nalm6 tumor cells at the ratio of 1:3, and the remaining tumor cells were analyzed by flowcytometry to evaluate killing activity. As shown in **FIG. 5**, all different 2A linkers between transmembrane domain and CD3 $\zeta$  signaling domain mediated effective killing activity, suggesting that CD3 $\zeta$  signaling domain retained with the CAR through uncleaved 2A linkers were still effective in driving killing activity.

The previous studies suggested that P2A and T2A peptide linkers could mediate very effective cleavage during protein expression, while F2A and E2A could mediate moderately effective cleavage. The results suggest that a 2A linker can be used to control the expression of a certain signaling domain retained with the whole CAR molecule, while still driving effective downstream signaling. More efficient 2A cleavage will result in less amount of the certain signaling domain downstream to the 2A linker retained with the whole CAR, therefore driving less downstream signaling elicited by the certain signaling domain downstream to the 2A linker to avoid over-signaling in the immune cells.

#### **Example 5: Reduction of Toxicity Associated with T Cells Expressing IL10 in Immune Deficient Mice**

Human T cells were activated and transduced with a lentivector comprising:

1. a coding sequence for a signal peptide (SP; SEQ ID NO: 22) and a coding sequence for the mature IL10 (IL10; SEQ ID NO: 19); and
2. a coding sequence for the SP, a coding sequence for P2A-linker, and a coding sequence for the mature IL10.

The resulting T cells were injected to immune deficient mice to monitor the body weight. As shown in **FIGS. 6A-6B**, insertion of the P2A linker between SP and the IL10 cytokine did not cause loss of body weight in the mice, whereas the format of SP and the IL10 cytokine coding sequence caused significant body weight loss in the mice. The analysis of blood IL10 levels by ELISA revealed significantly lower concentrations of IL10 in the mice injected with T cells expressing the element with the P2A linker between SP and the IL10 cytokine coding sequence.

The results suggest that using a self-cleavage peptide such as the P2A linker between a SP and a cytokine such as IL10 can be an effective approach to control the expression level of the cytokine and thus lower toxicity associated with overexpression of the cytokine in T cells.

5

**Example 6: Reduction of Toxicity Associated with T Cells Expressing IL18 in Immune Deficient Mice**

Human T cells were activated and transduced with a lentivector comprising:

1. a coding sequence for a signal peptide (SP; SEQ ID NO: 22) and a coding sequence  
10 for the mature IL18 (IL18; SEQ ID NO: 20); and
2. a coding sequence for SP, a coding sequence for the P2A linker, and a coding  
sequence for the mature IL18.

The resulting T cells were injected to immune deficient mice to monitor the body weight. As shown in **FIGS. 7A-7B**, the P2A linker between SP and the IL18 cytokine coding  
15 sequence did not cause significant loss of body weight in the mice, whereas the format of SP and the IL18 cytokine coding sequence caused significant body weight loss in the mice. The analysis of blood IL18 levels by ELISA revealed significantly lower concentrations of IL18 in the mice injected with T cells expressing the element with the P2A linker between SP and the  
20 P2A linker between SP and a coding sequence for a cytokine such as IL18 can be used as an effective approach to lower the toxicity associated with T cells expressing the cytokine such as IL18.

**Example 7: Reduction of Toxicity Associated with T Cells Expressing IL15 in Immune Deficient Mice**

25

Human T cells were activated and transduced with a lentivector comprising:

1. a coding sequence for a signal peptide (SP; SEQ ID NO: 22) and a coding sequence  
for mature IL15 (IL15; SEQ ID NO: 21); and
2. a coding sequence for SP, a coding sequence for the P2A linker, and a coding  
30 sequence for the mature IL15.

The resulting T cells were injected to immune deficient mice to monitor the body weight. As shown in **FIGS. 8A-8B**, the P2A linker between SP and the IL15 cytokine coding sequence did not cause significant loss of body weight in the mice, whereas the format of SP and the IL15 cytokine coding sequence caused significant body weight loss in the mice. The

analysis of blood IL15 levels by ELISA revealed significantly lower concentrations of IL15 in the mice injected with T cells expressing the element with the P2A linker between SP and the IL15 cytokine coding sequence. The results suggest that a self-cleavage peptide such as the P2A linker between SP and the IL15 cytokine coding sequence can be used as an effective approach to lower the toxicity associated with T cells expressing IL15.

**Example 8: Reduction of Downstream Cytokine Expression in Human T Cells**

Human T cells were activated and transduced with a lentivector comprising a coding sequence for a signal peptide (*e.g.*, SEQ ID NO: 22) and an anti-CD19 CAR (SEQ ID NO: 17), a P2A linker, and the following elements: a coding sequence for a signal peptide (SP; SEQ ID NO: 22), a coding sequence for the mature IL18, a coding sequence for a T2A linker, and a coding sequence for the mature IL10. The resulting T cells were cocultured with Nalm6 tumor cells at the ratio of 1:1.5, and the coculture supernatant was collected for ELISA to evaluate the concentrations of cytokines. As shown in **FIG. 9**, the result suggests that the a self-cleavage peptide such as the T2A linker between IL18 and IL10 can effectively reduce the level of IL10 downstream to the 2A linker as compared to IL18.

**Example 9: Reduction of Downstream Cytokine Expression in Immune Deficient Mice**

Human T cells were activated and transduced with a lentivector comprising a coding sequence for a signal peptide (SP; SEQ ID NO: 22), a coding sequence for a first T2A linker, a coding sequence for the mature IL18, a coding sequence for a second T2A linker, and a coding sequence for the mature IL10. The resulting T cells were injected to immune deficient mice. As shown in **FIG. 10**, the analysis of blood cytokine levels by ELISA revealed significantly lower concentration of IL10 as compared to IL18, suggesting that a self-cleavage peptide such as the T2A linker between IL18 and IL10 coding sequence can be used as an effective approach to lower the production of the cytokine downstream to the 2A linker.

**Sequence Table**

Exemplary amino acid sequences described herein are provided in **Table 1** below:

**Table 1. Amino Acid Sequences for Exemplary Polypeptides Described Herein**

| SEQ ID NO: | Name             | Sequence            |
|------------|------------------|---------------------|
| 1          | 2A core sequence | D-V/I-E-X-N-P-G-P   |
| 2          | P2A peptide-1    | ATNFSLLKQAGDVEENPGP |

|    |                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
|----|-----------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 3  | T2A peptide-1               | EGRGSLLTCGDVEENPGP                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| 4  | F2A peptide-1               | VKQTLNFDLLKLAGDVESNPGP                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| 5  | E2A peptide-1               | QCTNYALLKLAGDVESNPGP                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| 6  | P2A peptide-2               | GSGATNFSLLKQAGDVEENPGP                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| 7  | T2A peptide-2               | GSGEGRGSLLTCGDVEENPGP                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| 8  | F2A peptide-2               | GSGVKQTLNFDLLKLAGDVESNPGP                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| 9  | E2A peptide-2               | GSGQCTNYALLKLAGDVESNPGP                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
| 10 | P2A-T2A peptide             | GSGATNFSLLKQAGDVEENPGPGGGSGEGRGSLLTCGDVEENPGP                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
| 11 | Human insulin B chain       | FVNQHLCGSHLVEALYLVCGERGFFYTPKT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| 12 | Human insulin A chain       | GIVEQCCTSICSLYQLENYCN                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| 13 | Single chain human insulin* | FVNQHLCGSHLVEALYLVCGERGFFYTPKT <b>QRGGGGG</b><br><b>QR</b> GIVEQCCTSICSLYQLENYCN                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| 14 | Linker                      | QRGGGGGQR                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
| 15 | Human growth hormone        | FPTIPLSRLFDNAMLRAHRLHQLAFDTYQEFEEAYIPKE<br>QKYSFLQNPQTSLCFSESIPTSNREETQKSNLELLRISL<br>LLIQSWLEPVQFLRSVFANSLVYGASDSNVYDLLKDLE<br>EGIQTLMGRLEDGSPRTGQIFKQTYSKFDTNSHNDAL<br>LKNYGLLYCFRKMDMDKVETFLRIVQCRSVEGSCGF                                                                                                                                                                                                                                                                                                              |
| 16 | Anti-IFN $\gamma$ scFv      | EIVLTQSPGTLSSLSPGERATLSCRASQSVSSYLAWYQQ<br>KPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRL<br>EPEDFAVYYCQRSGGSFTFGPGTKVDIKGGGSGGGG<br>SGGGGSEVQLVQSGAEVKKPGESLKISCKGSGYNFTSY<br>WIGWVRQMPGKGLELMGHIYPGDS DTRYSPSFQGGQVTI<br>SADKSISTAYLQWSSLKASDTAMY YCGSGSYFYFDLW<br>GRGTLVTVSS                                                                                                                                                                                                                                                  |
| 17 | Anti-CD19 CAR(19BBZ)        | DIQMTQTSSLSASLGDRVTISCRASQDISKYLNWYQQ<br>KPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNL<br>EQEDIATYFCQQGNTLPYTFGGGKLEITGGGSGGGG<br>SGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDY<br>GVSWIRQPPRKGLEWLGVIWGSETTYNSALKSRLTIHK<br>DNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAM<br>DYWGQGTSTVVSSTTPAPRPPTPAPTIASQPLSLRPEA<br>CRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLLLSLV<br>ITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPE<br>EEEGGCELRVKFSRSADAPAYKQQNQLYNELNLGRR<br>EEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK<br>MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYD<br>ALHMQALPPR |
| 18 | Anti-CD19 CAR (19Z)         | DIQMTQTSSLSASLGDRVTISCRASQDISKYLNWYQQ<br>KPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNL<br>EQEDIATYFCQQGNTLPYTFGGGKLEITGGGSGGGG<br>SGGGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDY<br>GVSWIRQPPRKGLEWLGVIWGSETTYNSALKSRLTIHK<br>DNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAM<br>DYWGQGTSTVVSSTTPAPRPPTPAPTIASQPLSLRPEA<br>CRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLLLSLV                                                                                                                                                                                   |

|    |                           |                                                                                                                                                                                |
|----|---------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|    |                           | ITLYCRVKFSRSADAPAYKQGQNQLYNELNLGRREEYD<br>VLDKRRGRDPENMGKPRRKNPQEGLYNELQDKMAE<br>AYSEIGMKGERRRRGKHDGLYQGLSTATKDTYDALH<br>MQALPPR                                               |
| 19 | Human IL-10               | SPGQGTQSENSCTHFPGNLPNMLRDLRDAFSRVKTFQ<br>MKDQLDNLLLKESLLEDFKGYLGCQALSEMIQFYLEE<br>MPQAENQDPDIKAHVNSLGENLKTLRLRLRRCHRFLPC<br>ENKSKAVEQVKNAFNKLQEKGIYKAMSEFDIFINYIEA<br>YMTMKIRN |
| 20 | Human IL-18               | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTSDC<br>RDNAPRTIFIISMYKDSQPRGMAVTISVKCEKISTLSCEN<br>KIISFKEMNPPDNIKDTKSDIIFQRSVPGHDNKMQFESS<br>SYEGYFLACEKERDLFKLILKKEDELGDRSIMFTVQNE<br>D    |
| 21 | Human IL-15               | NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTA<br>MKCFLLELQVISLESGDASIHDTVENLIILANNSLSSNGN<br>VTESGCKECELEEKNIKEFLQSFVHIVQMFINTS                                                      |
| 22 | Signal Peptide            | MALPVTALLPLALLHAARP                                                                                                                                                            |
| 23 | V <sub>H</sub> of TGN1412 | QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYIHWV<br>RQAPGQGLEWIGCIYPGNVNTNYNEKFKDRATLTVDT<br>ISTAYMELSRLRSDDTAVYFCTRSHYGLDWNFDVWGQ<br>GTTVTVSS                                             |
| 24 | V <sub>L</sub> of TGN1412 | DIQMTQSPSSLSASVGDRVTITCHASQNIYVWLNWYQQ<br>KPGKAPKLLIYKASNLHTGVPSRFSGSGSGTDFTLTIS<br>SLQPEDFATYYCQQGQTYPTFGGGTKVEIK                                                             |
| 25 | V <sub>H</sub> of TGN1112 | QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYIHWV<br>RQAPGQGLEWIGCIYPGNVNTNYNEKFKDRATLTVDT<br>ISTAYMELSRLRSDDTAVYFCTRSHYGLDWNFDVWGQ<br>GTTVTVSS                                             |
| 26 | V <sub>L</sub> of TGN1112 | DIQMTQSPSSLSASVGDRVTITCHASQNIYVWLNWYQQ<br>KPGKAPKLLIYKASNLHTGVPSRFSGSGSGTDFTLTIS<br>SLQPEDFATYYCQQGQTYPTFGGGTKVEIK                                                             |

*\*linker italicized and bolded*

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination.

- 5 Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

- From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof,  
10 can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

## EQUIVALENTS

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of examples only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with “and/or” should be construed in the same fashion, i.e., “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A

and B (optionally including other elements); etc.

As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but  
5 also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (i.e., “one or the other but not both”) when  
10 preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one element  
15 selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or  
20 unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally  
25 including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of  
30 the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” “composed of,” and the like are to be understood to be open-ended, i.e., to mean including but

not limited to. Only the transitional phrases “consisting of” and “consisting essentially of” shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

**What Is Claimed Is:**

1. A population of genetically engineered cells, comprising a first exogenous nucleic acid, which comprises from 5' to 3':
  - 5 a first nucleotide sequence encoding a signal peptide;
  - a second nucleotide sequence, which encodes one or more self-cleaving peptides, optionally wherein the self-cleaving peptide is a 2A peptide; and
  - a third nucleotide sequence encoding a polypeptide, which is free of an N-terminal signal peptide;
  - 10 wherein the genetically engineered cells express the polypeptide at a lower level as compared to a population of counterpart genetically engineered cells comprising a nucleic acid encoding a counterpart polypeptide having an N-terminus signal peptide.
2. The population of genetically engineered cells of claim 1, wherein the
  - 15 polypeptide encoded by the third nucleotide is a blood factor, an antibody, a chimeric antigen receptor (CAR), a cytoplasmic signaling domain of an immune cell receptor, a hormone, an immune modulating polypeptide, or a combination thereof.
3. The population of genetically engineered cells of claim 2, wherein the
  - 20 polypeptide encoded by the third nucleotide sequence is an immune modulating polypeptide set forth in any one of (a)-(e) below:
    - (a) a cytokine selected from the group consisting of IL-2, IL-7, IL-9, IL-10, IL-15, IL-12, IL-17, IL-18, IL-21, IL-23, IL-24, IL-27, IL-33, and CCL19;
    - (b) an inhibitor of a checkpoint molecule selected from the group consisting of PD1, PDL1, LAG3, TIGIT, TIM3, and CTLA4, optionally wherein the checkpoint inhibitor is an
      - 25 anti-PD1/PDL1 antibody, an anti-LAG3 antibody, an anti-TIGIT antibody, an anti-TIM3 antibody, or an anti-CTLA4 antibody;
    - (c) an immune enhancer selected from the group consisting of 41BBL, anti-CD3 antibody, an anti-FcR antibody, an anti-41BB antibody, an anti-OX40 antibody, an anti-CD28
      - 30 antibody, and an anti-CD40 antibody;
    - (d) a cytokine antagonist selected from the group consisting of an anti-IL6 antibody, an anti-GM-CSF antibody, an anti-IFN- $\gamma$  antibody, an anti-TNF antibody, an anti-IL-1 antibody, and IL-1RA; and
    - (e) a cytoplasmic signaling domain of an immune cell receptor, which optionally is

selected from the group consisting of CD3, DAP12, FcR, CD79, CNAIP, CD28, 4-1BB, CTLA-4, PD1, TIGIT, LAG3, TIM3 and a cytokine receptor.

4. The population of genetically engineered cells of claim 2, wherein the  
5 polypeptide encoded by the third nucleotide sequence is a hormone, optionally wherein the hormone is an insulin or a growth hormone.

5. The population of genetically engineered cells of any one of claims 1-4, wherein  
the one or more self-cleaving peptides are selected from the group consisting of P2A, T2A,  
10 E2A, F2A, and a combination thereof, optionally wherein the one or more 2A self-cleaving peptides comprise a combination of P2A and T2A.

6. The population of genetically engineered cells of any one of claims 1-5, wherein  
the first nucleotide sequence encoding the signal peptide is directly linked to the second  
15 nucleotide sequence encoding the one or more self-cleaving peptides.

7. The population of genetically engineered cells of any one of claims 1-6, wherein  
the genetically engineered cells further express one or more proteins of interest, which  
optionally are different from the polypeptide encoded by the third nucleotide sequence.  
20

8. The population of genetically engineered cells of claim 7, wherein the one or  
more proteins of interest comprise a blood factor, a chimeric antigen receptor (CAR), an  
immune modulating polypeptide, an antibody, a cytokine, a hormone, a cell surface marker, or  
a combination thereof,  
25 optionally wherein the CAR comprises an extracellular domain, a hinge and a  
transmembrane domain, and optionally one or more signaling domains;  
optionally wherein the cell surface marker is CD20, a truncated epidermal growth  
factor receptor (EGFR), or receptor tyrosine kinase-like orphan receptor 1 (ROR1); and  
optionally the immune modulating polypeptide is a cytokine antagonist, which  
30 preferably is an antibody that binds IL-6, IL-1, GM-CSF, TNF, IFN- $\gamma$ , or a combination  
thereof.

9. The population of genetically engineered cells of claim 7 or claim 8, wherein the first exogenous nucleic acid comprises a fourth nucleotide sequence encoding the one or more proteins of interest.

5 10. The population of genetically engineered cells of claim 9, wherein the fourth nucleotide sequence is located between the first nucleotide sequence encoding the signal peptide and the second nucleotide sequence encoding the one or more self-cleaving peptides.

10 11. The population of genetically engineered cells of claim 9, wherein the fourth nucleotide sequence is located 5' to the first nucleotide sequence encoding the signal peptide, and/or 3' to the third nucleotide sequence encoding the one or more proteins of interest.

12. The population of genetically engineered cells of any one of claims 7-11, wherein the fourth nucleotide sequence comprises a self-cleaving peptide coding sequence, a promoter sequence, a protease cleavage site coding sequence, an mRNA splicing site, or an internal ribosomal entry sequence.

13. The population of genetically engineered cells of any one of claims 1-12, wherein the population of genetically engineered cells further comprises one or more additional exogenous nucleic acids, which encode the one or more proteins of interest.

14. The population of genetically engineered cells of any one of claims 1-13, wherein the genetically engineered cells comprise immune cells, islet cells, tumor cells, or stem cells.

25 15. The population of genetically engineered cells of claim 14, which comprises immune cells, wherein the immune cells comprise T-cells, tumor infiltrating lymphocytes, NK cells, dendritic cells, macrophages, B cells, neutrophils, eosinophils, basophils, mast cells, myeloid- derived suppressor cells, or a combination thereof

30 16. The population of genetically engineered cells of any one of claims 1-15, wherein the polypeptide encoded by the third nucleotide sequence is fused to an immunoglobulin Fc domain or an albumin domain.

17. The population of genetically engineered cells of any one of claims 9-16, wherein any one of the proteins of interest encoded by the fourth nucleotide sequence or encoded by the additional exogenous nucleic acid(s) are fused to an immunoglobulin Fc domain or an albumin domain.

5

18. A nucleic acid, comprising from 5' to 3':  
a first nucleotide sequence encoding a signal peptide;  
a second nucleotide sequence, which encodes one or more self-cleaving peptides,  
wherein optionally the self-cleaving peptide is a 2A peptide; and  
10 a third nucleotide sequence encoding a polypeptide, which is free of an N-terminal signal peptide,  
wherein when the nucleic acid is expressed in a cell, the cell expresses the polypeptide at a lower level as compared to a counterpart cell comprising a nucleic acid encoding a counterpart polypeptide that has an N-terminus signal peptide.

15

19. The nucleic acid of claim 18, which is set forth in any one of claims 2-13, 16, and 17.

20. The nucleic acid of claim 18, wherein the nucleic acid does not encode another  
20 signal peptide between the second and third nucleotide sequences.

21. The nucleic acid of any one of claims 18-20, which is a vector and/or an RNA, optionally wherein the vector is an expression vector.

22. The nucleic acid of claim 21, wherein the vector is a plasmid, retroviral vector, lentiviral vector, adenoviral vector, or adeno-associated viral vector.

23. A pharmaceutical composition comprising the population of genetically engineered cells of any one of claims 1-17 and/or the nucleic acids of any one of claims 18-22  
30 and a pharmaceutically acceptable carrier.

24. A method for treating a disease in a subject, comprising administering to a subject in need thereof an effective amount of the population of genetically engineered cells of any one of claims 1-17 and/or the nucleic acids of any one of claims 18-22, or the

pharmaceutical composition of claim 23.

25. The method of claim 24, wherein the disease is an autoimmune disorder, cancer,  
an infectious disease, an immune disorder, a fibrosis disease, or a senescence disease,  
5 optionally wherein the senescence disease is liver fibrosis, atherosclerosis, or natural aging.

26. A method for preparing genetically engineered cells, the method comprising:  
delivering the nucleic acid of any one of claims 18-22 to a population of cells to  
produce genetically engineered cells carrying the nucleic acid.

10