

COMPOSITIONS AND METHODS FOR THE CRYOPRESERVATION OF IMMUNE CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Patent Application No. 62/830,950, filed April 8, 2019, the entire contents of which are incorporated herein by reference.

SEQUENCE LISTING

[0001a] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 4, 2020, is named 106417-0376_SL.txt and is 22,030 bytes in size.

TECHNICAL FIELD

[0002] The present technology relates to compositions and methods for the cryopreservation of immune cells, such as peripheral blood mononuclear cells (PBMCs) by pre-treating the cells with a PTD-MYC fusion protein (*e.g.*, an HIV TAT-MYC fusion polypeptide) prior to freezing. Kits for use in practicing the methods are also provided herein.

BACKGROUND

[0003] Cryopreservation is a process in which cells are preserved by cooling them to low temperatures. At these low temperatures, biological activity, including the biochemical reactions that would lead to cell death under normal conditions, are effectively stopped. However, if not properly controlled, cryopreservation can lead to cell damage and a decrease in cell viability. Further, after the freeze-thaw process, cells normally need to be cultured to ensure proper recovery. Currently, there is an unmet need for cryopreservation methods that increase cell viability and recovery after the cryopreserved cells have thawed.

SUMMARY

[0004] In one aspect, the present disclosure provides a frozen composition that includes (a) a MYC fusion polypeptide, comprising (i) a protein transduction domain, (ii) a MYC polypeptide sequence; and (b) one or more peripheral blood mononuclear cells (PBMCs) isolated from a donor subject, wherein the composition exhibits increased cell viability compared to control PBMC cells isolated from the subject. In some examples, the protein

transduction domain sequence is a TAT protein transduction domain sequence. In some examples, the protein transduction domain is TAT_[48-57]. In some examples, the protein transduction domain is TAT_[57-48]. In some examples, the MYC fusion polypeptide comprises SEQ ID NO: 1. In some examples, the one or more peripheral blood mononuclear cells comprises a T-cell, a B-cell, an NK cell, a monocyte, a granulocyte, a macrophage, or any combination thereof. In some examples, the compositions further include a cell suspension medium. In some examples, the cell suspension medium comprises CHB media, CS5 media, or CS10 media. In some examples, the composition exhibits increased cell recovery when thawed as compared to control PBMCs in the absence of the MYC fusion polypeptide after a freeze-thaw cycle. In some examples, the composition exhibits increased expression of CD25 after cell activation as compared to control PBMCs in the absence of the MYC fusion polypeptide after a freeze-thaw cycle. In some examples, the present disclosure provides an immune cell bank comprising the frozen composition.

[0005] In one aspect, the present disclosure provides a method of cryopreserving peripheral blood mononuclear cells (PBMCs) that includes (a) contacting a composition comprising one or more PBMCs isolated from a donor subject with an effective amount of a MYC fusion polypeptide, comprising (i) a protein transduction domain; (ii) a MYC polypeptide sequence; and (b) cooling the PBMCs to a temperature sufficient to freeze the composition. In some examples, the protein transduction domain sequence is a TAT protein transduction domain sequence. In some examples, the protein transduction domain is TAT_[48-57]. In some examples, the protein transduction domain is TAT_[57-48]. In some examples, the MYC fusion polypeptide comprises SEQ ID NO: 1. In some examples, the one or more peripheral blood mononuclear cells comprises a T-cell, a B-cell, an NK cell, a monocyte, a granulocyte, or any combination thereof. In some examples, the method further includes suspending the PBMCs in a cell suspension medium. In some examples, the cell suspension medium comprises CHB media, CS5 media, or CS10 media.

[0006] In some examples, the composition comprising one or more PBMCs is contacted with the MYC fusion polypeptide at a concentration of about 0.5µg/mL to about

500 µg/mL. In some examples, the composition comprising one or more PBMCs is contacted with the MYC fusion polypeptide at a concentration of about 0.5µg/mL to about 10 µg/mL. In some examples, the composition comprising one or more PBMCs is contacted with the MYC fusion polypeptide for less than 24 hours prior to step (b). In some examples, the composition comprising one or more PBMCs is contacted with the MYC fusion polypeptide for about 1 hour prior to step (b). In some examples, the PBMCs are washed following step (a) and prior to step (b).

[0007] In some examples, the PBMCs are cooled using a controlled-rate cryogenic freezer. In some examples, the PBMCs are cooled at a rate of about -1°C per min. In some examples, the temperature sufficient to freeze the composition is about -80°C to about -190°C.

[0008] In some examples, the method further includes thawing of the cryopreserved cells, such that the cells exhibit one or more of increased cell viability, increased cell recovery, cell activation, or increased expression of CD25 after cell activation as compared to control PBMCs not contacted with an effective amount of the MYC fusion polypeptide.

[0009] Also provided herein are method of using cells that have been cryopreserved using a MYC fusion polypeptide.

[0010] Also provided herein are kits comprising the MYC fusion polypeptides, the MYC fusion polypeptide-modified immune cells, and/or the frozen composition of the present technology of any example described herein and instructions for use.

[0011] In one aspect, the present disclosure provides an immune cell bank comprising: (a) a MYC fusion polypeptide, comprising (i) a protein transduction domain; (ii) a MYC polypeptide sequence; and (b) one or more peripheral blood mononuclear cells (PBMCs) isolated from a donor subject.

[0011a] In another aspect, the present disclosure refers to an immune cell bank comprising the frozen composition as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1 illustrates the cell viability of immune cells (*e.g.*, peripheral blood mononuclear cells (PBMC)), following pre-treatment with a PTD-MYC fusion polypeptide

wherein -X- is a molecule that links the protein transduction domain and the MYC polypeptide sequence. In some embodiments, -X- is at least one amino acid.

[0097] In some embodiments, a fusion polypeptide disclosed herein comprises (a) a protein transduction domain; (b) a MYC polypeptide sequence; (c) at least two protein tags; and (d) optionally linker(s). In some embodiments, the fusion polypeptide is a polypeptide of Formula (III-VI):

protein transduction domain-X-MYC polypeptide sequence-X-protein tag 1-X-protein tag 2
(Formula (III)), or

protein transduction domain-MYC polypeptide sequence-X-protein tag 1-X-protein tag 2
(Formula (IV)), or

protein transduction domain-MYC polypeptide sequence-protein tag 1-X-protein tag 2
(Formula (V)), or

protein transduction domain-MYC polypeptide sequence-protein tag 1-protein tag 2
(Formula (VI)),

wherein -X- is a linker. In some embodiments, -X- is one or more amino acids.

[0098] In some embodiments, a fusion polypeptide disclosed herein comprises (a) a protein transduction domain; (b) a MYC polypeptide sequence; (c) a 6-histidine tag (SEQ ID NO: 8); (d) a V5 epitope tag; and (e) optionally linker(s). In some embodiments, the fusion polypeptide is a polypeptide of Formula (VII-XIV):

protein transduction domain-X-MYC polypeptide sequence-X-6-histidine tag-X-V5 epitope tag
(Formula (VII)), or

protein transduction domain-MYC polypeptide sequence-X-6-histidine tag-X-V5 epitope tag
(Formula (VIII)), or

protein transduction domain-MYC polypeptide sequence-6-histidine tag-X-V5 epitope tag
(Formula (IX)), or

protein transduction domain-MYC polypeptide sequence-6-histidine tag-V5 epitope tag

(Formula (X)),

protein transduction domain-X-MYC polypeptide sequence-X-V5 epitope tag-X-6-histidine tag (Formula (XI)), or

protein transduction domain-MYC polypeptide sequence-X-V5 epitope tag-X-6-histidine tag (Formula (XII)), or

protein transduction domain-MYC polypeptide sequence-V5 epitope tag-X-6-histidine tag (Formula (XIII)), or

protein transduction domain-MYC polypeptide sequence-V5 epitope tag-6-histidine tag (Formula (XIV)),

wherein -X- is a linker. In some embodiments, -X- is one or more amino acids.

[0099] As noted above, in some embodiments, the MYC fusion protein comprises one or more linker sequences. The linker sequences can be employed to link the protein transduction domain, MYC polypeptide sequence, V5 epitope tag and/or 6-histidine tag (SEQ ID NO: 8) of the fusion protein. In some embodiments, the linker comprises one or more amino acids. In some embodiments, the amino acid sequence of the linker comprises KGELNSKLE (SEQ ID NO: 12). In some embodiments, the linker comprises the amino acid sequence of RTG.

Protein Transduction Domain (PTD)

[0100] In some embodiments, the MYC fusion protein includes a protein transduction domain. Peptide transport provides an alternative for delivery of small molecules, proteins, or nucleic acids across the cell membrane to an intracellular compartment of a cell. One non-limiting example and well-characterized protein transduction domain (PTD) is a TAT-derived peptide. Frankel *et al.* (*see, e.g.*, U.S. Pat. No. 5,804,604, U.S. Pat. No. 5,747,641, U.S. Pat. No. 5,674,980, U.S. Pat. No. 5,670,617, and U.S. Pat. No. 5,652,122) demonstrated transport of a cargo protein (β -galactosidase or horseradish peroxidase) into a cell by conjugating a

of example, but not by way of limitation, in some embodiments, the linker sequence is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in length.

[0104] The MYC fusion protein of the present technology can be arranged in any desired order. For example, in some embodiments, the MYC fusion protein can be arranged in order of a) the protein transduction domain linked in frame to the MYC polypeptide, b) the MYC polypeptide linked in frame to the V5 domain, and c) the V5 domain linked in frame to the 6-histidine epitope tag (SEQ ID NO: 8). In some embodiments, the MYC fusion protein has an order of components of a) the MYC polypeptide linked in frame to the protein transduction domain, b) the protein transduction domain linked in frame to the V5 domain, and c) the V5 domain linked in frame to the 6-histidine epitope tag (SEQ ID NO: 8). In some embodiments, one or more additional amino acid sequences or linkers can be included between each of the sequences. In some embodiments, additional amino acids can be included at the start and/or end of the polypeptide sequences.

[0105] In some embodiments, the protein transduction domain is a TAT protein transduction domain. In some embodiments, the protein transduction domain is TAT_[48-57]. In some embodiments, the protein transduction domain is TAT_[57-48].

Protein Tag Domains

[0106] In some embodiments, the MYC fusion protein comprises a protein tag domain that comprises one or more amino acid sequences that facilitate purification of the fusion protein. In some embodiments, the protein tag domain comprises one or more of a polyhistidine tag, and an epitope tag. By way of example, but not by way of limitation, exemplary tags include one or more of a V5, a histidine-tag (*e.g.*, a 6-histidine tag (SEQ ID NO: 8)), HA (hemagglutinin) tags, FLAG tag, CBP (calmodulin binding peptide), CYD (covalent yet dissociable NorpD peptide), Strepll, or HPC (heavy chain of protein C). In some embodiments, the protein tag domain comprises about 10 to about 20 amino acids in length. In some embodiments, the protein tag domain comprises 2 amino acids to 40 amino acids in length, for example 6-20 amino acids in length. In some embodiments, the protein tag domain comprises 2 amino acids, 3 amino acids, 4 amino acids, 5 amino acids, 6 amino acids, 7 amino acids, 8 amino acids, 9 amino acids, 10 amino acids, 11 amino acids, 12 amino acids,

13 amino acids, 14 amino acids, 15 amino acids, 16 amino acids, 17 amino acids, 18 amino acids, 19 amino acids, 20 amino acids, 21 amino acids, 22 amino acids, 23 amino acids, 24 amino acids, 25 amino acids, 26 amino acids, 27 amino acids, 28 amino acids, 29 amino acids, 30 amino acids, 31 amino acids, 32 amino acids, 33 amino acids, 34 amino acids, 35 amino acids, 36 amino acids, 37 amino acids, 38 amino acids, 39 amino acids, or 40 amino acids. In some embodiments, two of the above listed tags (for example, V5 and the HIS-tag) are used together to form the protein tag domain.

[0107] In some embodiments, the histidine tag is a 6-histidine tag (SEQ ID NO: 8). In some embodiments, the histidine tag comprises the sequence HHHHHH (SEQ ID NO:8). In some embodiments, the fusion polypeptide disclosed herein comprises a V5 epitope tag. In some embodiments, the V5 tag comprises the amino acid sequence of: GKPIPNPLLGLDST (SEQ ID NO:9). In some embodiments, the V5 tag comprises the amino acid sequence of IPNPLLGLD (SEQ ID NO:10).

[0108] The protein tags can be added to the fusion protein disclosed herein by any suitable method. By way of example, but not by way of limitation, in some embodiments, a TAT-MYC polypeptide sequence is cloned into an expression vector encoding one or more protein tags, *e.g.*, a polyHis-tag and/or a V5 tag. In some embodiments, a polyhistidine tag and/or a V5 tag is added by PCR (*i.e.*, the PCR primers comprise a polyhistidine sequence and/ or V5 sequence).

Construction of PTD-MYC fusion polypeptides

[0109] PTD-MYC fusion polypeptides (*e.g.*, TAT-MYC fusion polypeptide) disclosed herein can be constructed by methods well known in the art. By way of example, but not by way of limitation, a nucleotide sequence encoding a TAT-MYC fusion polypeptide can be generated by PCR. In some embodiments, a forward primer for a human MYC sequence comprises an in frame N-terminal 9-amino-acid sequence of the TAT protein transduction domain (*e.g.*, RKKRRQRRR (SEQ ID NO: 13)). In some embodiments, a reverse primer for a human MYC sequence is designed to remove the stop codon. In some embodiments, the PCR product is cloned into any suitable expression vector. In some embodiments, the expression vector comprises a polyhistidine tag and a V5 tag.

[0110] In some embodiments, a fusion polypeptide disclosed herein comprises (a) TAT, and (b) c-MYC. In some embodiments, a fusion polypeptide disclosed herein comprises (a) TAT_[48-57], and (b) c-MYC. In some embodiments, a fusion polypeptide disclosed herein comprises (a) TAT_[57-48], and (b) c-MYC.

[0111] In some embodiments, a fusion polypeptide disclosed herein comprises (a) TAT, (b) c-MYC, (c) linker(s), (d) V5 tag, and (e) 6-histidine tag (SEQ ID NO: 8). In some embodiments, a fusion polypeptide disclosed herein comprises (a) TAT_[48-57], (b) c-MYC, (c) linker(s), (d) V5 tag, and (e) 6-histidine tag (SEQ ID NO: 8). In some embodiments, a fusion polypeptide disclosed herein comprises (a) TAT_[57-48], (b) c-MYC, (c) linker(s), (d) V5 tag, and (e) 6-histidine tag (SEQ ID NO: 8).

[0112] In some embodiments, the PTD-MYC fusion polypeptide comprises SEQ ID NO: 1; in some embodiments, the PTD-MYC fusion polypeptide is SEQ ID NO: 1.

MRKKRRQRRRPLNVSTNRNYDL DYDSVQPYFYCDEEENFYQQQQQSELQPPAPSE
DIWKKFELLPTPPLSPSRRSGLCSPSYVAVTPFSLRGDNDGGGGSFSTADQLEMVTEL
LGGMVNVQSFICDPDDETFIKNIIQDCMWSGFSAAKLVSEKLASYQAARKDSGSP
NPARGHSVCSTSSLYLQDLSAAASECIDPSVVFYPYPLNDSSSPKSCASQDSSAFSPSSD
SLLSSTESSPQGSPEPLVLHEETPPTTSSDSEEEQEDEEEIDVVSVEKRQAPGKRSESGS
PSAGGHSKPPHSPLVLKRCHVSTHQHNYAAPSTRKDYPAAKRVKLDSVRVLRQISN
NRKCTSPRSSDTEENVKRRTHNVLERQRRNELKRSFFALRDQIPELENNEKAPKVVIL
KKATAYILSVQAEEQKLISEEDLLRKRREQLKHKLEQLRKGELENSKLEGKPIPNPLLG
LDSTRTGHHHHHH (SEQ ID NO: 1).

[0113] The fusion protein can be modified during or after synthesis to include one or more functional groups. By way of example but not by way of limitation, the protein can be modified to include one or more of an acetyl, phosphate, acetate, amide, alkyl, and/or methyl group. This list is not intended to be exhaustive, and is exemplary only. In some embodiments, the protein includes at least one acetyl group.

[0114] A PTD-MYC fusion polypeptide can be generated by any suitable method known the art, *e.g.* by recombinant protein expression in a cell, such as a bacterial cell, an insect cell, or mammalian cell. In some embodiments, a PTD-MYC fusion polypeptide is recombinantly produced by microbial fermentation. In some embodiments microbial fermentation is

[0120] The PTD-MYC fusion polypeptide is typically expressed as cytosolic inclusion bodies in microbial cells. To harvest inclusion bodies, a cell pellet is collected by centrifugation of the fermentation culture following induction, frozen at -70°C or below, thawed and resuspended in disruption buffer. The cells are lysed by conventional methods, *e.g.*, sonication, homogenization, etc. The lysate is then resuspended in solubilization buffer, usually in the presence of urea at a concentration effective to solubilize proteins, *e.g.*, from around about 5M, 6M, 7M, 8M, 9M or greater. Resuspension can require mechanically breaking apart the pellet and stirring to achieve homogeneity. In some embodiments, the cell pellet is directly resuspended in urea buffer and mixed until homogenous. In some embodiments, the resuspension/solubilization buffer is 8M Urea, 50 mM Phosphate pH 7.5 and the suspension is passed through a homogenizer.

[0121] In some embodiments, the homogenized suspension is sulfonylated. For example, in some embodiments, the homogenized suspension is adjusted to include 200 mM Sodium Sulfite and 10 mM Sodium Tetrathionate. The solution is then mixed at room temperature until homogeneous. The mixed lysate is then mixed for an additional period of time to complete the sulfonylation (*e.g.*, at 2-8°C for ≥ 12 hours). The sulfonylated lysate was then centrifuged for an hour. The supernatant containing the sulfonylated PTD-MYC fusion polypeptides is then collected by centrifugation and the cell pellet discarded. The supernatant is then passed through a filter, *e.g.*, 0.22 μ m membrane filter to clarify the lysate.

[0122] The solubilized protein is then purified. Purification methods can include affinity chromatography, reverse phase chromatography, gel exclusion chromatography, and the like. In some embodiments, affinity chromatography is used. For example, the protein is provided with an epitope tag or histidine 6 tag (SEQ ID NO: 8) for convenient purification. In the present methods, exemplary PTD-MYC fusion polypeptide comprise histidine 6 tag (SEQ ID NO: 8) for purification using Ni affinity chromatography using Ni- resin.

[0123] In exemplary embodiments, the Ni- resin column is equilibrated in a buffer containing urea. In some embodiments, the equilibration buffer is 6M Urea, 50 mM Phosphate, 500 mM NaCl, and 10% Glycerol solution. The sulfonylated and clarified supernatant comprising the PTD-MYC fusion polypeptide is then loaded onto the Ni- resin column. The column is then washed with a wash buffer, *e.g.*, 6M Urea, 50mM Phosphate,