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(54) **Title**: MARROW INFILTRATING LYMPHOCYTES (MILS) AS A SOURCE OF T-CELLS FOR CHIMERIC ANTIGEN RECEPTOR (CAR) THERAPY

(57) **Abstract**: In some embodiments, marrow-infiltrating lymphocytes ("MILs") comprising a chimeric antigen receptor ("CAR") are provided. In some aspects, the embodiments relate to a method for making a recombinant MIL, comprising obtaining bone marrow comprising MILs; and transfecting, transforming, or transducing the MILs with a nucleic acid encoding a chimeric antigen receptor. In some aspects, the embodiments relate to a method for treating a condition in a subject, comprising administering to the subject a MIL comprising a CAR.

# MARROW INFILTRATING LYMPHOCYTES (MILs) AS A SOURCE OF T-CELLS FOR CHIMERIC ANTIGEN RECEPTOR (CAR) THERAPY

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/189,928, filed July 8, 2015, which is hereby incorporated by reference in its entirety.

## BACKGROUND

The large majority of patients with malignancies will die from their disease. One approach to treating these patients is to genetically modify MILs to target antigens expressed on tumor cells through the expression of chimeric antigen receptors ("CARs"). CARs are antigen receptors that are designed to recognize cell surface antigens in a human leukocyte antigen-independent manner. Outside of the successes with CD19-targeted approaches, attempts at using genetically modified cells expressing CARs to treat other malignancies have met with limited success.

## SUMMARY

In some embodiments, marrow-infiltrating lymphocytes ("MIL") comprising a chimeric antigen receptor ("CAR") are provided. In some embodiments, the CAR comprises an extracellular domain that can bind a ligand. In some embodiments, the CAR comprises an intracellular domain that can initiate an intracellular signaling cascade (*e.g.*, in the MIL).

In some embodiments, methods for treating a condition in a subject, comprising administering to the subject a MIL comprising a CAR are provided. In some embodiments, the method comprises administering to the subject a composition comprising a population of MILs, wherein each MIL of the population of MILs comprises a CAR.

In some embodiments, methods for making a recombinant MIL, comprising obtaining bone marrow comprising MILs; and transfecting, transforming, or transducing the MILs with a nucleic acid encoding a chimeric antigen receptor are provided. The bone marrow may be obtained from a subject, such as a subject with a neoplasm. The subject may be a human or a mouse.

## DETAILED DESCRIPTION

In some embodiments, provided herein are compositions and methods for treating cancer including but not limited to hematologic malignancies and solid tumors. Aspects relate to, but are not limited to, a strategy of adoptive cell transfer of marrow-infiltrating lymphocytes (MILs) transduced to express a chimeric antigen receptor (CAR). CARs are molecules that combine antibody-based specificity for a desired antigen (*e.g.*, tumor antigen) with a MIL receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-tumor cellular immune activity.

In some embodiments, the use of MILs genetically modified to stably express a desired CAR are provided. MILs expressing a CAR are referred to herein as CAR-MILs or CAR-modified MILs. In some embodiments, the cell can be genetically modified to stably express an antibody binding domain on its surface, conferring novel antigen specificity that is MHC independent. In some embodiments, the MIL is genetically modified to stably express a CAR that combines an antigen recognition domain of a specific antibody with an intracellular domain of the CD3ζ chain or FcγRI protein into a single chimeric protein.

In some embodiments, the CAR comprises an extracellular domain having an antigen recognition domain, a transmembrane domain, and a cytoplasmic domain. In some embodiments, the transmembrane domain that naturally is associated with one of the domains in the CAR is used. In some embodiments, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex. For example, the transmembrane domain may be a CD8α hinge domain.

With respect to the cytoplasmic domain, a CAR, for example, can be designed to comprise the CD28 and/or 4-1BB signaling domain by itself or be combined with any other desired cytoplasmic domain(s) useful in the context of the CAR. In some embodiments, the cytoplasmic domain of the CAR can be designed to further comprise the signaling domain of CD3ζ. For example, the cytoplasmic domain of the CAR can include but is not limited to CD3ζ, 4-1BB, and CD28 signaling modules, and combinations thereof. Accordingly, the embodiments provides CAR-MILs and methods of their use for adoptive therapy.

In some embodiments, the CAR-MILs can be generated by introducing a lentiviral vector comprising a desired CAR (*e.g.*, a CAR comprising anti-CD19, transmembrane

domain, and human 4-1BB) into the cells. The CAR-MILs are, for example, able to replicate *in vivo* resulting in long-term persistence that can lead to sustained tumor control.

In some embodiments, administering a genetically-modified MIL expressing a CAR for the treatment of a patient having a neoplasm using an infusion of CAR-MILs are provided. In some embodiments, autologous infusions are used in the treatment. Autologous MILs are collected from a patient in need of treatment and are activated and expanded using methods described herein and known in the art and then infused back into the patient.

In some embodiments, MILs expressing an anti-CD19 CAR including both CD3ζ and the 4-1BB costimulatory domain are used. In some instances, the CAR MILs infused into a patient can eliminate leukemia cells *in vivo* in patients. However, the embodiments are not limited to MILs that target CD19 or signal through CD3ζ and/or 4-1BB mediated pathways. For example, the embodiments include any antigen-binding moiety fused with one or more intracellular domains selected from the group consisting of a CD137 (4-1BB) signaling domain, a CD28 signaling domain, a CD3ζ signal domain, and any combination thereof.

DEFINITIONS

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

As used in this document, terms "comprise," "have," "has," and "include" and their conjugates, as used herein, mean "including but not limited to." While various compositions, and methods are described in terms of "comprising" various components or steps (interpreted as meaning "including, but not limited to"), the compositions, methods, and devices can also "consist essentially of" or "consist of" the various components and steps, and such terminology should be interpreted as defining essentially closed-member groups.

"Activation", as used herein, refers to the state of a MIL that has been sufficiently stimulated to induce detectable cellular proliferation. Activation can also be associated with induced cytokine production, and detectable effector functions. The term "activated MILs" refers to, among other things, MILs that are undergoing cell division.

The term "antibody," as used herein, refers to an immunoglobulin molecule which specifically binds with an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. The antibodies may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)2, as well as single chain antibodies and humanized antibodies.

The term "antibody fragment" refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. Examples of antibody fragments include, but are not limited to, Fab, Fab′, F(ab′)2, and Fv fragments, linear antibodies, scFv antibodies, and multispecific antibodies formed from antibody fragments.

The term "antigen" as used herein is defined as a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an "antigen" as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene. It is readily apparent that the embodiments include, but are not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are arranged in various combinations to elicit the desired immune response. Moreover, a skilled artisan will understand that an antigen need not be encoded by a "gene" at all. It is readily apparent that an antigen can be generated synthesized or can be derived from a biological sample. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a biological fluid.

The term "anti-tumor effect" as used herein, refers to a biological effect that can be manifested by a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in the number of metastases, an increase in life expectancy, or amelioration of various physiological symptoms associated with the cancerous condition. An "anti-tumor effect" can also be manifested by the ability of the peptides, polynucleotides, cells and antibodies to prevent the occurrence of tumor in the first place.

The term "auto-antigen" means any self-antigen which is mistakenly recognized by the immune system as being foreign. Auto-antigens comprise, but are not limited to, cellular proteins, phosphoproteins, cellular surface proteins, cellular lipids, nucleic acids, glycoproteins, including cell surface receptors.

The term "autoimmune disease" as used herein is defined as a disorder that results from an autoimmune response. An autoimmune disease is the result of an inappropriate and excessive response to a self-antigen. Examples of autoimmune diseases include but are not limited to, Addision's disease, alopecia greata, ankylosing spondylitis, autoimmune hepatitis, autoimmune parotitis, Crohn's disease, diabetes (Type I), dystrophic epidermolysis bullosa, epididymitis, glomerulonephritis, Graves' disease, Guillain-Barr syndrome, Hashimoto's disease, hemolytic anemia, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis, pemphigus vulgaris, psoriasis, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, spondyloarthropathies, thyroiditis, vasculitis, vitiligo, myxedema, pernicious anemia, ulcerative colitis, among others.

As used herein, the term "autologous" is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

"Allogeneic" refers to a graft derived from a different animal of the same species.

"Xenogeneic" refers to a graft derived from an animal of a different species.

The term "cancer" as used herein is defined as disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers include but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer and the like.

"Co-stimulatory ligand," as the term is used herein, includes a molecule on an antigen presenting cell (*e.g.*, an aAPC, dendritic cell, B cell, and the like) that specifically binds a cognate co-stimulatory molecule on a MIL, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a MIL response, including, but not limited to, proliferation, activation, differentiation, and the like. A co-stimulatory ligand can include, but is not limited to, CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD70, CD83, HLA-G, MICA, MICB, HVEM, lymphotoxin beta receptor,

3/TR6, ILT3, ILT4, HVEM, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also encompasses, inter alia, an antibody that specifically binds with a co-stimulatory molecule present on a MIL, such as, but not limited to, CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83.

A "co-stimulatory molecule" refers to the cognate binding partner on a MIL that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the MIL, such as, but not limited to, proliferation. Co-stimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and a Toll ligand receptor.

A "co-stimulatory signal", as used herein, refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to MIL proliferation and/or upregulation or downregulation of key molecules.

A "disease" is a state of health of a subject wherein the subject cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate. In contrast, a "disorder" in a subject is a state of health in which the subject is able to maintain homeostasis, but in which the subject's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the subject's state of health.

An "effective amount" as used herein, means an amount which provides a therapeutic or prophylactic benefit.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

As used herein "endogenous" refers to any material from or produced inside an organism, cell, tissue or system.

As used herein, the term "exogenous" refers to any material introduced from or produced outside an organism, cell, tissue or system.

The term "expression" as used herein is defined as the transcription and/or translation of a particular nucleotide sequence driven by its promoter.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an *in vitro* expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

"Homologous" refers to the sequence similarity or sequence identity between two polypeptides or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, *e.g.*, if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared ×100. For example, if 6 of 10 of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

The term "immunoglobulin" or "Ig," as used herein is defined as a class of proteins, which function as antibodies. Antibodies expressed by B cells are sometimes referred to as the BCR (B cell receptor) or antigen receptor. The five members included in this class of proteins are IgA, IgG, IgM, IgD, and IgE.

"Isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

As used herein, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

A "lentivirus" as used herein refers to a genus of the Retroviridae family. Lentiviruses are unique among the retroviruses in being able to infect non-dividing cells; they can deliver a significant amount of genetic information into the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. HIV, SIV, and FIV are all examples of lentiviruses. Vectors derived from lentiviruses offer the means to achieve significant levels of gene transfer *in vivo*.

The term "marrow infiltrating lymphocyte" ("MIL") as used herein refers to a lymphocyte derived from the bone marrow. Marrow infiltrating lymphocytes ("MILs") have many distinguishable differences from peripheral blood lymphocytes as well as tumor infiltrating lymphocytes ("TILs"). The bone marrow ("BM") microenvironment is a special immunologic niche due to the richness of antigen presenting cells ("APC"). The presence of these antigen presenting cells allows for the processing and presenting of antigen to sustain the higher levels of central memory cells that are found in the bone marrow compartment. (Li JM et al J Immunol. 2009 Dec 15;183(12):7799-809). These MILs express memory markers such as CD45RO+ and CD62L+ and there are more memory MILs than memory cells found in the PBL. (Noonan K et al Clin Cancer Res. 2012 Mar 1;18(5):1426-34). Furthermore, MILs are not just the "TILs" of hematologic malignancies because of their ability to continuously prime memory cells to antigen (Beckhove P et al J Clin Invest. 2004 Jul 1; 114(1): 67–76; Castiglioni P et al 6 J Immunol 2008; 180:4956-4964). MILs also express more CXCR4 than their PBL counterparts due to the cognate antigen stromal derived factor type 1 ("SDF1") that is expressed in great amounts in the bone marrow stroma (Noonan K et al Cancer Res. 2005 Mar 1;65(5):2026-34). The expression of 41BB is also increased in MILs compared to PBLs, likely due to the hypoxic nature of the BM micro-environment. Further, MILs can be harvested and expanded from all patients, in contrast with TILs(Noonan, K et al Sci Transl Med. 2015 May 20;7(288):288ra78). TILs are found in only about 50% of patients, and only about 25% of patients comprise expandable TILs. In contrast to peripheral blood lymphocytes (PBLs), MILs possess a broad endogenous antigenic repertoire which account for their intrinsic tumor specificity – a feature which is completely absent in PBLs (Noonan et al Clin Cancer Res).

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

The term "operably linked" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

The term "overexpressed" tumor antigen or "overexpression" of the tumor antigen is intended to indicate an abnormal level of expression of the tumor antigen in a cell from a disease area like a solid tumor within a specific tissue or organ of the patient relative to the level of expression in a normal cell from that tissue or organ. Patients having solid tumors or a hematological malignancy characterized by overexpression of the tumor antigen can be determined by standard assays known in the art.

"Parenteral" administration of an immunogenic composition includes, *e.g.*, subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

The terms "patient," "subject," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether *in vitro* or in situ, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as

proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

The term "promoter" as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

A "constitutive" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

By the term "stimulation," is meant a primary response induced by binding of a stimulatory molecule (*e.g.*, a TCR/CD3 complex) with its cognate ligand thereby mediating a signal transduction event, such as, but not limited to, signal transduction via the TCR/CD3 complex. Stimulation can mediate altered expression of certain molecules, such as downregulation of TGF-β, and/or reorganization of cytoskeletal structures, and the like.

A "stimulatory molecule," as the term is used herein, means a molecule on a MIL that specifically binds with a cognate stimulatory ligand present on an antigen presenting cell.

A "stimulatory ligand," as used herein, means a ligand that when present on an antigen presenting cell (*e.g.*, an aAPC, a dendritic cell, a B-cell, and the like) can specifically bind with a cognate binding partner (referred to herein as a "stimulatory molecule") on a MIL, thereby mediating a primary response by the MIL, including, but not limited to, activation, initiation of an immune response, proliferation, and the like. Stimulatory ligands are well-known in the art and encompass, inter alia, an MHC Class I molecule loaded with a peptide, an anti-CD3 antibody, a superagonist anti-CD28 antibody, and a superagonist anti-CD2 antibody.

The term "subject" is intended to include living organisms in which an immune response can be elicited (*e.g.*, mammals). Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof.

The term "therapeutic" as used herein means a treatment and/or prophylaxis. A therapeutic effect is obtained by suppression, remission, or eradication of a disease state.

The term "therapeutically effective amount" refers to the amount of the subject compound that will elicit the biological or medical response of a tissue, system, or subject that is being sought by the researcher, veterinarian, medical doctor, or other clinician. The term "therapeutically effective amount" includes that amount of a compound that, when administered, is sufficient to prevent development of, or alleviate to some extent, one or more of the signs or symptoms of the disorder or disease being treated. The therapeutically effective amount will vary depending on the compound, the disease and its severity and the age, weight, etc., of the subject to be treated.

To "treat" a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject.

The term "transfected" or "transformed" or "transduced" as used herein refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A "transfected" or "transformed" or "transduced" cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

The phrase "under transcriptional control" or "operatively linked" as used herein means that the promoter is in the correct location and orientation in relation to a

polynucleotide to control the initiation of transcription by RNA polymerase and expression of the polynucleotide.

A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like . Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

DESCRIPTION

The present disclosure provides compositions and methods for treating cancer among other diseases. The cancer may be a hematological malignancy, a solid tumor, a primary or a metastasizing tumor. The cancer may be a hematological malignancy, such as Chronic Lymphocytic Leukemia ("CLL"). Other diseases treatable using the compositions and methods described and provided for herein include viral, bacterial, and parasitic infections as well as autoimmune diseases.

In some embodiments, the cell (*i.e.*, MIL) engineered to express a CAR wherein the CAR-MIL exhibits an antitumor property is provided. The CAR can, for example, be engineered to comprise an extracellular domain having an antigen-binding domain fused to an intracellular signaling domain of the MIL antigen receptor complex ζ chain (*e.g.*, CD3ζ). The CAR, for example, when expressed in a MIL, is able to redirect antigen recognition based on the antigen-binding specificity. In some embodiments, the antigen is CD19 because this antigen is expressed on malignant B cells. However, the embodiments are not limited to targeting CD19. Rather, the embodiments include any antigen-binding moiety that when bound to its cognate antigen, affects a tumor cell so that the tumor cell fails to grow, is prompted to die, or otherwise is affected so that the tumor burden in a patient is diminished or eliminated. The antigen-binding moiety may be fused with an intracellular domain from one or more of a costimulatory molecule and a ζ chain. In some embodiments, the antigen-binding moiety is fused with one or more intracellular domains selected from the group of a CD137 (4-1BB) signaling domain, a CD28 signaling domain, a

CD3ζ signal domain, and any combination thereof. The antigen-binding moiety may also be fused with an intracellular domain such as CD134 (OX40).

In some embodiments, the CAR comprises a CD137 (4-1BB) signaling domain. Without being bound to any particular theory, this is because the embodiments are partly based on the discovery that CAR-mediated T-cell responses can be further enhanced with the addition of costimulatory domains.

## I. CHIMERIC ANTIGEN RECEPTORS

Provided herein are chimeric antigen receptors (CARs) comprising an extracellular and intracellular domain. The extracellular domain comprises a target-specific binding element otherwise referred to as an antigen-binding moiety. The intracellular domain or otherwise the cytoplasmic domain may comprise a costimulatory signaling region and/or a portion of a ζ chain. The costimulatory signaling region refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. Costimulatory molecules are cell surface molecules other than antigens receptors or their ligands that are required for an efficient response of lymphocytes to antigen.

A spacer domain may be incorporated between the extracellular domain and the transmembrane domain of the CAR or between the cytoplasmic domain and the transmembrane domain of the CAR. As used herein, the term "spacer domain" generally means a stretch of amino acids that functions to link the transmembrane domain to either the extracellular domain or the cytoplasmic domain in the polypeptide chain. A spacer domain may comprise up to 300 amino acids, preferably 2 to 100 amino acids, such as 25 to 50 amino acids.

## II. EXTRACELLULAR DOMAINS

In some embodiments, the CAR comprises a target-specific binding element otherwise referred to as an antigen-binding moiety. The choice of moiety depends upon the type and number of ligands that define the surface of a target cell. For example, the antigen-binding domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Thus, examples of cell surface markers that may act as ligands for the antigen-binding domain in a CAR include those associated with viral, bacterial, and parasitic infections, autoimmune disease, and

cancer cells. For example, the ligand may be the protein of a bacterium, virus, or parasite. Similarly, the ligand may be a protein that is upregulated on the surface of a cancer cell.

In some embodiments, a CAR can be engineered to target a tumor antigen of interest by way of engineering a desired antigen-binding moiety that specifically binds to an antigen on a tumor cell. As used herein, "tumor antigen" or "hyperporoliferative disorder antigen" or "antigen associated with a hyperproliferative disorder," refers to antigens that are common to specific hyperproliferative disorders such as cancer. The antigens discussed herein are merely included by way of example. The list is not intended to be exclusive and further examples will be readily apparent to those of skill in the art.

Tumor antigens are proteins that are produced by tumor cells that elicit an immune response, particularly T-cell mediated immune responses. The selection of the antigen-binding moiety will depend on the particular type of cancer to be treated. Tumor antigens are well known in the art and include, for example, a glioma-associated antigen, carcinoembryonic antigen (CEA), β-human chorionic gonadotropin, alpha-fetoprotein (AFP), lectin-reactive AFP, thyroglobulin, RAGE-1, MN-CA IX, human telomerase reverse transcriptase, RU1, RU2 (AS), intestinal carboxyl esterase, mutant hsp70-2, M-CSF, prostase, prostate-specific antigen (PSA), PAP, NY-ESO-1, LAGE-1a, p53, prostein, PSMA, Her2/neu, survivin, telomerase, prostate-carcinoma tumor antigen-1 (PCTA-1), MAGE, ELF2M, neutrophil elastase, ephrinB2, CD22, insulin growth factor (IGF)-I, IGF-II, IGF-I receptor, and mesothelin.

In some embodiments, the tumor antigen comprises one or more antigenic cancer epitopes associated with a malignant tumor. Malignant tumors express a number of proteins that can serve as target antigens for an immune attack. These molecules include but are not limited to tissue-specific antigens such as MART-1, tyrosinase, and GP 100 in melanoma and prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA) in prostate cancer. Other target molecules belong to the group of transformation-related molecules such as the oncogene HER-2/Neu/ErbB-2. Yet another group of target antigens are onco-fetal antigens such as carcinoembryonic antigen (CEA). In B-cell lymphoma, the tumor-specific idiotype immunoglobulin constitutes a truly tumor-specific immunoglobulin antigen that is unique to the individual tumor. B-cell differentiation antigens such as CD19, CD20 and CD37 are other candidates for target antigens in B-cell lymphoma. Some of these antigens (*e.g.*, CEA, HER-2, CD19, CD20, idiotype) have been used as targets for passive immunotherapy with monoclonal antibodies with limited success.

The type of tumor antigen referred to may also be a tumor-specific antigen (TSA) or a tumor-associated antigen (TAA). A TSA is unique to tumor cells and does not occur on other cells in the body. A TAA associated antigen is not unique to a tumor cell and instead is also expressed on a normal cell under conditions that fail to induce a state of immunologic tolerance to the antigen. The expression of the antigen on the tumor may occur under conditions that enable the immune system to respond to the antigen. TAAs may be antigens that are expressed on normal cells during fetal development when the immune system is immature and unable to respond or they may be antigens that are normally present at extremely low levels on normal cells but which are expressed at much higher levels on tumor cells.

Non-limiting examples of TSA or TAA antigens include the following: Differentiation antigens such as MART-1/MelanA (MART-I), gp100 (Pmel 17), tyrosinase, TRP-1, TRP-2 and tumor-specific multilineage antigens such as MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, p15; overexpressed embryonic antigens such as CEA; overexpressed oncogenes and mutated tumor-suppressor genes such as p53, Ras, HER-2/neu; unique tumor antigens resulting from chromosomal translocations; such as BCR-ABL, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR; and viral antigens, such as the Epstein Barr virus antigens EBVA and the human papillomavirus (HPV) antigens E6 and E7. Other large, protein-based antigens include TSP-180, MAGE-4, MAGE-5, MAGE-6, RAGE, NY-ESO, p185erbB2, p180erbB-3, c-met, nm-23H1, PSA, TAG-72, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, beta-Catenin, CDK4, Mum-1, p 15, p 16, 43-9F, 5T4, 791Tgp72, alpha-fetoprotein, beta-HCG, BCA225, BTAA, CA 125, CA 15-3\CA 27.29\BCAA, CA 195, CA 242, CA-50, CAM43, CD68\P1, CO-029, FGF-5, G250, Ga733\EpCAM, HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB/70K, NY-CO-1, RCAS1, SDCCAG16, TA-90\Mac-2 binding protein\cyclophilin C-associated protein, TAAL6, TAG72, TLP, and TPS.

In a some embodiments, the antigen-binding moiety portion of the CAR targets an antigen that includes but is not limited to CD19, CD20, CD22, ROR1, Mesothelin, CD33/IL3Ra, c-Met, PSMA, Glycolipid F77, EGFRvIII, GD-2, MY-ESO-1 TCR, MAGE A3 TCR, and the like.

Depending on the desired antigen to be targeted, a CAR can be engineered to include the appropriate antigen bind moiety that is specific to the desired antigen target. For example, if CD19 is the desired antigen that is to be targeted, an antibody for CD19 can

be used as the antigen bind moiety for incorporation into the CAR. Thus, in some embodiments, the antigen-binding moiety portion of the CAR targets CD19.

The extracellular domain of a CAR may comprise, for example, a single-chain variable fragment ("scFv") that binds to any one of the targets described herein.

The extracellular domain can be any antigen-binding polypeptide, a wide variety of which are known in the art. In some instances, the antigen-binding domain is a single chain Fv ("scFv"). Other antibody based recognition domains (cAb VHH (camelid antibody variable domains) and humanized versions, IgNAR VH (shark antibody variable domains) and humanized versions, sdAb VH (single domain antibody variable domains) and "camelized" antibody variable domains are suitable for use. In some instances, T-cell receptor (TCR) based recognition domains such as single chain TCR (scTv, single chain two-domain TCR containing ν νβ) are also suitable for use.

Other extracellular domains known in the art may also be used in embodiments (*see, e.g.*, PCT Patent Application Publication No. WO 2014/127261; U.S. Patent No. 8,975,071, hereby incorporated by reference).

III. TRANSMEMBRANE DOMAINS

With respect to the transmembrane domain, the CAR can be designed to comprise a transmembrane domain that is fused to the extracellular domain of the CAR. In some embodiments, the transmembrane domain that naturally is associated with one of the domains in the CAR is used. In some instances, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

The transmembrane domain may be derived either from a natural source or the transmembrane domain may be designed (*e.g.*, from a stretch of 18 to 30 hydrophobic amino acids, such as alanine, valine, leucine, and isoleucine, which form an α-helix). Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions of particular use may be derived from (*i.e.*, comprise at least the transmembrane region(s) of) the α, β, or ζ chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, or CD154. Alternatively the transmembrane domain may be designed, in which case it will comprise predominantly hydrophobic residues such

as leucine and valine. For a designed transmembrane domain, phenylalanine, tryptophan, and/or tyrosine may be found near the membrane/water interface. Optionally, a short oligo- or polypeptide linker between 2 and 10 amino acids in length may link the transmembrane domain and the cytoplasmic signaling domain of the CAR. A glycine-serine spacer provides a particularly suitable linker.

IV. INTRACELLULAR DOMAIN

The cytoplasmic domain or otherwise the intracellular signaling domain of the CAR is responsible for activation of at least one of the normal effector functions of a MIL. The term "effector function" refers to a specialized function of a cell. An effector function of a MIL, for example, may be cytolytic activity or helper activity including the secretion of cytokines. Thus the term "intracellular signaling domain" refers to the portion of a protein which transduces the effector function signal and directs the cell to perform a specialized function. While an entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire intracellular domain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The term intracellular signaling domain is thus meant to include any truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal.

Some non-limiting examples of intracellular signaling domains for use in the CAR include the cytoplasmic sequences of the T-cell receptor (TCR) and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or variant of these sequences and any synthetic sequence that has the same functional capability.

Signals generated through the TCR alone are insufficient for full activation of a lymphocyte, and a secondary or co-stimulatory signal is also required. Thus, MIL activation is mediated by two distinct classes of cytoplasmic signaling: those that initiate antigen-dependent primary activation through the TCR (primary cytoplasmic signaling sequences) and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal (secondary cytoplasmic signaling sequences).

Primary cytoplasmic signaling sequences regulate primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary cytoplasmic

signaling sequences that act in a stimulatory manner may contain signaling motifs that are known as immunoreceptor tyrosine-based activation motifs or ITAMs.

Examples of ITAM containing primary cytoplasmic signaling sequences that can be used include, but are not limited to, those derived from TCR ζ, FcR gamma, FcR beta, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b, and CD66d. In some embodiments, the cytoplasmic signaling molecule of the CAR comprises a cytoplasmic signaling sequence derived from CD3ζ.

In some embodiments, the cytoplasmic domain of the CAR can be designed to comprise the CD3ζ signaling domain by itself or combined with any other desired cytoplasmic domain(s) useful in the context of a CAR. For example, the cytoplasmic domain of the CAR may comprise a portion of a CD3ζ chain and a costimulatory signaling region. The costimulatory signaling region refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. In some embodiments, the costimulatory molecule is a cell surface molecule other than an antigen receptor or their ligands that is required for an efficient response by lymphocytes to an antigen. Examples of such molecules include CD27, CD28, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and the like. Thus, while some embodiments may be exemplified with 4-1BB as the co-stimulatory signaling element, other costimulatory elements can also be used.

The cytoplasmic signaling sequences within the cytoplasmic signaling portion of a CAR may be linked to each other in a random or specified order. Optionally, short oligo- or polypeptide linkers, preferably between 2 and 10 amino acids in length may form the linkage. A glycine-serine spacer provides a particularly suitable linker.

In some embodiments, the cytoplasmic domain is designed to comprise the signaling domain of CD3ζ and the signaling domain of CD28. In some embodiments, the cytoplasmic domain is designed to comprise the signaling domain of CD3ζ and the signaling domain of 4-1BB. In some embodiments, the cytoplasmic domain is designed to comprise the signaling domain of CD3ζ and the signaling domain of CD28 and 4-1BB.

In some embodiments, the cytoplasmic domain in the CAR is designed to comprise the signaling domain of 4-1BB and the signaling domain of CD3ζ.

V. VECTORS

The expression of natural or synthetic nucleic acids encoding CARs is typically achieved by operably linking a nucleic acid encoding the CAR polypeptide or portions thereof to a promoter, and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration eukaryotes. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

Vectors derived from retroviruses such as the lentivirus are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors have the added advantage over vectors derived from onco-retroviruses such as murine leukemia viruses in that they can transduce non-proliferating cells, such as hepatocytes. They also have the added advantage of low immunogenicity.

The nucleic acid sequences coding for the desired molecules can be obtained using recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the gene of interest can be produced synthetically, rather than cloned.

The expression constructs may also be used for nucleic acid immunization and gene therapy, using standard gene delivery protocols. Methods for gene delivery are known in the art (*see, e.g.*, U.S. Pat. Nos. 5,399,346, 5,580,859, 5,589,466, hereby incorporated by reference). In some embodiments, the embodiments provide a gene therapy vector. The nucleic acid sequence may also be inserted using gene editing techniques such as, but not limited to, CRISPR.

The nucleic acid can be cloned into a number of types of vectors. For example, the nucleic acid can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

An expression vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Green & Sambrook (Molecular Cloning: A Laboratory Manual, (4th ed., 2012)), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and

lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers, (*e.g.*, WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193).

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. In some embodiments, adenovirus vectors are used. In some embodiments, lentivirus vectors are used.

Additional regulatory elements (*e.g.*, promoters and enhancers) regulate the frequency of transcriptional initiation. Typically, these are located 30-100,000 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements is frequently flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased by 50 bp apart before activity begins to decline. Depending on the promoter, individual elements can function either cooperatively or independently to activate transcription.

One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. Another example of a suitable promoter is Elongation Growth Factor-1α (EF-1α). However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the creatine kinase promoter. The promoters are not limited to constitutive promoters. Inducible promoters can also be used. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence that is operatively linked when such expression is desired, or turning off the expression when

expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

In order to assess the expression of a CAR polypeptide or portions thereof, the expression vector to be introduced into a cell can 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 be transfected or infected through viral vectors. In other aspects, 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 regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, *e.g.*, enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (*e.g.*, Ui-Tei et al., 2000 FEBS Letters 479: 79-82). In some embodiments, the reporter gene is mCherry.

Methods of introducing and expressing genes into a cell are well known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, *e.g.*, mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art (*see, e.g.*, Green & Sambrook, Molecular Cloning: A Laboratory Manual, (4th ed., 2012)).

Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors,

have become a widely used method for inserting genes into mammalian cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses, adeno-associated viruses, and the like (*see, e.g.*, U.S. Pat. Nos. 5,350,674 and 5,585,362).

Chemical means for introducing a nucleic acid into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (*e.g.*, an artificial membrane vesicle).

In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (*in vitro*, *ex vivo*, or *in vivo*). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained, or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA, or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape.

## VI. MARROW INFILTRATING LYMPHOCYTES

Prior to expansion and genetic modification of the MILs, a source of MILs is obtained from a subject. In patients with any of a number of types of cancer, including hematologic malignancies and solid tumors, T cells can easily be obtained from the bone marrow microenvironment with heightened tumor specificity as compared to peripheral blood (*see, e.g.*, U.S. Patent Application Publication No. U.S. 2011/0223146, hereby incorporated by reference). By comparing T cells obtained from these two different compartments from a subject having a hematological malignancy, oligoclonal restriction of marrow infiltrating lymphocytes (MILs) obtained from marrow aspirates is observed. Methods, such as those including anti-CD3/CD28 antibody-conjugated magnetic beads,

may be used to activate and expand the bone marrow cells *in vitro* to generate activated MILs. The activated MILs show a greater expansion and enhanced tumor activity as compared to peripheral blood lymphocytes in all patients examined. These findings suggest that: 1) the marrow is a reservoir of tumor-specific T cells; 2) MILs can be activated and expanded in all patients studied (as compared to the limited numbers observed in metastatic melanoma); 3) these cells traffic to the bone marrow upon infusion; 4) persist for up to 200 days following adoptive transfer in NOD/SCID mice; and that 5) activated MILs are capable of eradicating pre-established disease and targeting myeloma stem cell precursors thus implying a broad antigenic recognition.

The T-cells, which represent a minority of the total bone marrow cell population may be expanded in the presence of almost complete bone marrow. To assure maximal tumor -T cell contact, the aspirated bone marrow may be fractionated on a Lymphocyte Separation Medium density gradient and cells may be collected almost to the level of the red cell pellet. This separation method removes substantially only the red blood cells and the neutrophils, providing nearly complete bone marrow, and results in the collection of both T cells as well as tumor cells. T-cells may be expanded without a T-cell specific separation step, and without a tumor cell separation step. Cell type specific separation steps include, for example, cell labeling using antibodies or other cell-type specific detectable labels, and sorting using fluorescence activated cell sorting (FACS). In some embodiments, the methods can be practiced without such labeling and cell sorting methods.

For activation with beads, bead-T cell contact is preferably maximized during the first 24-48 hours of culture. As the T-cells represent only a minority of the total cells in the population, contact of the T-cells with the antibody coated beads is promoted by the use of a sufficient number of beads to cells, in the range of about 1:1 to about 5:1 beads to cells, preferably about 2:1 to 4:1 beads to cells, more preferably about 2.5:1 to 3.5: 1 beads to cells. These ratios are applicable for the disclosed beads, and a change in the size of the beads and/ or the density of antibodies on the beads can alter the bead:cell ratio.

In some embodiments, a device may be utilized for culturing the cells, providing a smooth, rigid, rounded bottom surface to promote collection of the cells and beads by gravity in close proximity (*see, e.g.*, U.S. Patent Application Publication No. U.S. 2011/0223146, hereby incorporated by reference). The device includes an enclosed cell container that rests on a support. During at least the first 3 days of culture in the presence of the beads, the container is preferably stationary (*i.e.*, no rocking or rotation) to further

promote contact between the beads and the cells. These steps and conditions are preferable for maximizing the expansion of tumor-specific MILs using beads, to allow for the production of sufficient cells to be therapeutically useful. Further, these culture conditions promote growth of the T cells without promoting growth of the tumor cells.

Several attributes of MILs make them suitable candidates for immunotherapy. Specifically, under the conditions described herein, they expand more rapidly upon stimulation than PBLs and often maintain a skewed T-cell repertoire upon activation, possibly suggesting augmented tumor specificity. Whereas the unactivated MILs show profound hyporesponsiveness toward autologous tumor, the ability to activate and expand T cells and markedly enhance their tumor reactivity argues against deletional tolerance as a presumptive mechanism mediating T-cell unresponsiveness in this setting. Furthermore, activated MILs show tumor specificity with little cross-reactivity towards nonmalignant hematopoietic elements, have a higher expression of CXCR-4, and possess a greater responsiveness to SDF-I, suggesting an increased migratory ability of MILs to the bone marrow. Taken together, these findings show the ability to activate and expand marrow-infiltrating T cells with a memory/effector phenotype that seem to target the broad range of tumor antigens present on both mature terminally differentiated plasma cells as well as their precursors and possess chemokine receptors that would seem to facilitate trafficking to the bone marrow compartment — features that would be necessary for maximizing antitumor immunity of adoptive immunotherapy.

Activation and expansion of MILs was based on two previously reported phenomena: the enhanced tumor specificity of tumor-infiltrating lymphocytes (Rosenberg et al. Science 1986;233:1318) and the demonstration of tumor-reactive T cells in the bone marrow of patients with melanoma (Letsch et al. Cancer Res 2003;63:5582-6), breast cancer (Feuerer et al. Nat Med 2001;7:452), and multiple myeloma - a disease in which the bone marrow also represents the tumor microenvironment (Dhodapkar et al. Proc Natl Acad Sci U S A 2002;99:13009).

The ability to activate and expand MILs as a means of overcoming their unresponsiveness and significantly increasing their tumor specificity compared with activated PBLs is provided herein. The presence of tumor in the bone marrow microenvironment may play a critical role in preserving the antigen specificity of activated MILs. Several hypotheses may explain the increased reactivity of activated MILs over activated PBLs. Without being bound by mechanism, it is suggested that the persistence of

antigen in the bone marrow may be essential for the maintenance of a memory response. Anti-CD3/CD28 antibody-coated bead activation may be reversing tolerance in the bone marrow T-cell population. Similarly, plate bound and/or soluble CD3 and/or CD28 may be used for activation. The means of activating MILs, however, is not particularly limiting, and any suitable method of activation may be used in various embodiments. As demonstrated herein, the tumor specificity of activated MILs was dependent on the presence of antigen during T-cell activation. Further, the bone marrow is a functional lymphoid organ capable of mounting both a primary immune response and a secondary responses via reactive lymphoid follicles in the presence of danger signals (infection, inflammation, autoimmunity, and cancer).

T cells in myeloma patients show considerable skewing of the VB T-cell receptor repertoire. Such skewing suggests either the selective outgrowth of T cells with marked tumor specificity or results from the profound underlying T-cell defects characteristic of patients with a significant tumor burden. In the latter case, a benefit of polyclonal stimulation of PBLs with the anti-CD3/CD28 antibody-conjugated magnetic beads is the ability to restore a normal T-cell repertoire and thus correct any underlying T-cell defects. In contrast, if the oligoclonal expression of specific VB families reflects the presence of T cells with tumor specificity, activation and expansion of this pool of T cells with maintained antitumor activity and T-cell receptor repertoire skewing may be preferable. As demonstrated herein, PBLs normalized their VB T-cell repertoire upon activation and expansion with anti-CD3/CD28 antibody-conjugated magnetic beads, whereas MILs maintained the VB restriction. Considering the enhanced tumor-specific response of activated MILs, their skewed T-cell repertoire may be suggestive of greater tumor recognition. Without being bound by mechanism, it may be important to conserve and possibly increase the degree of VB skewing during T-cell expansion.

The activation and expansion of MILs with anti-CD3/CD28 antibody-conjugated magnetic beads generates potent antitumor activity and the persistence of antigen during this expansion may be of significant importance in maintaining (and augmenting) the tumor specificity. Dhodapkar et al. (2002) have also studied the role of MILs in myeloma patients. Similar to our findings, freshly isolated MILs or PBLs showed no activity upon stimulation with autologous tumor or tumor peptides. However, whereas that study saw no significant differences between T cells obtained from the peripheral blood and the marrow compartment in the enzyme-linked immunospot assay following 12 to 16 days of

incubation with tumor-pulsed dendritic cells, a 10-fold greater antitumor response of activated MILs over activated PBLs was observed in our system in all assays examined. These discrepant results may be related to potency of anti-CD3/CD28 bead stimulation as compared with dendritic cell activation of MILs. Without being bound by mechanism, what seems to be an increase in frequency of tumor-reactive T cells in the activated and expanded MILs cultures may reflect the breaking of tolerance and restoration of function of tumor-reactive T cells. Furthermore, stimulation of MILs within the bone marrow microenvironment is another important factor that may explain these results.

Enrichment of a MIL population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. One method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4+ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8.

For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (*e.g.*, particles such as beads) can be varied. In certain embodiments, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (*i.e.*, increase the concentration of cells), to ensure maximum contact of cells and beads.

In some embodiments, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of MILs and surface (*e.g.*, particles such as beads), interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles.

Also provided herein is the collection of samples comprising MILs from a subject at a time period prior to when the expanded cells as described herein might be needed. As such, the source of the cells to be expanded can be collected at any time point necessary, and desired cells, such as MILs, isolated and frozen for later use in MIL therapy for any number of diseases or conditions that would benefit from MIL therapy, such as those described herein. In some embodiments a sample comprising MILs is taken from a generally healthy subject. In some embodiments, a sample comprising MILs is taken from a generally healthy subject who is at risk of developing a disease, but who has not yet developed a disease, and the cells of interest are isolated and frozen for later use. In some

embodiments, the MILs may be expanded, frozen, and used at a later time. In some embodiments, samples are collected from a patient shortly after diagnosis of a particular disease as described herein but prior to any treatments. In some embodiments, the cells are isolated from a sample comprising MILs from a subject prior to any number of relevant treatment modalities, including but not limited to treatment with agents such as natalizumab, efalizumab, antiviral agents, chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies, cytoxan, fludarabine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin). In some embodiments, the cells are isolated for a patient and frozen for later use in conjunction with (*e.g.*, before, simultaneously or following) bone marrow or stem cell transplantation, MIL ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In some embodiments, the cells are isolated prior to and can be frozen for later use for treatment following B-cell ablative therapy such as agents that react with CD20, *e.g.*, Rituxan.

In some embodiments, MILs are obtained from a patient directly following treatment. In this regard, it has been observed that following certain cancer treatments, in particular treatments with drugs that damage the immune system, shortly after treatment during the period when patients would normally be recovering from the treatment, the quality of MILs obtained may be optimal or improved for their ability to expand *ex vivo*. Likewise, following *ex vivo* manipulation using the methods described herein, these cells may be in a preferred state for enhanced engraftment and *in vivo* expansion. Thus, the MILs may be collected during this recovery phase.

Whether prior to or after genetic modification of the MILs to express a desirable CAR, the MILs can be activated and expanded generally using methods as described, for example, in U.S. Pat. Nos. 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005 (hereby incorporated by reference).

In some embodiments, the MILs are expanded by contact with a surface having attached thereto an agent that stimulates a CD3/TCR complex associated signal and a ligand that stimulates a co-stimulatory molecule on the surface of the MILs. In particular, MIL populations may be stimulated as described herein, such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (*e.g.*, bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the MILs, a ligand that binds the accessory molecule is used. For example, a population of MILs can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the MILs. To stimulate proliferation of either CD4+ MILs or CD8+ MILs, an anti-CD3 antibody and an anti-CD28 antibody. Examples of an anti-CD28 antibody include 9.3, B-T3, XR-CD28 (Diaclone, Besancon, France) can be used as can other methods commonly known in the art (Berg et al., Transplant Proc. 30(8):3975-3977, 1998; Haanen et al., J. Exp. Med. 190(9):13191328, 1999; Garland et al., J. Immunol Meth. 227(1-2):53-63, 1999).

In certain embodiments, the primary stimulatory signal and the co-stimulatory signal for the MIL may be provided by different protocols. For example, the agents providing each signal may be in solution or coupled to a surface. When coupled to a surface, the agents may be coupled to the same surface (*i.e.*, in "cis" formation) or to separate surfaces (*i.e.*, in "trans" formation). Alternatively, one agent may be coupled to a surface and the other agent in solution. In some embodiments, the agent providing the co-stimulatory signal is bound to a cell surface and the agent providing the primary activation signal is in solution or coupled to a surface. In certain embodiments, both agents can be in solution. In some embodiments, the agents may be in soluble form, and then cross-linked to a surface, such as a cell expressing Fc receptors or an antibody or other binding agent which will bind to the agents. (*see generally* U.S. Patent Application Publication Nos. 20040101519 and 20060034810, hereby incorporated by reference, especially for artificial antigen presenting cells (aAPCs) that are contemplated for use in activating and expanding MILs).

In some embodiments, the two agents are immobilized on beads, either on the same bead, *i.e.*, "cis," or to separate beads, *i.e.*, "trans." By way of example, the agent providing the primary activation signal is an anti-CD3 antibody or an antigen-binding fragment thereof and the agent providing the co-stimulatory signal is an anti-CD28 antibody or

antigen-binding fragment thereof; and both agents are co-immobilized to the same bead in equivalent molecular amounts. In some embodiments, a 1:1 ratio of each antibody bound to the beads for CD4+ MIL expansion and MIL growth is used. In some embodiments, a ratio of anti CD3:CD28 antibodies bound to the beads is used such that an increase in MIL expansion is observed as compared to the expansion observed using a ratio of 1:1. In some embodiments an increase of from about 1 to about 3 fold is observed as compared to the expansion observed using a ratio of 1:1. In some embodiments, the ratio of CD3:CD28 antibody bound to the beads ranges from 100:1 to 1:100 and all integer values there between. In some embodiments, more anti-CD28 antibody is bound to the particles than anti-CD3 antibody, *i.e.*, the ratio of CD3:CD28 is less than one. In some embodiments, the ratio of anti CD28 antibody to anti CD3 antibody bound to the beads is greater than 2:1. In some embodiments, a 1:100 CD3:CD28 ratio of antibody bound to beads is used. In some embodiments, a 1:75 CD3:CD28 ratio of antibody bound to beads is used. In some embodiments, a 1:50 CD3:CD28 ratio of antibody bound to beads is used. In some embodiments, a 1:30 CD3:CD28 ratio of antibody bound to beads is used. In some embodiments, a 1:10 CD3:CD28 ratio of antibody bound to beads is used. In some embodiments, a 1:3 CD3:CD28 ratio of antibody bound to the beads is used. In some embodiments, a 3:1 CD3:CD28 ratio of antibody bound to the beads is used.

Ratios of particles to cells from 1:500 to 500:1 and any integer values in between may be used to stimulate MILs. As those of ordinary skill in the art can readily appreciate, the ratio of particles to cells may depend on particle size relative to the target cell. For example, small sized beads could only bind a few cells, while larger beads could bind many. In certain embodiments the ratio of cells to particles ranges from 1:100 to 100:1 and any integer values in-between and in further embodiments the ratio comprises 1:9 to 9:1 and any integer values in between, can also be used to stimulate MILs. The ratio of anti-CD3- and anti-CD28-coupled particles to cell that result in MIL stimulation can vary as noted above, however certain values include, but are not limited to, 1:100, 1:50, 1:40, 1:30, 1:20, 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, and 15:1. In some embodiments, the ratio is at least 1:1 particles per cell. In some embodiments, a ratio of particles to cells of 1:1 or less is used. In some embodiments, a particle: cell ratio is 1:5. In some embodiments, the ratio of particles to cells can be varied depending on the day of stimulation. For example, in some embodiments, the ratio of particles to cells is from 1:1 to 10:1 on the first day and additional particles are added to the

cells every day or every other day thereafter for up to 10 days, at final ratios of from 1:1 to 1:10 (based on cell counts on the day of addition). In some embodiments, the ratio of particles to cells is 1:1 on the first day of stimulation and adjusted to 1:5 on the third and fifth days of stimulation. In some embodiments, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:5 on the third and fifth days of stimulation. In some embodiments, the ratio of particles to cells is 2:1 on the first day of stimulation and adjusted to 1:10 on the third and fifth days of stimulation. In some embodiments, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:10 on the third and fifth days of stimulation. One of skill in the art will appreciate that a variety of other ratios may also be used. For example, ratios will vary depending on particle size and on cell size and type.

In some embodiments, the MILs are combined with agent-coated beads, the beads and the cells are subsequently separated, and then the cells are cultured. In some embodiments, prior to culture, the agent-coated beads and cells are not separated but are cultured together. In some embodiments, the beads and cells are first concentrated by application of a force, such as a magnetic force, resulting in increased ligation of cell surface markers, thereby inducing cell stimulation.

By way of example, cell surface proteins may be ligated by allowing paramagnetic beads to which anti-CD3 and anti-CD28 are attached (3×28 beads) to contact the MILs. In some embodiments the cells and beads (for example, DYNABEADS® M-450 CD3/CD28 T paramagnetic beads at a ratio of 1:1) are combined in a buffer, preferably PBS (without divalent cations such as, calcium and magnesium). Those of ordinary skill in the art can readily appreciate any cell concentration may be used. For example, the target cell may be very rare in the sample and comprise only 0.01% of the sample or the entire sample (*i.e.*, 100%) may comprise the target cell of interest. Accordingly, any cell number can be used. In certain embodiments, it may be desirable to significantly decrease the volume in which particles and cells are mixed together (*i.e.*, increase the concentration of cells), to ensure maximum contact of cells and particles. For example, in some embodiments, a concentration of about 2 billion cells/ml is used. In some embodiments, greater than 100 million cells/ml is used. In some embodiments, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In some embodiments, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In some embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can

result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative cells. Such populations of cells may have therapeutic value and would be desirable to obtain in certain embodiments.

In some embodiments, the mixture may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. In some embodiments, the mixture may be cultured for 21 days. In some embodiments the beads and the MILs are cultured together for about eight days. In some embodiments, the beads and MILs are cultured together for 2-3 days. Several cycles of stimulation may also be desired such that culture time of MILs can be 60 days or more. Conditions appropriate for MIL culture include an appropriate media (*e.g.*, Minimal Essential Media or RPMI Media 1640 or, X-vivo 15, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (*e.g.*, fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-γ, IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGFβ, and TNF-α or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, AIM-V, DMEM, MEM, α-MEM, F-12, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of MILs. Antibiotics, *e.g.*, penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (*e.g.*, 37°C.) and atmosphere (*e.g.*, air plus 5% $CO_2$).

In addition to CD4 and CD8 markers, other phenotypic markers vary significantly, but in large part, reproducibly during the course of the cell expansion process. Thus, such reproducibility enables the ability to tailor an activated MIL product for specific purposes.

Additionally, methods for preparing tumor infiltrating lymphocytes may be used to prepare MILs. For example, high does IL-2 growth conditions may be used to generate "young" TILs, and these methods are applicable to preparing MILs (*see, e.g.*, U.S. Patent No. 8,383,099, hereby incorporated by reference).

In some embodiments, the MILs can also be activated and/or expanded under hypoxic conditions. An example of growing the MILs under hypoxic conditions can

found, for example, in WO2016037054, which is hereby incorporated by reference in its entirety.

In some embodiments, the method may comprise removing cells in the bone marrow, lymphocytes, and/or marrow infiltrating lymphocytes ("MILs") from the subject; incubating the cells in a hypoxic environment, thereby producing activated MILs; and administering the activated MILs to the subject. The cells can also be activated in the presence of anti-CD3/anti-CD28 antibodies and cytokines as described herein. Cyotkines can also be used to activate the MILs as described herein. A nucleic acid molecule encoding the CAR, such as one of those described herein, can be transfected or infected into a cell before or after the MIL is incubated in a hypoxic environment.

The hypoxic environment may comprise less than about 21 % oxygen, such as less than about 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, or less than about 3% oxygen. For example, the hypoxic environment may comprise about 0% oxygen to about 20% oxygen, such as about 0% oxygen to about 19% oxygen, about 0% oxygen to about 18% oxygen, about 0% oxygen to about 17% oxygen, about 0% oxygen to about 16% oxygen, about 0% oxygen to about 15% oxygen, about 0% oxygen to about 14% oxygen, about 0% oxygen to about 13% oxygen, about 0% oxygen to about 12% oxygen, about 0% oxygen to about 11% oxygen, about 0% oxygen to about 10% oxygen, about 0% oxygen to about 9% oxygen, about 0% oxygen to about 8% oxygen, about 0% oxygen to about 7% oxygen, about 0% oxygen to about 6% oxygen, about 0% oxygen to about 5% oxygen, about 0% oxygen to about 4% oxygen, or about 0% oxygen to about 3% oxygen. In some embodiments, the hypoxic environment comprises about 1 % to about 7% oxygen. In some embodiments, the hypoxic environment is about 1% to about 2% oxygen. In some embodiments, the hypoxic environment is about 0.5% to about 1.5% oxygen. In some embodiments, the hypoxic environment is about 0.5% to about 2% oxygen. The hypoxic environment may comprise about 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 1 1%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or about 0% oxygen. In some embodiments, the hypoxic environment comprises about 7%, 6%, 5%, 4%, 3%, 2%, or 1% oxygen.

Incubating MILs in a hypoxic environment may comprise incubating the MILs, *e.g.*, in tissue culture medium, for at least about 1 hour, such as at least about 12 hours, 18 hours, 24 hours, 30 hours, 36 hours, 42 hours, 48 hours, 60 hours, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 1 1 days, 12 days, 13 days, or even at least about 14 days.

Incubating may comprise incubating the MILs for about 1 hour to about 30 days, such as about 1 day to about 20 days, about 1 day to about 14 days, or about 1 day to about 12 days. In some embodiments, incubating MILs in a hypoxic environment comprises incubating the MILs in a hypoxic environment for about 2 days to about 5 days. The method may comprise incubating MILs in a hypoxic environment for about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 day, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the method comprises incubating the MILs in a hypoxic environment for about 3 days. In some embodiments, the method comprises incubating the MILs in a hypoxic environment for about 2 days to about 4 days. In some embodiments, the method comprises incubating the MILs in a hypoxic environment for about 3 days to about 4 days.

In some embodiments, the method further comprises incubating the MILs in a normoxic environment, *e.g.*, after incubating the MILs in a hypoxic environment.

The normoxic environment may comprise at least about 21% oxygen. The normoxic environment may comprise about 5% oxygen to about 30% oxygen, such as about 10% oxygen to about 30% oxygen, about 15% oxygen to about 25% oxygen, about 18% oxygen to about 24% oxygen, about 19% oxygen to about 23% oxygen, or about 20% oxygen to about 22% oxygen. In some embodiments, the normoxic environment comprises about 21% oxygen.

Incubating MILs in a normoxic environment may comprise incubating the MILs, *e.g.*, in tissue culture medium, for at least about 1 hour, such as at least about 12 hours, 18 hours, 24 hours, 30 hours, 36 hours, 42 hours, 48 hours, 60 hours, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or even at least about 14 days. Incubating may comprise incubating the MILs for about 1 hour to about 30 days, such as about 1 day to about 20 days, about 1 day to about 14 days, about 1 day to about 12 days, or about 2 days to about 12 days.

In some embodiments, the cell is transfected or infected with a nucleic acid molecule encoding a CAR described herein after being placed in a normoxic environment or before it is placed in a normoxic environment.

In some embodiments, the MILs are obtained by extracting a bone marrow sample from a subject and culturing/incubating the cells as described herein. In some embodiments, the bone marrow sample is centrifuged to remove red blood cells. In some embodiments, the bone marrow sample is not subject to, or obtained by, apheresis. In some

embodiments, the bone marrow sample does not comprise peripheral blood lymphocytes ("PBL") or the bone marrow sample is substantially free of PBLs. These methods select for cells that are not the same as what have become to be known as TILs. Thus, a MIL is not a TIL.

In some embodiments, the cells can then be plated in a plate, flask, or bag. In some embodiments, hypoxic conditions can be achieved by flushing either the hypoxic chamber or cell culture bag for 3 minutes with a 95% Nitrogen and 5% $CO_2$ gas mixture. This can lead to, for example, 1-2% or less $O_2$ gas in the receptacle. Cells can be then cultured as described herein or as in the examples of WO2016037054, which is hereby incorporated by reference.

In some embodiments, a hypoxic MIL comprising a CAR as described herein is provided. In some embodiments, the hypoxic MIL is in an environment of about 0.5% to about 5% oxygen gas. In some embodiments, the hypoxic MIL is in an environment of about 1% to about 2% oxygen gas. In some embodiments, the hypoxic MIL is in an environment of about 1% to about 3% oxygen gas. In some embodiments, the hypoxic MIL is in an environment of about 1% to about 4% oxygen gas. A hypoxic MIL is a MIL that has been incubated in a hypoxic environment, such as those described herein, for a period of time, such as those described herein. As described herein, the hypoxic MIL can also be activated in the presence of anti-CD3/anti-CD28 beads or other similar activating reagents. Thus, a hypoxic MIL comprising a CAR can also be an activated-hypoxic MIL.

## VII. METHODS OF TREATMENT

In some embodiments a cell (*e.g.*, MIL) expressing a CAR is provided. The cell (or a parent cell) may be transfected with a vector comprising a nucleotide sequence encoding the CAR. The vector may be a lentiviral vector (LV). For example, the LV encodes a CAR that combines an antigen recognition domain of a specific antibody with an intracellular domain of CD3ζ, CD28, 4-1BB, or any combinations thereof. Therefore, in some instances, the transduced MIL can elicit a CAR-mediated T-cell response.

Provided herein are the uses of a CAR to redirect the specificity of a primary MIL to a tumor antigen. Thus, in some embodiments, methods for stimulating a MIL-mediated immune response to a target cell population or tissue in a mammal comprising the step of administering to the subject a MIL that expresses a CAR, wherein the CAR comprises a binding moiety that specifically interacts with a predetermined target, a ζ chain portion

comprising for example the intracellular domain of human CD3ζ, and a costimulatory signaling region are provided.

In some embodiments, cellular therapies are provided where MILs are genetically modified to express a CAR and the CAR-MIL is infused to a recipient in need thereof. The infused cell is able to kill tumor cells (or other targets) in the recipient. Unlike antibody therapies, CAR-MILs are able to replicate *in vivo* resulting in long-term persistence that can lead to sustained tumor control.

In some embodiments, the CAR-MILs can undergo robust *in vivo* MIL expansion and can persist for an extended amount of time.

Cancers that may be treated include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized tumors. The cancers may comprise non-solid tumors (such as hematological tumors, for example, leukemias and lymphomas) or may comprise solid tumors. Types of cancers to be treated with the CARs include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies *e.g.*, sarcomas, carcinomas, and melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included.

Hematologic cancers are cancers of the blood or bone marrow. Examples of hematological (or hematogenous) cancers include leukemias, including acute leukemias (such as acute lymphocytic leukemia, acute myelocytic leukemia, acute myelogenous leukemia and myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia), chronic leukemias (such as chronic myelocytic (granulocytic) leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia), polycythemia vera, lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma (indolent and high grade forms), multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, myelodysplastic syndrome, hairy cell leukemia and myelodysplasia.

Solid tumors are abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors can be benign or malignant. Different types of solid tumors are named for the type of cells that form them (such as sarcomas, carcinomas, and lymphomas). Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell

carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytomas sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma (such as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma craniopharyogioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, neuroblastoma, retinoblastoma and brain metastases).

In some embodiments, the antigen bind moiety portion of the CAR is designed to treat a particular cancer. For example, the CAR designed to target CD19 can be used to treat cancers and disorders including but are not limited to pre-B ALL (pediatric indication), adult ALL, mantle cell lymphoma, diffuse large B-cell lymphoma, salvage post allogenic bone marrow transplantation, and the like.

In some embodiments, the CAR can be designed to target CD22 to treat diffuse large B-cell lymphoma.

In some embodiments, cancers and disorders include but are not limited to pre-B ALL (pediatric indication), adult ALL, mantle cell lymphoma, diffuse large B-cell lymphoma, salvage post allogenic bone marrow transplantation, and the like can be treated using a combination of CARs that target CD19, CD20, CD22, and ROR1.

In some embodiments, the CAR can be designed to target mesothelin to treat mesothelioma, pancreatic cancer, ovarian cancer, and the like.

In some embodiments, the CAR can be designed to target CD33/IL3Ra to treat acute myelogenous leukemia and the like.

In some embodiments, the CAR can be designed to target c-Met to treat triple negative breast cancer, non-small cell lung cancer, and the like.

In some embodiments, the CAR can be designed to target PSMA to treat prostate cancer and the like.

In some embodiments, the CAR can be designed to target Glycolipid F77 to treat prostate cancer and the like.

In some embodiments, the CAR can be designed to target EGFRvIII to treat gliobastoma and the like.

In some embodiments, the CAR can be designed to target GD-2 to treat neuroblastoma, melanoma, and the like.

In some embodiments, the CAR can be designed to target NY-ESO-1 TCR to treat myeloma, sarcoma, melanoma, and the like.

In some embodiments, the CAR can be designed to target MAGE A3 TCR to treat myeloma, sarcoma, melanoma, and the like.

However, the embodiments should not be construed to be limited to solely to the antigen targets and diseases disclosed herein. Rather, the embodiments should be construed to include any antigenic target that is associated with a disease where a CAR can be used to treat the disease.

The CAR-modified MILs may also serve as a type of vaccine for *ex vivo* immunization and/or *in vivo* therapy in a subject, such as a human.

With respect to *ex vivo* immunization, at least one of the following occurs *in vitro* prior to administering the cell into a mammal: i) expansion of the cells, ii) introducing a nucleic acid encoding a CAR to the cells, and/or iii) cryopreservation of the cells. In some embodiments, all of the steps are performed prior to administering the cells into a mammal.

*Ex vivo* procedures are well known in the art and are discussed more fully below. Briefly, cells are isolated from a mammal (such as a human) and genetically modified (*i.e.*, transduced or transfected *in vitro*) with a vector expressing a CAR disclosed herein. The CAR-MIL can be administered to a mammalian recipient to provide a therapeutic benefit. The mammalian recipient may be a human and the CAR- MIL can be autologous with respect to the recipient. Alternatively, the cells can be allogeneic, syngeneic or xenogeneic with respect to the recipient.

In addition to using a cell-based vaccine in terms of *ex vivo* immunization, also provided herein are compositions and methods for *in vivo* immunization to elicit an immune response directed against an antigen in a patient.

Generally, the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised. In some embodiments, the CAR-modified MILs are used in the treatment of CCL. In some embodiments, the cells are used in the treatment of patients at risk for developing CCL. Thus, methods are provided for the treatment or prevention of CCL comprising administering to a subject in need thereof, a therapeutically effective amount of the CAR-modified MILs.

The CAR-modified MILs may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components such as IL-2 or other cytokines or cell populations. Briefly, pharmaceutical compositions may comprise a target cell population as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (*e.g.*, aluminum hydroxide); and preservatives. In some embodiments, compositions are formulated for intravenous administration.

Pharmaceutical compositions may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

When "an immunologically effective amount", "an anti-tumor effective amount", "an tumor-inhibiting effective amount", or "therapeutic amount" is indicated, the precise amount of the compositions to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the MILs described herein may be administered at a dosage of $10^4$ to $10^9$ cells/kg body weight, preferably $10^5$ to $10^6$ cells/kg body weight, including all integer values within those ranges. MIL compositions may also be administered multiple times at these dosages. The cells can be administered by using infusion techniques that are commonly known in immunotherapy (see, *e.g.*, Rosenberg et al., New Eng. J. of Med. 319:1676, 1988). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

The administration of the subject compositions may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally. In some embodiments, the MIL compositions are administered to a patient by intradermal or subcutaneous

injection. In some embodiments, the MIL compositions are administered by intravenous injection. The compositions of MILs may, for example, be injected directly into a tumor, lymph node, or site of infection.

In some embodiments, cells activated and expanded using the methods described herein, or other methods known in the art where MILs are expanded to therapeutic levels, are administered to a patient in conjunction with (*e.g.*, before, simultaneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral therapy, cidofovir and interleukin-2, Cytarabine (also known as ARA-C) or natalizumab treatment for MS patients or efalizumab treatment for psoriasis patients or other treatments for PML patients. In some embodiments, the MILs may be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAM PATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludaribine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin) (Liu et al., Cell 66:807-815, 1991; Henderson et al., Immun 73:316-321, 1991; Bierer et al., Curr. Opin. Immun 5:763-773, 1993). In some embodiments, the cell compositions are administered to a patient in conjunction with (*e.g.*, before, simultaneously or following) bone marrow transplantation, MIL ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In some embodiments, the cell compositions are administered following B-cell ablative therapy such as agents that react with CD20, *e.g.*, Rituxan. For example, in some embodiments, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In some embodiments, following the transplant, subjects receive an infusion of the expanded immune cells described herein. In some embodiments , expanded cells are administered before or following surgery.

The dosage for treatments to be administered to a patient will vary with the precise nature of the condition being treated and the recipient of the treatment. The scaling of dosages for human administration can be performed according to art-accepted practices. The dose for CAMPATH, for example, will generally be in the range 1 to about 100 mg for

an adult patient, usually administered daily for a period between 1 and 30 days. In some embodiments, the daily dose is 1 to 10 mg per day although in some instances larger doses of up to 40 mg per day may be used (described in U.S. Pat. No. 6,120,766).

VIII. SUBJECTS

The subject may be any organism that comprises MILs. For example, the subject may be selected from rodents, canines, felines, porcines, ovines, bovines, equines, and primates. The subject may be a mouse or a human.

The subject may have a neoplasm. The neoplasm may be a benign neoplasm, a malignant neoplasm, or a secondary neoplasm. The neoplasm may be cancer. The neoplasm may be a lymphoma or a leukemia, such as chronic lymphocytic leukemia ("CLL") or acute lymphoblastic leukemia ("ALL"). The subject may have a glioblastoma, medulloblastoma, breast cancer, head and neck cancer, kidney cancer, ovarian cancer, Kaposi's sarcoma, acute myelogenous leukemia, and B-lineage malignancies. The subject may have multiple myeloma.

The subject may have acute myelogenous leukemia, adenocarcinoma, osteosarcoma, lymphoblastic leukemia, lymphoma, B-cell lymphomas, B-cell Non-Hodgkin's Lymphoma, a B-lineage lymphoid malignancy, breast cancer, ovarian cancer, cervical cancer, colorectal cancer, epithelial cancer, a glioblastoma, glioma, Hodgkin lymphoma, indolent B-cell lymphoma, leukemia, lymphoma, lung cancer, mantel cell lymphoma, medulloblastoma, melanoma, neuroblastoma, prostate cancer, follicular lymphoma, renal cell carcinoma, rhabdomyosarcoma.

**EXAMPLES**

The following examples are illustrative, but not limiting, of the methods and compositions described herein. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in therapy and that are obvious to those skilled in the art are within the spirit and scope of the embodiments.

**Example 1: MIL-CAR is used to treat B-Cell Lymphoma**

A MIL is obtained from a subject with B-Cell Lymphoma. Briefly, after the marrow sample is obtained from the subject, the cells are incubated under hypoxic conditions in the presence of anti-CD3/anti-CD28 beads and cytokines as described in WO2016037054, which is hereby incorporated by reference. A nucleic acid molecule encoding a CAR, comprising the extracellular domain of CD19, the transmembrane domain

of CD19, and the intracellular domains of CD3ζ and 4-1BB is transfected into the MIL. The cells are then grown under normoxic conditions and allowed to expand. The activated and expanded MILs are administered to the subject with B-Cell Lymphoma. The subject's B-Cell Lymphoma is put into remission. In summary, the embodiments and examples provided herein demonstrate that cells expressing a CAR can be effectively used to treat cancer.

**Example 2: MIL-CAR is used to treat Multiple Myeloma**

A MIL is obtained from a subject with multiple myeloma. Briefly, after the marrow sample is obtained from the subject, the cells are incubated under hypoxic conditions in the presence of anti-CD3/anti-CD28 beads and cytokines as described in WO2016037054, which is hereby incorporated by reference. A nucleic acid molecule encoding a CAR, comprising the extracellular domain of CD38, the transmembrane domain of CD8, and the intracellular domains of CD3ζ and 4-1BB is transfected into the MIL. The cells are then grown under normoxic conditions and allowed to expand. The activated and expanded MILs are administered to the subject with multiple myeloma. The subject's multiple myeloma is put into remission.

Any U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications, including CAS numbers, referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety.

What is claimed:

1. A cell, comprising a chimeric antigen receptor ("CAR"), wherein:
the cell is a marrow infiltrating lymphocyte ("MIL");
the CAR comprises an extracellular domain that can bind a ligand; and
the CAR comprises an intracellular domain that can initiate an intracellular signaling cascade.

2. The cell of claim 1, wherein the cell is $CD3^+$.

3. The cell of claim 1 or 2, wherein the cell is $CD4^+$.

4. The cell of any one of the preceding claims, wherein the cell is $CD8^+$.

5. The cell of any one of the preceding claims, wherein the cell is CD45RO+.

6. The cell of any one of the preceding claims, wherein the cell is CD62L+.

7. The cell of any one of the preceding claims, wherein the cell is CXCR4+.

8. The cell of any one of the preceding claims, wherein the cell is $4\text{-}1BB^+$.

9. The cell of any one of the preceding claims, wherein the cell is interferon $\gamma^+$.

10. The cell of any one of the preceding claims, wherein the cell is $CD138^+$.

11. The cell of any one of the preceding claims, wherein the cell is $CD33^+$.

12. The cell of any one of the preceding claims, wherein the cell is $CD34^-$.

13. The cell of any one of the preceding claims, wherein the ligand is a molecule expressed on a neoplastic cell.

14. The cell of claim 13, wherein the ligand is glioma-associated antigen, carcinoembryonic antigen (CEA), β-human chorionic gonadotropin, alpha-fetoprotein (AFP), lectin-reactive AFP, thyroglobulin, RAGE-1, MN-CA IX, human telomerase reverse transcriptase, RU1, RU2 (AS), intestinal carboxyl esterase, mutant hsp70-2, M-CSF, prostase, prostate-specific antigen ("PSA"), prostatic acid phosphatase ("PAP"), NY-ESO-1, LAGE-1a, p53, prostein, PSMA, Her2/neu, survivin, telomerase, prostate-carcinoma tumor antigen-1 (PCTA-1), MAGE, ELF2M, neutrophil elastase, ephrinB2, CD22, insulin growth factor (IGF)-I, IGF-II, IGF-I receptor, mesothelin, MART-1, tyrosinase, GP 100, HER-2/Neu/ErbB-2, CD19, CD20, CD37, MART-1/MelanA ("MART-I"), gp100 (Pmel 17), TRP-1, TRP-2, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, p15, p53, Ras, BCR-ABL, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR, EBVA, E6, E7, TSP-180, MAGE-4, MAGE-5, MAGE-6, RAGE, NY-ESO, p185erbB2, p180erbB-3, c-met, nm-23H1, PSA, TAG-72, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, beta-Catenin, CDK4, Mum-1, p 15, p 16, 43-9F, 5T4, 791Tgp72, alpha-fetoprotein, beta-HCG, BCA225, BTAA, CA 125, CA 15-3\CA 27.29\BCAA, CA 195, CA 242, CA-50, CAM43, CD68\P1, CO-029, FGF-5, G250, Ga733\EpCAM, HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB/70K, NY-CO-1, RCAS1, SDCCAG16, TA-90\Mac-2 binding protein\cyclophilin C-associated protein, TAAL6, TAG72, TLP, or TPS.

15. The cell of claim 13, wherein the ligand is CD19, CD20, CD22, ROR1, Mesothelin, CD33/IL3Ra, c-Met, PSMA, Glycolipid F77, EGFRvIII, GD-2, MY-ESO-1 TCR, or MAGE A3 TCR.

16. The cell of claim 13, wherein the ligand is α-folate receptor, carbonic anhydrase 9 ("CAIX"), CD19, CD20, CD22, CD30, CD33, CD44, CD44v6, CD44v7, CD44v7, carcinoembryonic antigen ("CEA"), epidermal growth factor-2 ("EGF-2"), epithelial glycoprotein 40 ("EGF-40"), receptor tyrosine-protein kinase erbB-2 (HER2; Neu; CD340), receptor tyrosine-protein kinase erbB-3 (HER3), receptor tyrosine-protein kinase erbB-4 (HER4), folate-binding protein ("FBP"), fetal acetylcholine receptor, GD2, GD3, interleukin-13 receptor subunit alpha-2 ("IL-13Rα2"), kinase insert domain receptor ("KDR"; CD309), κ-light chain, Lewis Yantigen ("LeY"), L1 cell adhesion molecule, MAGE-A1, mesothelin, mucin 1, cell surface associated ("MUC1"), prostate stem cell

antigen, prostate-specific membrane antigen, tumor-associated glycoprotein 72 ("TAG-72"), or VEGF-R2.

17. The cell of any one of the preceding claims, wherein the ligand is a molecule expressed by a pathogen.

18. The cell of claim 17, wherein the pathogen is a virus, bacterium, fungus, parasite, or viroid.

19. The cell of any one of the preceding claims, wherein the extracellular domain of the CAR comprises a single-chain variable fragment ("scFv") domain.

20. The cell of any one of the preceding claims, wherein the intracellular domain of the CAR comprises the intracellular signaling domain of CD3ζ, 4-1BB, and/or CD28.

21. A method for treating a condition in a subject, comprising administering to the subject the cell of any one of the preceding claims.

22. The method of claim 21, wherein the method comprises administering to the subject a plurality of cells according to any one of claims 1 to 20.

23. A method for making a recombinant MIL, comprising:
obtaining bone marrow comprising MILs; and
transfecting, transforming, or transducing the MILs with a nucleic acid encoding a chimeric antigen receptor.

24. The method of claim 23, wherein the bone marrow is obtained from a subject.

25. The method of claim 24, wherein the subject has a neoplasm.

26. The method of claim 24, wherein the subject has an autoimmune disease.

27. The method of claim 24, wherein the subject has an infection caused by a pathogen.

28. The method of any one of claims 23 to 27, further comprising activating the MILs.

29. The method of any one of claims 23 to 28, further comprising expanding the MILs.

30. The method of any one of claims 23 to 29, wherein the method comprises making a plurality of recombinant MILs.

31. The method of claim 23, further comprising incubating the MILs under hypoxic conditions prior to transfecting, transforming, or transducing the MILs with the nucleic acid encoding the chimeric antigen receptor.

32. The method of claim 31, wherein the hypoxic conditions comprise about 0.5% to about 5% oxygen gas.

33. The method of claim 31, wherein the hypoxic conditions comprise about 1% to about 2% oxygen gas.

34. The method of 31, further comprising incubating the MILs under normoxic conditions after transfecting, transforming, or transducing the MILs with a nucleic acid encoding a chimeric antigen receptor.

35. The method of claim 31, further comprising contacting the MILs with anti-CD3/anti-CD28 beads while incubating the MILS under hypoxic conditions.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/041521

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2016.01) C12N 5/078, C12N 5/10, C07K 19/00, A61K 38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (2016.01) C12N, C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Databases consulted: THOMSON INNOVATION, CAPLUS, BIOSIS, EMBASE, MEDLINE, Google Scholar
Search terms used: marrow infiltrating lymphocytes, chimeric antigen receptor,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | NOONAN, Kimberly A., et al. Adoptive transfer of activated marrow-infiltrating lymphocytes induces measurable antitumor immunity in the bone marrow in multiple myeloma. Science translational medicine, 2015, 7.288: 288ra78-288ra78 Retrieved from the internet: < http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4634889/> DOI: 10.1126/scitranslmed.aaa7014<br>20 May 2015 (2015/05/20)<br>page 8 forth paragraph | 1-35 |
| Y | MAUS, Marcela V., et al. Antibody-modified T cells: CARs take the front seat for hematologic malignancies. Blood, 2014, 123.17: 2625-2635. Retrieved from the internet: <http://www.bloodjournal.org/content/123/17/2625.full.pdf> doi:10.1182/blood-2013-11-492231<br>24 Apr 2014 (2014/04/24)<br>the whole document | 1-35 |
| P,Y | WO WO2016037054 A1 UNIV JOHNS HOPKINS<br>10 Mar 2016 (2016/03/10)<br>page 6 lines 19-20, examples | 31-35 |

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
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**INTERNATIONAL SEARCH REPORT**
Information on patent family members

International application No.
PCT/US2016/041521

| Patent document cited search report | Publication date | Patent family member(s) | Publication Date |
|---|---|---|---|
| WO WO2016037054 A1 | 10 Mar 2016 | NONE | |

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*A61K 38/17*(2006.01)

权利要求书2页 说明书23页

(54)发明名称

作为用于嵌合抗原受体(CAR)疗法的T细胞来源的骨髓浸润淋巴细胞(MIL)

(57)摘要

在一些实施方案中,提供包含嵌合抗原受体(“CAR”)的骨髓浸润淋巴细胞(“MIL”)。在一些方面,所述实施方案涉及用于制备重组MIL的方法,所述方法包括获得包含MIL的骨髓;并用编码嵌合抗原受体的核酸转染、转化或转导MIL。在一些方面,所述实施方案涉及用于在受试者中治疗病症的方法,所述方法包括给予受试者包含CAR的MIL。

CN 108026510 A

1.一种包含嵌合抗原受体(“CAR”)的细胞,其中:

所述细胞为骨髓浸润淋巴细胞(“MIL”);

所述CAR包含可结合配体的胞外结构域;和

所述CAR包含可启动胞内信号传导级联的胞内结构域。

2.权利要求1的细胞,其中所述细胞为$CD3^+$。

3.权利要求1或2的细胞,其中所述细胞为$CD4^+$。

4.前述权利要求中任一项的细胞,其中所述细胞为$CD8^+$。

5.前述权利要求中任一项的细胞,其中所述细胞为CD45RO+。

6.前述权利要求中任一项的细胞,其中所述细胞为CD62L+。

7.前述权利要求中任一项的细胞,其中所述细胞为CXCR4+。

8.前述权利要求中任一项的细胞,其中所述细胞为$4\text{-}1BB^+$。

9.前述权利要求中任一项的细胞,其中所述细胞为干扰素$\gamma^+$。

10.前述权利要求中任一项的细胞,其中所述细胞为$CD138^+$。

11.前述权利要求中任一项的细胞,其中所述细胞为$CD33^+$。

12.前述权利要求中任一项的细胞,其中所述细胞为$CD34^-$。

13.前述权利要求中任一项的细胞,其中所述配体为赘生细胞上表达的分子。

14. 权利要求13的细胞,其中所述配体为神经胶质瘤相关抗原、癌胚抗原(CEA)、β-人绒毛膜促性腺激素、甲胎蛋白(AFP)、凝集素反应性AFP、甲状腺球蛋白、RAGE-1、MN-CA IX、人端粒酶逆转录酶、RU1、RU2 (AS)、肠羧酸酯酶、突变hsp70-2、M-CSF、prostase、前列腺特异性抗原(“PSA”)、前列腺酸性磷酸酶(“PAP”)、NY-ESO-1、LAGE-1a、p53、prostein、PSMA、Her2/neu、存活素、端粒酶、前列腺癌肿瘤抗原-1 (PCTA-1)、MAGE、ELF2M、嗜中性粒细胞弹性蛋白酶、肝配蛋白B2、CD22、胰岛素生长因子(IGF)-I、IGF-II、IGF-I受体、间皮素、MART-1、酪氨酸酶、GP 100、HER-2/Neu/ErbB-2、CD19、CD20、CD37、MART-1/MelanA (“MART-I”)、gp100 (Pmel 17)、TRP-1、TRP-2、MAGE-1、MAGE-3、BAGE、GAGE-1、GAGE-2、p15、p53、Ras、BCR-ABL、E2A-PRL、H4-RET、IGH-IGK、MYL-RAR、EBVA、E6、E7、TSP-180、MAGE-4、MAGE-5、MAGE-6、RAGE、NY-ESO、p185erbB2、p180erbB-3、c-met、nm-23H1、PSA、TAG-72、CA 19-9、CA 72-4、CAM 17.1、NuMa、K-ras、β-连环蛋白、CDK4、Mum-1、p 15、p 16、43-9F、5T4、791Tgp72、甲胎蛋白,β-HCG、BCA225、BTAA、CA 125、CA 15-3\CA 27.29\BCAA、CA 195、CA 242、CA-50、CAM43、CD68\P1、CO-029、FGF-5、G250、Ga733\EpCAM、HTgp-175、M344、MA-50、MG7-Ag、MOV18、NB/70K、NY-CO-1、RCAS1、SDCCAG16、TA-90\Mac-2结合蛋白\亲环蛋白C-相关蛋白、TAAL6、TAG72、YLP或TPS。

15. 权利要求13的细胞,其中所述配体为CD19、CD20、CD22、ROR1、间皮素、CD33/IL3Ra、c-Met、PSMA、糖脂F77、EGFRvIII、GD-2、MY-ESO-1 TCR或MAGE A3 TCR。

16. 权利要求13的细胞,其中所述配体为α-叶酸受体、碳酸酐酶9 (“CAIX”)、CD19、CD20、CD22、CD30、CD33、CD44、CD44v6、CD44v7、CD44v7、癌胚抗原(“CEA”)、表皮生长因子-2(“EGF-2“)、上皮糖蛋白40 (“EGF-40”)、受体酪氨酸蛋白激酶erbB-2 (HER2;Neu;CD340)、受体酪氨酸蛋白激酶erbB-3 (HER3)、受体酪氨酸蛋白激酶 erbB-4 (HER4)、叶酸结合蛋白(“FBP”)、胎儿型乙酰胆碱受体、GD2、GD3、白细胞介素-13受体亚基α-2 (“IL-13Rα2”)、激酶插入结构域受体(“KDR”CD139)、κ-轻链、Lewis Y抗原(“LeY”)、L1细胞粘附分子、MAGE-A1、

间皮素、细胞表面缔合的粘蛋白1 (“MUC1”)、前列腺干细胞抗原、前列腺特异性膜抗原、肿瘤相关糖蛋白72 (“TAG-72”)或VEGF-R2。

17.前述权利要求中任一项的细胞,其中所述配体为由病原体表达的分子。

18.权利要求17的细胞,其中所述病原体为病毒、细菌、真菌、寄生虫或类病毒。

19.前述权利要求中任一项的细胞,其中所述CAR的胞外结构域包含单链可变片段(“scFv”)结构域。

20.前述权利要求中任一项的细胞,其中所述CAR的胞内结构域包含CD3ξ、4-1BB和/或CD28的胞内信号传导结构域。

21.一种用于在受试者中治疗病症的方法,所述方法包括给予受试者前述权利要求中任一项的细胞。

22.权利要求21的方法,其中所述方法包括给予受试者多种权利要求1-20中任一项的细胞。

23. 一种用于制备重组MIL的方法,所述方法包括:

获得包含MIL的骨髓;和

用编码嵌合抗原受体的核酸转染、转化或转导所述MIL。

24.权利要求23的方法,其中所述骨髓得自受试者。

25.权利要求24的方法,其中所述受试者患有赘生物。

26.权利要求24的方法,其中所述受试者患有自身免疫性疾病。

27.权利要求24的方法,其中所述受试者患有由病原体引起的感染。

28.权利要求23-27中任一项的方法,所述方法进一步包括激活MIL。

29.权利要求23-28中任一项的方法,所述方法进一步包括扩繁MIL。

30.权利要求23-29中任一项的方法,其中所述方法包括制备多种重组MIL。

31.权利要求23的方法,所述方法进一步包括在用编码嵌合抗原受体的核酸转染、转化或转导MIL之前,于低氧条件下温育MIL。

32.权利要求31的方法,其中所述低氧条件包含约0.5%-约5%的氧气。

33.权利要求31的方法,其中所述低氧条件包含约1%-约2%的氧气。

34.权利要求31的方法,所述方法进一步包括在用编码嵌合抗原受体的核酸转染、转化或转导MIL之后,于常氧条件下温育MIL。

35.权利要求31的方法,所述方法进一步包括在于低氧条件下温育MIL的同时使MIL与抗CD3/抗CD28珠接触。

# 作为用于嵌合抗原受体(CAR)疗法的T细胞来源的骨髓浸润淋巴细胞(MIL)

[0001] 相关申请的交叉参考

该申请要求2015年7月8日递交的美国临时申请第62/189928号的优先权,其在此通过参照以其全部结合。

[0002] 背景

绝大多数恶性肿瘤患者将死于其疾病。一种治疗这些患者的方法是遗传修饰MIL以通过嵌合抗原受体("CAR")的表达来靶向肿瘤细胞上表达的抗原。CAR为抗原受体,其被设计为以不依赖于人白细胞抗原的方式识别细胞表面抗原。除了以CD19靶向方法取得的成功以外,使用表达CAR的遗传修饰细胞治疗其他恶性肿瘤的尝试仅取得有限的成功。

[0003] 概述

在一些实施方案中,提供包含嵌合抗原受体("CAR")的骨髓浸润淋巴细胞("MIL")。在一些实施方案中,CAR包含可结合配体的胞外结构域。在一些实施方案中,CAR包含可启动胞内信号传导级联的胞内结构域(例如在MIL中)。

[0004] 在一些实施方案中,提供用于在受试者中治疗病症的方法,包括给予受试者包含CAR的MIL。在一些实施方案中,方法包括给予受试者包含MIL群的组合物,其中MIL群中的每一个MIL包含CAR。

[0005] 在一些实施方案中,提供用于制备重组MIL的方法,包括获得包含MIL的骨髓,并用编码嵌合抗原受体的核酸转染、转化或转导MIL。骨髓可得自受试者,比如患有赘生物(neoplasm)的受试者。受试者可为人或小鼠。

[0006] 详述

在一些实施方案中,本文提供用于治疗包括但不限于血液恶性肿瘤和实体肿瘤的癌症的组合物和方法。各方面涉及但不限于被转导以表达嵌合抗原受体(CAR)的骨髓浸润淋巴细胞(MIL)的过继细胞转移策略。CAR为将基于抗体的针对期望的抗原(例如肿瘤抗原)的特异性与激活MIL受体的胞内结构域组合以产生呈现特异性抗肿瘤细胞免疫活性的嵌合蛋白的分子。

[0007] 在一些实施方案中,提供被遗传修饰以稳定表达期望的CAR的MIL的用途。表达CAR的MIL本文称为CAR-MIL或CAR-修饰的MIL。在一些实施方案中,可对细胞进行遗传修饰以在其表面稳定表达抗体结合结构域,赋予独立于MHC的新颖抗原特异性。在一些实施方案中,对MIL进行遗传修饰以稳定表达将特异性抗体的抗原识别结构域与CD3ζ链或FcγRI蛋白的胞内结构域组合成单一嵌合蛋白的CAR。

[0008] 在一些实施方案中,CAR包含具有抗原识别结构域的胞外结构域、跨膜结构域和细胞质结构域。在一些实施方案中,使用天然与CAR中的结构域之一缔合的跨膜结构域。在一些实施方案中,可选择或通过氨基酸取代来修饰跨膜结构域,以避免这样的结构域结合于相同或不同表面膜蛋白的跨膜结构域,以使与受体复合物的其他成员的相互作用最小化。例如,跨膜结构域可为CD8α铰链结构域。

[0009] 就细胞质结构域而言,CAR例如可被设计为本身包含CD28和/或4-1BB信号传导结

构域，或者与在CAR的情况下有用的任何其他期望的细胞质结构域组合。在一些实施方案中，CAR的细胞质结构域可被设计为进一步包含CD3ζ的信号传导结构域。例如，CAR的细胞质结构域可包括但不限于 CD3ζ、4-1BB和CD28信号传导模块及其组合。因此，实施方案提供CAR-MIL及其用于过继疗法的方法。

[0010] 在一些实施方案中，CAR-MIL可通过将包含期望的CAR (例如包含抗CD19、跨膜结构域和人4-1BB的CAR)的慢病毒载体引入到细胞中而产生。例如，CAR-MIL能够在体内复制，导致长期持续存在，这可导致持续的肿瘤控制。

[0011] 在一些实施方案中，提供使用CAR-MIL输注给予表达CAR的遗传修饰的MIL用于治疗赘生物患者。在一些实施方案中，治疗中使用自体输注。自体MIL收集自需要治疗的患者，并使用本文描述的和本领域已知的方法激活并扩繁(expand)，并然后输注回患者体内。

[0012] 在一些实施方案中，使用表达包括CD3ζ和4-1BB共刺激结构域的抗CD19 CAR的MIL。在某些情况下，输注到患者体内的CAR MIL可消除患者体内的白血病细胞。然而，实施方案不限于靶向CD19或通过CD3ζ和/或4-1BB介导的途径传导信号的MIL。例如，实施方案包括与选自CD137 (4-1BB)信号传导结构域、CD28信号传导结构域、CD3ζ信号结构域及其任何组合的一种或多种胞内结构域融合的任何抗原结合部分。

[0013] 定义

冠词“一”和“一个”本文用于指文章的一个或多于一个(即至少一个)语法对象。举例来说，“一个元素”意指一个元素或多于一个元素。

[0014] 如该文件中使用的那样，本文使用的术语“包含”、“具有”、“有”和“包括”及其变化形式意指“包括但不限于”。尽管各种组合物和方法就“包含”各种组分或步骤而言进行描述(解释为意指“包括但不限于”)，但组合物、方法和装置也可“实质上由...(各种组分和步骤)组成”或“由..... (各种组分和步骤)组成”，并且这样的术语应该解释为定义实质上封闭式成员组。

[0015] 本文使用的“激活”指的是已充分刺激以诱导可检测的细胞增殖的MIL的状态。激活也可与诱导的细胞因子产生和可检测的效应子功能相关联。术语“激活的MIL”尤其指正在经历细胞分裂的MIL。

[0016] 本文使用的术语“抗体”指的是与抗原特异性结合的免疫球蛋白分子。抗体可为源自天然来源或源自重组来源的完整免疫球蛋白，并且可为完整免疫球蛋白的免疫反应性部分。抗体可以多种形式存在，包括例如多克隆抗体、单克隆抗体、Fv、Fab和F(ab)2以及单链抗体和人源化抗体。

[0017] 术语“抗体片段”指的是完整抗体的一部分，并且指的是完整抗体的抗原决定可变区。抗体片段的实例包括但不限于Fab、Fab’、F(ab’)2和Fv片段、线性抗体、scFv抗体和由抗体片段形成的多特异性抗体。

[0018] 本文使用的术语“抗原”定义为引发免疫反应的分子。该免疫反应可能涉及抗体产生或特定免疫活性细胞的激活或两者。技术人员将会理解，任何大分子(包括几乎所有的蛋白质或肽)都可用作抗原。此外，抗原可源自重组或基因组DNA。技术人员将会理解，包含编码引发免疫反应的蛋白质的核苷酸序列或部分核苷酸序列的任何DNA因此编码如该术语在本文中使用的“抗原”。此外，本领域技术人员将会理解，抗原不需要仅由基因的全长核苷酸序列编码。显而易见的是，实施方案包括但不限于使用多于一种基因的部分核苷酸序列，并

且这些核苷酸序列以多种组合排列以引发期望的免疫反应。此外，技术人员将会理解抗原根本不需要由“基因”编码。显而易见的是，抗原可被合成产生或者可源自生物样本。这样的生物样本可包括但不限于组织样本、肿瘤样本、细胞或生物流体。

[0019] 本文使用的术语“抗肿瘤作用”指的是可通过肿瘤体积的减少、肿瘤细胞数目的减少、转移数目的减少、预期寿命的增加或与癌性病症相关的各种生理症状的改善而表现的生物效应。“抗肿瘤作用”首先也可通过肽、多核苷酸、细胞和抗体预防肿瘤发生的能力来表现。

[0020] 术语“自体抗原(auto-antigen)”意指任何被免疫系统误认为是外源的自身抗原(self-antigen)。自体抗原包括但不限于细胞蛋白、磷蛋白、细胞表面蛋白、细胞脂质、核酸、糖蛋白，包括细胞表面受体。

[0021] 本文使用的术语“自身免疫性疾病”定义为由自身免疫反应引起的障碍。自身免疫性疾病为对自身抗原的不适当和过度反应的结果。自身免疫性疾病的实例包括但不限于艾迪生病、斑秃(alopecia greata)、强直性脊柱炎、自身免疫性肝炎、自身免疫性腮腺炎、克罗恩病、糖尿病(I型)、营养不良性大疱性表皮松解症、附睾炎、肾小球肾炎、格雷夫斯病、格-巴二氏综合征、桥本病、溶血性贫血、系统性红斑狼疮、多发性硬化症、重症肌无力、寻常型天疱疮、牛皮癣、风湿热、类风湿性关节炎、结节病、硬皮病、斯耶格伦综合征、脊柱关节病、甲状腺炎、血管炎、白癜风、粘液性水肿、恶性贫血、溃疡性结肠炎等。

[0022] 本文使用的术语“自体的”意思是指任何源自相同个体的材料，其后来被重新引入到该个体。

[0023] “同种异体的”指的是源自相同物种的不同动物的移植物。

[0024] “异种异体的”指的是源自不同物种的动物的移植物。

[0025] 本文使用的术语“癌症”定义为特征为异常细胞的快速和不受控制的生长的疾病。癌细胞可局部传播或通过血流和淋巴系统传播到身体其他部位。各种癌症的实例包括但不限于乳腺癌、前列腺癌、卵巢癌、宫颈癌、皮肤癌、胰腺癌、结直肠癌、肾癌、肝癌、脑癌、淋巴瘤、白血病、肺癌等。

[0026] 如本文中使用的术语“共刺激配体”包括特异性结合MIL上的同源共刺激分子，从而除了由例如TCR/CD3复合物与负载有肽的MHC分子的结合提供的初级信号以外还提供介导MIL反应(包括但不限于增殖、激活、分化等)的信号的抗原呈递细胞(例如aAPC、树突状细胞、B细胞等)上的分子。共刺激配体可包括但不限于CD7、B7-1 (CD80)、B7-2 (CD86)、PD-L1、PD-L2、4-1BBL、OX40L、诱导型共刺激配体(ICOS- L)、细胞间粘附分子(ICAM)、CD30L、CD40、CD70、CD83、HLA-G、MICA、MICB、HVEM、淋巴毒素β受体、3/TR6、ILT3、ILT4、HVEM、结合Toll配体受体的激动剂或抗体和与B7-H3特异性结合的配体。共刺激配体还尤其包括与存在于MIL上的共刺激分子特异性结合的抗体，所述共刺激分子比如但不限于 CD27、CD28、4-1BB、OX40、CD30、CD40、PD-1、ICOS、淋巴细胞功能相关抗原-1 (LFA-1)、CD2、CD7、LIGHT、NKG2C、B7-H3及与CD83特异性结合的配体。

[0027] “共刺激分子”指的是在MIL上的同源结合配偶体，其与共刺激配体特异性结合，从而介导MIL的共刺激反应比如但不限于增殖。共刺激分子包括但不限于 MHC I类分子、BTLA和Toll配体受体。

[0028] 本文使用的“共刺激信号”指的是与初级信号比如TCR/CD3连接组合导致MIL增殖

和/或关键分子的上调或下调的信号。

[0029] “疾病”为其中受试者不能维持体内平衡，并且其中如果疾病没有得到改善则动物的健康继续恶化的受试者的健康状态。相比之下，受试者的“障碍”为其中受试者能够维持体内平衡，但其中受试者的健康状态不如在不存在障碍的情况下那样有利的健康状态。如果不进行治疗，障碍不一定会造成受试者的健康状态进一步下降。

[0030] 本文使用的“有效量”意指提供治疗或预防益处的量。

[0031] “编码”指的是用作在生物过程中合成具有确定的核苷酸序列(即rRNA、tRNA和mRNA)或确定的氨基酸序列及由此生成的生物学特性的其他聚合物和大分子的模板的多核苷酸(比如基因、cDNA或mRNA)中特定核苷酸序列的固有特性。因此，如果对应于该基因的mRNA的转录和翻译在细胞或其他生物系统中产生蛋白质，则该基因编码蛋白质。编码链(其核苷酸序列与mRNA序列一致，并且通常以序列表提供)和非编码链(用作基因或cDNA转录的模板)两者可称为编码蛋白质或者该基因或cDNA的其他产物。

[0032] 本文使用的“内源性的”指的是来自生物体、细胞、组织或系统的或者在其内部产生的任何材料。

[0033] 本文使用的术语“外源性的”指的是自生物体、细胞、组织或系统的外部引入的或者产生的任何材料。

[0034] 本文使用的术语“表达”定义为由其启动子驱动的特定核苷酸序列的转录和/或翻译。

[0035] “表达载体”指的是包含重组多核苷酸的载体，所述重组多核苷酸包含有效连接于待表达的核苷酸序列的表达控制序列。表达载体包含足够的用于表达的顺式作用元件，用于表达的其他元件可由宿主细胞或在体外表达系统中提供。表达载体包括本领域已知的所有那些表达载体，比如掺入重组多核苷酸的粘粒、质粒(例如裸露的或在脂质体中含有的)和病毒(例如慢病毒、逆转录病毒、腺病毒和腺伴随病毒)。

[0036] “同源的”指的是两个多肽之间或两个核酸分子之间的序列相似性或序列一致性。当两个比较序列两者中的一个位置被相同的碱基或氨基酸单体亚基占据时，例如如果两个DNA分子中每一个中的一个位置被腺嘌呤占据，则分子在该位置为同源的。两个序列之间的同源性百分比为两个序列共有的匹配或同源位置的数目除以比较位置的数目×100的函数。例如，如果两个序列中10个位置中的6个匹配或同源，则这两个序列为60%同源的。举例来说，DNA序列ATTGCC与TATGGC共有50%同源性。通常，当两个序列比对给出最大同源性时进行比较。

[0037] 本文使用的术语“免疫球蛋白”或“Ig”定义为一类起抗体作用的蛋白质。由B细胞表达的抗体有时称为BCR (B细胞受体)或抗原受体。包括在该类蛋白质中的5个成员为IgA、IgG、IgM、IgD和IgE。

[0038] “分离的”意指从天然状态改变或去除。例如，天然存在于活动物体内的核酸或肽不是“分离的”，但是与其天然状态的共存物质部分或完全分离的相同核酸或肽是“分离的”。分离的核酸或蛋白质可以实质上纯化的形式存在，或者可存在于非天然环境比如宿主细胞中。

[0039] 如本文使用的那样，对于通常存在的核酸碱基使用以下缩写。“A”指的是腺苷，“C”指的是胞嘧啶，“G”指的是鸟苷，“T”指的是胸苷和“U”指的是尿苷。

[0040] 本文使用的“慢病毒”指的是逆转录病毒科的属。慢病毒为逆转录病毒中在能够感染非分裂细胞方面是独特的，它们可将大量的遗传信息递送至宿主细胞的DNA中，因此它们是基因递送载体的最有效方法之一。HIV、SIV和FIV全部为慢病毒的实例。源自慢病毒的载体提供实现显著水平的体内基因转移的手段。

[0041] 本文使用的术语“骨髓浸润淋巴细胞”(“MIL”)指的是源自骨髓的淋巴细胞。骨髓浸润淋巴细胞(“MIL”)与外周血淋巴细胞以及肿瘤浸润淋巴细胞(“TIL”)具有许多可区分的差异。由于抗原呈递细胞(“APC”)的丰富性，骨髓(“BM”)微环境为一种特殊的免疫小生境。这些抗原呈递细胞的存在使得能够处理和呈递抗原以维持在骨髓隔室中发现的较高水平的中央型记忆细胞。(Li JM等人 J Immunol. 2009 Dec 15; 183(12):7799-809)。这些MIL表达记忆标记物比如CD45RO+和CD62L+，并且存在比PBL中发现的记忆细胞更多的记忆MIL (Noonan K等人 Clin Cancer Res. 2012 Mar 1; 18(5):1426-34)。此外，MIL由于其连续针对抗原致敏记忆细胞的能力而不仅仅是血液恶性肿瘤的“TIL”(Beckhove P等人 J Clin Invest. 2004 Jul 1; 114(1): 67-76; Castiglioni P等人 6 J Immunol 2008; 180:4956-4964)。由于在骨髓基质中大量表达的同源抗原基质衍生的1型因子(“SDF1”)，MIL也比其PBL对应物表达更多的CXCR4 (Noonan K等人 Cancer Res. 2005 Mar 1; 65(5):2026-34)。与PBL相比，MIL中41BB的表达也增加，这可能是由于BM微环境的低氧性质。进一步地，与TIL形成对比，MIL可自所有患者收获和扩繁(Noonan, K等人 Sci Transl Med. 2015 May 20; 7(288):288ra78)。TIL仅在约50%的患者中存在，和仅约25%的患者包含可扩繁的TIL。与外周血淋巴细胞(PBL)形成对比，MIL具有广泛的内源性抗原库，这解释了其内在肿瘤特异性--一种PBL中完全不存在的特征(Noonan等人 Clin Cancer Res)。

[0042] 除非另外规定，否则“编码氨基酸序列的核苷酸序列”包括为彼此的简并版本且编码相同氨基酸序列的所有核苷酸序列。编码蛋白质和RNA的核苷酸序列可包括内含子。

[0043] 术语“有效连接”指的是调控序列与异源核酸序列之间导致后者表达的功能性连接。例如，当第一核酸序列与第二核酸序列置于功能性关系时，第一核酸序列与第二核酸序列有效连接。例如，如果启动子影响编码序列的转录或表达，则启动子有效连接于编码序列。通常，有效连接的DNA序列为连续的，并且在必要时在相同的阅读框中连接两个蛋白质编码区。

[0044] 术语“过度表达的”肿瘤抗原或肿瘤抗原的“过度表达”旨在表明相对于来自该组织或器官的正常细胞中的表达水平，来自疾病区域像患者的特定组织或器官内的实体肿瘤的细胞中的肿瘤抗原的异常表达水平。通过本领域已知的标准测定可确定患有特征为肿瘤抗原的过度表达的实体肿瘤或血液恶性肿瘤的患者。

[0045] 免疫原性组合物的“肠胃外”给予包括例如皮下(s.c.)、静脉内(i.v.)、肌内(i.m.)或胸骨内注射或输注技术。

[0046] 术语“患者”、“受试者”、“个体”等本文可互换使用，并且指的是任何动物或其细胞，无论是体外还是原位，均适合于本文描述的方法。在某些非限制性的实施方案中，患者、受试者或个体为人。

[0047] 本文使用的术语“肽”、“多肽”和“蛋白质”可互换使用，并且指的是由经肽键共价连接的氨基酸残基组成的化合物。蛋白质或肽必须含有至少两个氨基酸，并且对可构成蛋白质或肽序列的氨基酸的最大数目没有限制。多肽包括包含两个或更多个经肽键彼此连接

的氨基酸的任何肽或蛋白质。如本文使用的那样，该术语指的是两条短链(其在本领域中通常也例如称为肽、寡肽和寡聚物)和指较长的链(其在本领域中通常称为蛋白质)，其存在许多类型。“多肽”包括例如生物活性片段、实质上同源的多肽、寡肽、同型二聚体、异型二聚体、多肽变体、修饰的多肽、衍生物、类似物、融合蛋白等。多肽包括天然肽、重组肽、合成肽或其组合。

[0048] 本文使用的术语“启动子”定义为启动多核苷酸序列的特异性转录需要的由细胞的合成机构或引入的合成机构识别的DNA序列。

[0049] 本文使用的术语“启动子/调控序列”意指有效连接于启动子/调控序列的基因产物表达需要的核酸序列。在某些情况下，该序列可为核心启动子序列，并且在其他情况下，该序列也可包括基因产物表达需要的增强子序列和其他调控元件。启动子/调控序列可例如为以组织特异性方式表达基因产物的启动子/调控序列。

[0050] “组成型”启动子为当与编码或指定基因产物的多核苷酸有效连接时，造成基因产物于细胞的大多数或全部生理条件下在细胞中产生的核苷酸序列。

[0051] “诱导型”启动子为当与编码或指定基因产物的多核苷酸有效连接时，造成基因产物实质上仅在细胞中存在对应于启动子的诱导子时才在细胞中产生的核苷酸序列。

[0052] “组织特异性的”启动子为当与由基因编码或指定的多核苷酸有效连接时，造成基因产物实质上仅在细胞为对应于启动子的组织类型的细胞时才在细胞中产生的核苷酸序列。

[0053] 所谓的术语“刺激”意味着通过刺激分子(例如TCR/CD3复合物)与其同源配体结合，从而介导信号转导事件比如但不限于经TCR/CD3复合物的信号转导而诱导的初级反应。刺激可介导某些分子的表达改变，比如TGF-β下调和/或细胞骨架结构的重组等。

[0054] 如同该术语在本文中使用的那样，“刺激分子”意指与存在于抗原呈递细胞上的同源刺激配体特异性结合的MIL上的分子。

[0055] 本文使用的“刺激配体”意指当存在于抗原呈递细胞(例如aAPC、树突状细胞、B细胞等)上时可与MIL上的同源结合配偶体(本文称为“刺激分子”)特异性结合，从而介导MIL的初级反应(包括但不限于激活、免疫反应的启动、增殖等)的配体。刺激配体为本领域熟知的，并且尤其包括负载有肽的MHC I类分子、抗CD3抗体、超级激动剂(superagonist)抗CD28抗体和超级激动剂抗CD2抗体。

[0056] 术语“受试者”旨在包括其中可引发免疫反应的活生物体(例如哺乳动物)。受试者的实例包括人、狗、猫、小鼠、大鼠及其转基因物种。

[0057] 本文使用的术语“治疗的”意指治疗和/或预防。治疗作用通过抑制、缓解或根除疾病状态来获得。

[0058] 术语“治疗有效量”指的是将引发研究人员、兽医、医生或其他临床医生正在探寻的组织、系统或受试者的生物或医学反应的受试化合物的量。术语“治疗有效量”包括当给予时足以预防所治疗的障碍或疾病的一种或多种体征或症状的发展或在某种程度上减轻的化合物的量。治疗有效量将依化合物、待治疗的受试者的疾病及其严重程度和年龄、体重等而变化。

[0059] 如同该术语在本文中使用的那样，“治疗”疾病意指减少受试者经历的疾病或障碍的至少一种体征或症状的频率或严重程度。

[0060] 本文使用的术语“转染的”或“转化的”或“转导的”指的是外源性核酸藉以转移或引入到宿主细胞中的过程。“转染的”或“转化的”或“转导的”细胞为已经用外源性核酸转染、转化或转导的细胞。细胞包括原代受试者细胞及其后代。

[0061] 本文使用的短语“在转录控制下”或“有效连接”意指启动子相对于多核苷酸处于正确的位置和方向，以控制RNA聚合酶的转录起始和多核苷酸的表达。

[0062] “载体”为包含分离的核酸并可用于将分离的核酸递送至细胞内部的物质的组合物。许多载体为本领域已知的，包括但不限于线性多核苷酸、与离子性或两亲性化合物缔合的多核苷酸、质粒和病毒。因此，术语“载体”包括自主复制质粒或病毒。该术语还应解释为包括促进核酸转移到细胞中的非质粒和非病毒化合物，比如聚赖氨酸化合物、脂质体等。病毒载体的实例包括但不限于腺病毒载体、腺伴随病毒载体、逆转录病毒载体等。

[0063] 描述

本公开提供用于治疗癌症等疾病的组合物和方法。癌症可为血液恶性肿瘤、实体肿瘤、原发性或转移性肿瘤。癌症可为血液恶性肿瘤，比如慢性淋巴细胞白血病(“CLL”)。可使用本文描述和提供的组合物和方法治疗的其他疾病包括病毒、细菌和寄生虫感染以及自身免疫性疾病。

[0064] 在一些实施方案中，提供被工程改造以表达CAR的细胞(即MIL)，其中CAR-MIL呈现抗肿瘤特性。CAR可例如被工程改造以包含具有与MIL抗原受体复合物ζ链(例如CD3ζ)的胞内信号传导结构域融合的抗原结合结构域的胞外结构域。CAR例如当在MIL中表达时能够基于抗原结合特异性重定向抗原识别。在一些实施方案中，抗原为CD19，因为该抗原在恶性B细胞上表达。然而，实施方案不限于靶向CD19。更确切地，实施方案包括任何抗原结合部分，其当结合于其同源抗原时影响肿瘤细胞以致肿瘤细胞不能生长、被促使死亡或者以其他方式受到影响以致减少或消除患者的肿瘤负担。抗原结合部分可与来自共刺激分子和ζ链中的一种或多种的胞内结构域融合。在一些实施方案中，抗原结合部分与选自CD137 (4-1BB)信号传导结构域、CD28信号传导结构域、CD3ζ信号结构域及其任何组合的一种或多种胞内结构域融合。抗原结合部分也可与胞内结构域比如CD134 (OX40)融合。

[0065] 在一些实施方案中，CAR包含CD137 (4-1BB)信号传导结构域。不受任何特定理论的束缚，这是因为实施方案部分地基于以下发现：CAR介导的T细胞反应可随着添加共刺激结构域而进一步增强。

[0066] I．嵌合抗原受体

本文提供包含胞外和胞内结构域的嵌合抗原受体(CAR)。胞外结构域包含另外称为抗原结合部分的靶标特异性结合元件。胞内结构域或者细胞质结构域可包含共刺激信号传导区和/或ζ链的一部分。共刺激信号传导区指的是包含共刺激分子的胞内结构域的CAR的一部分。共刺激分子为除了抗原受体或其配体以外的淋巴细胞对抗原有效反应需要的细胞表面分子。

[0067] 可在CAR的胞外结构域与跨膜结构域之间或在CAR的细胞质结构域与跨膜结构域之间掺入间隔结构域。本文使用的术语“间隔结构域”通常意指起将跨膜结构域连接至多肽链的胞外结构域或细胞质结构域的作用的一段氨基酸。间隔结构域可包含多达300个氨基酸，优选地2-100个氨基酸，比如25-50个氨基酸。

[0068] II．胞外结构域

在一些实施方案中,CAR包含另外称为抗原结合部分的靶标特异性结合元件。部分的选择取决于定义靶细胞表面的配体类型和数目。例如,可选择抗原结合结构域来识别起与特定疾病状态相关的靶细胞上的细胞表面标记物作用的配体。因此,可起CAR中抗原结合结构域的配体作用的细胞表面标记物的实例包括与病毒、细菌和寄生虫感染、自身免疫性疾病和癌细胞相关的那些细胞表面标记物。例如,配体可为细菌、病毒或寄生虫的蛋白质。类似地,配体可为在癌细胞表面上调的蛋白质。

[0069] 在一些实施方案中,可通过工程改造特异性结合于肿瘤细胞上的抗原的期望的抗原结合部分来工程改造CAR以靶向目标肿瘤抗原。本文使用的"肿瘤抗原"或"过度增殖性障碍抗原"或"与过度增殖性障碍相关的抗原"指的是对特定过度增殖性障碍比如癌症常见的抗原。本文讨论的抗原仅作为实例被包括。列表不旨在为排他性的,并且进一步的实例对本领域技术人员为易于显而易见的。

[0070] 肿瘤抗原为由引发免疫反应(特别是T细胞介导的免疫反应)的肿瘤细胞产生的蛋白质。抗原结合部分的选择取决于待治疗的癌症的特定类型。肿瘤抗原为本领域熟知的,并且包括例如神经胶质瘤相关抗原、癌胚抗原(CEA)、β-人绒毛膜促性腺激素、甲胎蛋白(AFP)、凝集素反应性AFP、甲状腺球蛋白、RAGE-1、MN-CA IX、人端粒酶逆转录酶、RU1、RU2(AS)、肠羧酸酯酶、突变hsp70-2、M-CSF、prostase、前列腺特异性抗原(PSA)、PAP、NY-ESO-1、LAGE-1a、p53、prostein、PSMA,Her2/neu、存活素、端粒酶、前列腺癌肿瘤抗原-1 (PCTA-1)、MAGE、ELF2M、嗜中性粒细胞弹性蛋白酶、肝配蛋白B2、CD22、胰岛素生长因子(IGF)-II、IGF-1受体和间皮素。

[0071] 在一些实施方案中,肿瘤抗原包含与恶性肿瘤相关的一种或多种抗原性癌症表位。恶性肿瘤表达许多可用作免疫攻击的靶抗原的蛋白质。这些分子包括但不限于组织特异性抗原比如黑素瘤中的MART-1、酪氨酸酶和GP100及前列腺癌中的前列腺酸性磷酸酶(PAP)和前列腺特异性抗原(PSA)。其他靶分子属于转化相关分子组,比如致癌基因HER-2/Neu/ErbB-2。又一组靶抗原为瘤胎抗原,比如癌胚抗原(CEA)。在B细胞淋巴瘤中,肿瘤特异性独特型免疫球蛋白构成对个体肿瘤独特的真正的肿瘤特异性免疫球蛋白抗原。B细胞分化抗原比如CD19、CD20和CD37为B细胞淋巴瘤中靶抗原的其他候选物。这些抗原中的一些(例如CEA、HER-2、CD19、CD20、独特型)已被用作单克隆抗体被动免疫疗法的靶标,但成功的有限。

[0072] 所提及的肿瘤抗原的类型也可为肿瘤特异性抗原(TSA)或肿瘤相关抗原(TAA)。TSA对肿瘤细胞为独特的,并且不发生在体内的其他细胞上。TAA相关抗原对肿瘤细胞不是独特的,而是在不能诱导对抗原免疫耐受状态的条件下也在正常细胞上表达。抗原在肿瘤上的表达可发生于使得免疫系统对抗原作出反应的条件下。当免疫系统不成熟并且不能反应时,TAA可为在胎儿发育期间于正常细胞上表达的抗原,或者其可为通常以极低水平存在于正常细胞但是以高得多的水平在肿瘤细胞上表达的抗原。

[0073] TSA或TAA抗原的非限制性实例包括以下:分化抗原比如MART-1/MelanA (MART-1)、gp100 (Pmel 17)、酪氨酸酶、TRP-1、TRP-2和肿瘤特异性多谱系抗原比如MAGE-1、MAGE-3、BAGE、GAGE-1、GAGE-2、p15;过度表达的胚胎抗原比如CEA;过度表达的致癌基因和突变的肿瘤抑制基因比如p53、Ras、HER-2/neu;自染色体易位生成的独特的肿瘤抗原比如BCR-ABL、E2A-PRL、H4-RET、IGH-IGK、MYL-RAR;和病毒抗原比如EB病毒抗原EBVA和人乳头状瘤病

毒(HPV)抗原E6和E7。其他大的基于蛋白质的抗原包括TSP-180、MAGE-4、MAGE-5、MAGE-6、RAGE、NY-ESO、p185erbB2、p180erbB-3、c-met、nm-23H1、PSA、TAG-72、CA 19-9、CA 72-4、CAM 17.1、NuMa、K-ras、β-连环蛋白、CDK4、Mum-1、p 15、p 16、43-9F、5T4、791Tgp72、甲胎蛋白、β-HCG、BCA225、BTAA、CA 125、CA 15-3\CA 27.29\BCAA、CA 195、CA 242、CA-50、CAM43、CD68\P1、CO-029、FGF-5、G250、Ga733\EpCAM、HTgp-175、M344、MA-50、MG7-Ag、MOV18、NB/70K、NY-CO-1、RCAS1、SDCCAG16、TA-901\Mac-2结合蛋白\亲环蛋白C-相关蛋白、TAAL6、TAG72、YLP和TPS。

[0074] 在一些实施方案中,CAR的抗原结合部分靶向包括但不限于 CD19、CD20、CD22、ROR1、间皮素、CD33/IL3Ra、c-Met、PSMA、糖脂F77、EGFRvIII、GD-2、MY-ESO-1 TCR、MAGE A3 TCR等的抗原。

[0075] 依期望的待靶向的抗原而定,CAR可被工程改造为包括对期望的抗原靶标特异性的合适的抗原结合部分。例如,如果CD19为期望的待靶向的抗原,则针对CD19的抗体可用作抗原结合部分掺入到CAR中。因此,在一些实施方案中,CAR的抗原结合部分靶向CD19。

[0076] CAR的胞外结构域可包含例如结合于任何一种本文描述的靶标的单链可变片段(“scFv”)。

[0077] 胞外结构域可为任何抗原结合多肽,其广泛种类为本领域已知的。在某些情况下,抗原结合结构域为单链Fv (“scFv”)。其他基于抗体的识别结构域(cAb VHH (骆驼抗体可变结构域)和人源化版本、IgNAR VH (鲨鱼抗体可变结构域)和人源化版本、sdAb VH (单结构域抗体可变结构域)和“骆驼化”抗体可变结构域适合使用。在某些情况下,基于T细胞受体(TCR)的识别结构域比如单链TCR (scTv,含有v vβ的单链双结构域TCR)也适合使用。

[0078] 本领域已知的其他胞外结构域也可用于实施方案中(参见例如PCT专利申请公开号WO 2014/127261、美国专利第8975071号,在此通过参照结合)。

[0079] III. 跨膜结构域

就跨膜结构域而言,CAR可被设计为包含与CAR的胞外结构域融合的跨膜结构域。在一些实施方案中,使用天然与CAR中的一个结构域缔合的跨膜结构域。在某些情况下,可选择或通过氨基酸取代来修饰跨膜结构域,以避免这样的结构域与相同或不同表面膜蛋白的跨膜结构域结合,以使与受体复合物的其他成员的相互作用最小化。

[0080] 跨膜结构域可源自天然来源或者跨膜结构域可被设计(例如自形成α-螺旋的一段18-30个疏水性氨基酸,比如丙氨酸、缬氨酸、亮氨酸和异亮氨酸)。当来源为天然的,结构域可源自任何膜结合的或跨膜蛋白质。特定用途的跨膜区可源自(即至少包含其跨膜区)T细胞受体的α、β或ζ链、CD28、CD3ε、CD45、CD4、CD5、CD8、CD9、CD16、CD22、CD33、CD37、CD64、CD80、CD86、CD134、CD137或CD154。或者跨膜结构域可被设计,在这种情况下,其将主要包含疏水性残基比如亮氨酸和缬氨酸。对于设计的跨膜结构域,可在膜/水界面附近存在苯丙氨酸、色氨酸和/或酪氨酸。任选地,长度在2-10个氨基酸之间的短的寡肽或多肽接头可连接CAR的跨膜结构域和细胞质信号传导结构域。甘氨酸-丝氨酸间隔子提供特别合适的接头。

[0081] IV. 胞内结构域

CAR的细胞质结构域或者胞内信号传导结构域负责激活MIL的至少一种正常效应子功能。术语“效应子功能”指的是细胞的特化功能。MIL的效应子功能例如可为细胞溶解活性或辅助细胞活性,包括细胞因子的分泌。因此,术语“胞内信号传导结构域”指的是转导效应子

功能信号并引导细胞实施特化功能的蛋白质部分。尽管可使用整个胞内信号传导结构域，但在许多情况下没有必要使用整个胞内结构域。就使用胞内信号传导结构域的截短部分而言，可使用这样的截短部分代替完整的链，只要其转导效应子功能信号。术语胞内信号传导结构域因此意指包括足以转导效应子功能信号的胞内信号传导结构域的任何截短部分。

[0082] 用于CAR的胞内信号传导结构域的一些非限制性实例包括T细胞受体(TCR)的细胞质序列和共同起作用以启动抗原受体接合后的信号转导的共受体，以及这些序列的任何衍生物或变体和具有相同功能性能力的任何合成序列。

[0083] 仅通过TCR产生的信号不足以完全激活淋巴细胞，还需要次级或共刺激信号。因此，MIL激活通过两种不同种类的细胞质信号传导介导：通过TCR启动抗原依赖性初级激活的那些信号传导(初级细胞质信号传导序列)和以抗原非依赖性方式起作用以提供次级或共刺激信号的那些信号传导(次级细胞质信号传导序列)。

[0084] 初级细胞质信号传导序列以刺激方式或以抑制方式调节TCR复合物的初级激活。以刺激方式起作用的初级细胞质信号传导序列可含有称为基于免疫受体酪氨酸的激活基序或ITAM的信号传导基序。

[0085] 可使用的含有初级细胞质信号传导序列的ITAM的实例包括但不限于源自TCRζ、FcRγ、FcRβ、CD3γ、CD3δ、CD3ε、CD5、CD22、CD79a、CD79b和CD66d的那些ITAM。在一些实施方案中，CAR的细胞质信号传导分子包含源自CD3ζ的细胞质信号传导序列。

[0086] 在一些实施方案中，CAR的细胞质结构域可被设计为本身包含CD3ζ信号传导结构域或者与在CAR的情况下有用的任何其他期望的细胞质结构域组合。例如，CAR的细胞质结构域可包含CD3ζ链的一部分和共刺激信号传导区。共刺激信号传导区指的是包含共刺激分子的胞内结构域的CAR的一部分。在一些实施方案中，共刺激分子为除了抗原受体或其配体以外的淋巴细胞对抗原有效反应需要的细胞表面分子。这样的分子的实例包括CD27、CD28、4-1BB (CD137)、OX40、CD30、CD40、PD-1、ICOS、淋巴细胞功能相关抗原-1 (LFA-1)、CD2、CD7、LIGHT、NKG2C、B7-H3等。因此，尽管一些实施方案可用4-1BB作为共刺激信号传导元件来举例说明，但是也可使用其他共刺激元件。

[0087] CAR的细胞质信号传导部分内的细胞质信号传导序列可以随机或指定的顺序彼此连接。任选地，短的寡肽或多肽接头(优选地长度在2-10个氨基酸之间)可形成连接。甘氨酸-丝氨酸间隔子提供特别合适的接头。

[0088] 在一些实施方案中，细胞质结构域被设计为包含CD3ζ的信号传导结构域和CD28的信号传导结构域。在一些实施方案中，细胞质结构域被设计为包含CD3ζ的信号传导结构域和4-1BB的信号传导结构域。在一些实施方案中，细胞质结构域被设计为包含CD3ζ的信号传导结构域和CD28与4-1BB的信号传导结构域。

[0089] 在一些实施方案中，CAR中的细胞质结构域被设计为包含4-1BB的信号传导结构域和CD3ζ的信号传导结构域。

[0090] V. 载体

编码CAR的天然或合成核酸的表达一般地通过将编码CAR多肽或其部分的核酸有效连接至启动子，并将该构建体掺入到表达载体中来实现。载体可适合于复制和整合真核生物。典型的克隆载体含有转录和翻译终止子、起始序列和可用于调节期望的核酸序列表达的启动子。

[0091] 源自逆转录病毒比如慢病毒的载体为实现长期基因转移的合适工具，因为它们使得转基因能够长期稳定整合及其在子细胞中繁殖。慢病毒载体具有超过源自致癌逆转录病毒比如鼠白血病病毒的载体的额外优点，因为其可转导非增殖细胞，比如肝细胞。它们也具有免疫原性低的额外优点。

[0092] 编码期望的分子的核酸序列可使用本领域已知的重组方法获得，比如通过筛选表达基因的细胞库、通过自已知包含基因的载体衍生基因或者通过使用标准技术直接自含有基因的细胞和组织分离。或者，目标基因可合成而不是克隆。

[0093] 使用标准基因递送方案，表达构建体也可用于核酸免疫和基因疗法。用于基因递送的方法为本领域已知的(参见例如美国专利第5399346、5580859、5589466号，在此通过参照结合)。在一些实施方案中，实施方案提供基因疗法载体。核酸序列也可使用基因编辑技术插入，比如但不限于CRISPR。

[0094] 核酸可被克隆到许多类型的载体中。例如，核酸可被克隆到包括但不限于以下的载体中：质粒、噬菌粒、噬菌体衍生物、动物病毒和粘粒。特别目标载体包括表达载体、复制载体、探针产生载体和测序载体。

[0095] 表达载体可以病毒载体的形式提供给细胞。病毒载体技术为本领域熟知的，并且例如在Green&Sambrook (Molecular Cloning: A Laboratory Manual)，(第4版, 2012))和其他病毒学和分子生物学手册中描述。可用作载体的病毒包括但不限于逆转录病毒、腺病毒、腺伴随病毒、疱疹病毒和慢病毒。通常，合适的载体含有在至少一种生物体中功能性的复制起点、启动子序列、合宜的限制性核酸内切酶位点和一种或多种选择标记物(例如WO 01/96584、WO 01/29058和美国专利第6326193号)。

[0096] 已经开发了许多基于病毒的系统用于基因转移到哺乳动物细胞中。例如，逆转录病毒为基因递送系统提供一种便利的平台。可使用本领域已知的技术将选择的基因插入到载体中并包装在逆转录病毒颗粒中。然后可将重组病毒分离并在体内或离体递送至受试者的细胞。在一些实施方案中，使用腺病毒载体。在一些实施方案中，使用慢病毒载体。

[0097] 另外的调控元件(例如启动子和增强子)调节转录起始的频率。一般地，这些位于起始位点上游30-100000 bp处，尽管最近已经显示出许多启动子在起始位点下游也含有功能元件。启动子元件之间的间隔通常是灵活的，以致当元件颠倒或相对于彼此移动时保持启动子功能。在胸苷激酶(tk)启动子中，启动子元件之间的间隔可在活性开始下降之前相距增加50 bp。依启动子而定，单个元件可合作或独立地起作用以激活转录。

[0098] 合适的启动子的一个实例为立即早期巨细胞病毒(CMV)启动子序列。该启动子序列为能够驱动与其有效连接的任何多核苷酸序列的高水平表达的强组成型启动子序列。合适的启动子的另一个实例为延伸生长因子-1α (EF-1α)。然而，也可使用其他组成型启动子序列，包括但不限于猿猴病毒40 (SV40)早期启动子、小鼠乳腺肿瘤病毒(MMTV)、人免疫缺陷病毒(HIV)长末端重复序列(LTR)启动子、MoMuLV 启动子、禽白血病病毒启动子、EB病毒立即早期启动子、劳斯肉瘤病毒启动子以及人基因启动子，比如但不限于肌动蛋白启动子、肌球蛋白启动子、血红蛋白启动子和肌酸激酶启动子。启动子不限于组成型启动子。诱导型启动子也可使用。诱导型启动子的使用提供一种分子开关，其能够在期望这样的表达时开启有效连接的多核苷酸序列的表达，或者在不期望表达时关闭表达。诱导型启动子的实例包括但不限于金属硫蛋白启动子、糖皮质激素启动子、黄体酮启动子和四环素启动子。

[0099] 为了评价CAR多肽或其部分的表达，待引入到细胞中的表达载体也可含有选择标记物基因或报告基因或两者，以促进自试图通过病毒载体转染或感染的细胞群鉴别和选择表达细胞。在其他方面，选择标记物可携带在单独的DNA片段上并用于共转染过程。选择标记物和报告基因两者侧翼可为合适的调控序列以使得在宿主细胞中表达。有用的选择标记物包括例如抗生素抗性基因，比如neo等。

[0100] 报告基因用于鉴定潜在转染的细胞和用于评估调控序列的功能性。通常，报告基因为不存在于接受者生物体或组织中或者由接受者生物体或组织表达并且编码多肽(其表达通过一些可易于检测的性质例如酶活性表现)的基因。在将DNA引入到接受者细胞后，于合适的时间测定报告基因的表达。合适的报告基因可包括编码萤光素酶、β-半乳糖苷酶、氯霉素乙酰转移酶、分泌型碱性磷酸酶的基因或绿色荧光蛋白基因(例如Ui-Tei等人, 2000 FEBS Letters 479: 79-82)。在一些实施方案中，报告基因为mCherry。

[0101] 将基因引入到细胞中和表达的方法为本领域熟知的。在表达载体的情况下，载体可通过本领域的任何方法易于引入到宿主细胞例如哺乳动物、细菌、酵母或昆虫细胞中。例如，表达载体可通过物理、化学或生物学手段转移至宿主细胞中。

[0102] 用于将多核苷酸引入到宿主细胞中的物理方法包括磷酸钙沉淀、脂质体转染、粒子轰击、显微注射、电穿孔等。用于产生包含载体和/或外源性核酸的细胞的方法为本领域熟知的(参见例如Green & Sambrook, Molecular Cloning: A Laboratory Manual, (第4版, 2012))。

[0103] 用于将目标多核苷酸引入到宿主细胞中的生物学方法包括使用DNA和RNA载体。病毒载体，并且尤其是逆转录病毒载体，已经成为用于将基因插入到哺乳动物细胞中的广泛使用的方法。其他病毒载体可源自慢病毒、痘病毒、I型单纯疱疹病毒、腺病毒、腺伴随病毒等(参见例如美国专利第5350674和5585362号)。

[0104] 用于将核酸引入到宿主细胞中的化学手段包括胶体分散系统，比如大分子复合物、纳米胶囊、微球、珠和基于脂质的系统，包括水包油乳液、胶束、混合胶束和脂质体。用作体外和体内递送载体的示例性胶体系统为脂质体(例如人工膜囊泡)。

[0105] 在使用非病毒递送系统的情况下，示例性的递送载体为脂质体。考虑使用脂质制剂用于将核酸引入到宿主细胞中(体外、离体或体内)。另一方面，核酸可与脂质缔合。与脂质缔合的核酸可被包封在脂质体的水性内部、散布在脂质体的脂质双分子层内、经与脂质体和寡核苷酸两者缔合的连接分子连接于脂质体、俘获在脂质体中、与脂质体复合、分散在含有脂质的溶液中、与脂质混合、与脂质组合、作为悬浮液在脂质中含有、含有胶束或与胶束复合、或以其他方式与脂质缔合。脂质、脂质/DNA或脂质/表达载体缔合的组合物不限于溶液中的任何特定结构。例如，它们可以双分子层结构存在，如胶束或“塌陷”结构。它们也可简单地散布在溶液中、可能形成大小或形状不均匀的聚集体。

[0106] VI. 骨髓浸润淋巴细胞

在MIL的扩繁和遗传修饰之前，MIL的来源得自受试者。在患有许多类型癌症中的任何一种(包括血液恶性肿瘤和实体肿瘤)的患者中，与外周血相比，T细胞可易于得自骨髓微环境，其具有提高的肿瘤特异性(参见例如美国专利申请公开号US 2011/0223146，在此通过参照结合)。通过比较得自患有血液恶性肿瘤的受试者的这两种不同隔室的T细胞，观察到得自骨髓抽吸物的骨髓浸润淋巴细胞(MIL)的寡克隆限制。方法(比如包括抗CD3/CD28抗体

缀合的磁珠的那些方法)可用于体外激活并扩繁骨髓细胞以产生激活的MIL。与所有被检查患者的外周血淋巴细胞相比,激活的MIL显示出更大的扩繁和增强的肿瘤活性。这些发现提示:1) 骨髓为肿瘤特异性T细胞的储库;2) 在所研究的所有患者中,MIL可被激活并扩繁(与在转移性黑素瘤中观察到的有限数目相比);3) 这些细胞在输注时进入骨髓; 4) 在NOD/SCID小鼠中过继转移后持续长达200天;并且5) 激活的MIL能够根除预先建立的疾病并靶向骨髓瘤干细胞前体,因此意味着广泛的抗原识别。

[0107] 代表总骨髓细胞群的少数部分的T细胞可在存在几乎完整骨髓的情况下扩繁。为了确保最大的肿瘤-T细胞接触,抽出的骨髓可基于淋巴细胞分离培养基密度梯度分级分离,并可将细胞收集到几乎达到红细胞沉淀的水平。这种分离方法实质上仅去除红细胞和嗜中性粒细胞,提供几乎完整的骨髓,并导致收集T细胞以及肿瘤细胞两者。T细胞可在没有T细胞特异性分离步骤和没有肿瘤细胞分离步骤的情况下扩繁。细胞类型特异性分离步骤包括例如使用抗体或其他细胞类型特异性检测标记进行的细胞标记和使用荧光激活细胞分选(FACS)进行分选。在一些实施方案中,所述方法可在没有这样的标记和细胞分选方法的情况下实践。

[0108] 为了用珠激活,珠-T细胞接触优选地在培养的最初24-48小时期间达到最大。因为T细胞仅代表群中总细胞的少数部分,因此通过使用足够数目的珠与细胞来促进T细胞与抗体包被的珠接触,其范围为约1:1-约5:1的珠与细胞,优选地为约2:1-4:1的珠与细胞,更优选地为约2.5:1至3.5:1的珠与细胞。这些比率适用于所公开的珠,并且珠大小和/或珠上抗体密度的变化可改变珠:细胞比率。

[0109] 在一些实施方案中,装置可用于培养细胞,提供光滑、刚性、圆形的底部表面以促进通过重力极接近地收集细胞和珠(参见例如美国专利申请公开号US2011/0223146,在此通过参照结合)。该装置包括放置在支架上的封闭的细胞容器。在存在珠的情况下至少第一个3天培养期间,容器优选地静止(即不摇动或旋转)以进一步促进珠与细胞之间的接触。这些步骤和条件对于使用珠使肿瘤特异性MIL的扩繁达到最大,以使得能够产生足够的治疗有用的细胞为优选的。进一步地,这些培养条件促进T细胞的生长而不促进肿瘤细胞的生长。

[0110] MIL的几种属性使其成为免疫疗法的合适候选物。具体地讲,在本文描述的条件下,它们在刺激时比PBL更快速地扩繁,并且在激活时通常维持偏态的T细胞库,可能提示肿瘤特异性增强。尽管未激活的MIL对自体肿瘤显示出显著的低反应性,但是激活并扩繁T细胞并显著增强其肿瘤反应性的能力显示缺失耐受性(deletional tolerance)作为这种情况下介导T细胞无反应性的推定机制是站不住脚的。此外,激活的MIL显示肿瘤特异性,对非恶性造血元件的交叉反应性小,CXCR-4的表达更高,并且对SDF-I具有更大的反应性,提示MIL向骨髓的迁移能力增加。综合考虑,这些研究结果显示激活并扩繁具有记忆/效应表型的骨髓浸润T细胞的能力,所述T细胞似乎靶向在成熟的终末分化浆细胞及其前体两者上存在的广泛范围的肿瘤抗原,并且具有趋化因子受体,这似乎促进进入到骨髓隔室-使过继免疫疗法的抗肿瘤免疫力达到最大所必要的特征。

[0111] MIL的激活并扩繁基于两个先前报道的现象:肿瘤浸润淋巴细胞的肿瘤特异性增强(Rosenberg等人 Science 1986; 233:1318),以及证实患有黑素瘤(Letsch等人 Cancer Res 2003; 63:5582-6)、乳腺癌(Feuerer等人 Nat Med 2001; 7:452)和多发性骨髓瘤(一

种骨髓也代表肿瘤微环境的疾病)(Dhodapkar等人 Proc Natl Acad Sci U S A 2002;99:13009)的患者的骨髓中存在肿瘤反应性T细胞。

[0112] 本文提供激活并扩繁MIL的能力作为克服其无反应性并且与激活的PBL相比显著增加其肿瘤特异性的手段。肿瘤在骨髓微环境中的存在可能在保留激活的MIL的抗原特异性方面起关键作用。几种假设可能解释激活的MIL的反应性增加超过激活的PBL。不受机制的束缚,提示抗原在骨髓中的持续存在可能对维持记忆反应(memory response)是必要的。抗CD3/CD28抗体包被的珠激活可能会逆转骨髓T细胞群的耐受性。类似地,平板结合的和/或可溶性CD3和/或CD28可用于激活。然而,激活MIL的手段不是特别限制的,并且任何合适的激活方法可用于各种实施方案中。如本文证实的那样,激活的MIL的肿瘤特异性取决于T细胞激活期间抗原的存在。进一步地,骨髓为一种功能性淋巴器官,能够在存在危险信号(感染、炎症、自身免疫和癌症)的情况下经反应性淋巴样滤泡产生初级免疫反应和次级反应两者。

[0113] 骨髓瘤患者的T细胞显示出VB T细胞受体库的相当的偏态。这样的偏态提示具有显著肿瘤特异性的T细胞的选择性生长或来自具有显著肿瘤负担的患者的明显基础性T细胞缺陷特征的结果。在后者情况下,用抗CD3/CD28抗体缀合的磁珠多克隆刺激PBL的益处为恢复正常T细胞库,并因此纠正任何基础性T细胞缺陷的能力。相比之下,如果特定VB家族的寡克隆表达反映存在具有肿瘤特异性的T细胞,则具有所维持的抗肿瘤活性和T细胞受体库偏态的该T细胞池的激活和扩繁可能为优选的。如本文证实的那样,在用抗CD3/CD28抗体缀合的磁珠激活并扩繁时,PBL标准化其VB T细胞库,而MIL维持VB限制。考虑到激活的MIL的肿瘤特异性反应增强,其偏态T细胞库可能提示更大的肿瘤识别。不受机制的束缚,在T细胞扩繁期间保持并可能地增加VB偏态的程度可能是重要的。

[0114] 用抗CD3/CD28抗体缀合的磁珠激活并扩繁MIL产生有效的抗肿瘤活性,并且在该扩繁期间抗原的持续存在在维持(和增强)肿瘤特异性方面具有重要意义。Dhodapkar等人(2002)也研究了MIL在骨髓瘤患者中的作用。与我们的研究结果类似,新鲜分离的MIL或PBL在用自体肿瘤或肿瘤肽刺激时未显示活性。然而,尽管该研究在用肿瘤脉冲的树突状细胞温育12-16天后,在酶联免疫斑点测定中自外周血与骨髓隔室获得的T细胞之间没见有显著差异,但是在检察的所有测定中,在我们的系统中观察到激活的MIL的抗肿瘤反应是激活的PBL的10倍大。与MIL的树突状细胞激活相比,这些不一致的结果可能与抗CD3/CD28珠刺激的效力有关。不受机制的束缚,似乎激活并扩繁的MIL培养物中肿瘤反应性T细胞的频率增加可能反映耐受性的破坏和肿瘤反应性T细胞功能的恢复。此外,在骨髓微环境内刺激MIL为可解释这些结果的另一个重要因素。

[0115] 通过负向选择富集MIL群可通过针对负向选择的细胞独特的表面标记物的抗体组合来完成。一种方法为经负磁性免疫吸附或流式细胞术进行细胞分选和/或选择,其使用针对负向选择的细胞上存在的细胞表面标记物的单克隆抗体的混合物。例如,为了通过负向选择富集CD4 +细胞,单克隆抗体混合物一般地包括针对CD14、CD20、CD11b、CD16、HLA-DR和CD8的抗体。

[0116] 为了通过正向或负向选择分离期望的细胞群,细胞和表面(例如颗粒比如珠)的浓度可以变化。在某些实施方案中,可以合乎需要的是显著减少其中珠和细胞混合在一起的体积(即增加细胞浓度),以确保细胞与珠的最大接触。

[0117] 在一些实施方案中，可以合乎需要的是使用较低浓度的细胞。通过显著稀释MIL与表面(例如颗粒比如珠)的混合物，使颗粒与细胞之间的相互作用最小化。这选择表达大量的待结合到颗粒上的期望抗原的细胞。

[0118] 本文也提供在可能需要如本文所述的扩繁的细胞之前的时间段自受试者收集包含MIL的样本。因此，可在必要的任何时间点收集待扩繁的细胞来源，并将期望的细胞(比如MIL)分离和冷冻供以后在MIL疗法中用于将得益于MIL疗法的任何数目的疾病或病症，比如本文描述的那些疾病或病症。在一些实施方案中，包含MIL的样本取自普通健康受试者。在一些实施方案中，包含MIL的样本取自处于发展疾病的风险但是尚未发展成疾病的普通健康受试者，并将目标细胞分离并冷冻供以后使用。在一些实施方案中，MIL可被扩繁、冷冻并在以后的时间使用。在一些实施方案中，在诊断如本文描述的特定疾病后不久，但在任何治疗之前，自患者收集样本。在一些实施方案中，在任何数目的相关治疗模式之前(包括但不限于用以下药物治疗)，自来自受试者的包含MIL的样本分离细胞：比如那他珠单抗、依法珠单抗、抗病毒药物、化学疗法、放射；免疫抑制药物比如环孢菌素、硫唑嘌呤、甲氨蝶呤、霉酚酸酯和FK506；抗体或其他免疫清除药物(immunoablative agent)比如CAMPATH、抗CD3抗体、环磷酰胺、氟达拉滨、环孢菌素、FK506、雷帕霉素、麦考酚酸、类固醇、FR901228和照射。这些药物抑制钙依赖性磷酸酶钙调磷酸酶(环孢菌素和FK506)或抑制对生长因子诱导的信号传导重要的p70S6激酶(雷帕霉素)。在一些实施方案中，为患者分离细胞并冷冻用于以后与骨髓或干细胞移植、使用化疗药物比如氟达拉滨的MIL消除疗法(ablative therapy)、外束放射疗法(XRT)、环磷酰胺或抗体比如OKT3或CAMPATH一起(例如在之前、同时或之后)使用。在一些实施方案中，细胞在之前被分离并可被冷冻用于以后在B细胞消除疗法，比如与CD20反应的药物例如Rituxan之后的治疗。

[0119] 在一些实施方案中，MIL在治疗之后直接得自患者。在这方面，已经观察到在某些癌症治疗(特别是用损害免疫系统的药物治疗)之后，在患者正常自治疗恢复期间的治疗后不久，所获得的MIL的质量可能是最佳的或者其离体扩繁的能力得以改善。同样地，在使用本文描述的方法进行离体操作之后，这些细胞可处于增强植入和体内扩繁的优选状态。因此，可在该恢复阶段收集MIL。

[0120] 无论是在对MIL进行遗传修饰以表达合乎需要的CAR之前还是之后，MIL通常可使用例如在美国专利第6352694、6534055、6905680、6692964、5858358、6887466、6905681、7144575、7067318、7172869、7232566、7175843、5883223、6905874、6797514、6867041号和美国专利申请公开号20060121005中描述的方法来激活并扩繁(在此通过参照结合)。

[0121] 在一些实施方案中，MIL通过与连接有刺激CD3/TCR复合物相关信号的药物和刺激MIL表面上的共刺激分子的配体的表面接触而扩繁。具体地讲，MIL群可如本文描述的那样进行刺激，比如通过与抗CD3抗体或其抗原结合片段或固定在表面上的抗CD2抗体接触，或者通过与连同钙离子载体一起的蛋白激酶C激活剂(例如苔藓抑素)接触。为了共刺激MIL表面上的辅助分子，使用结合辅助分子的配体。例如，可在适合于刺激MIL增殖的条件下使MIL群与抗CD3抗体和抗CD28抗体接触。刺激CD4+MIL或CD8+MIL的增殖，抗CD3抗体和抗CD28抗体。抗CD28抗体的实例包括9.3、B-T3、XR-CD28 (Diaclone, Besancon, France)，可如本领域通常已知的其他方法那样使用(Berg等人, Transplant Proc. 30(8):3975-3977, 1998; Haanen等人, J. Exp. Med. 190(9):13191328, 1999; Garland等人, J. Immunol

Meth. 227(1-2):53-63, 1999)。

[0122] 在某些实施方案中,MIL的初级刺激信号和共刺激信号可由不同的方案提供。例如,提供每种信号的药物可处于溶液中或偶联于表面。当偶联于表面时,药物可偶联于相同的表面(即以“顺式”构成)或偶联于分开的表面(即以“反式”构成)。或者,可将一种药物偶联于表面并且另一种药物在溶液中。在一些实施方案中,提供共刺激信号的药物结合于细胞表面,并且提供初级激活信号的药物处于溶液中或偶联于表面。在某些实施方案中,两种药物均可处于溶液中。在一些实施方案中,药物可以溶液形式存在,并然后交联至表面,比如表达Fc受体或抗体或其他将结合于药物的结合剂的细胞(通常参见美国专利申请公开号20040101519和20060034810,在此通过参照结合,尤其是对于考虑用于激活并扩繁MIL的人工抗原呈递细胞(aAPC))。

[0123] 在一些实施方案中,两种药物固定在珠上,或者在相同的珠上,即“顺式”,或者至分开的珠上,即“反式”。举例来说,提供初级激活信号的药物为抗CD3抗体或其抗原结合片段,并且提供共刺激信号的药物为抗CD28抗体或其抗原结合片段;并且两种药物以等同的分子数量共固定于相同的珠上。在一些实施方案中,使用1:1比率的每种抗体结合于珠用于CD4+MIL扩繁和MIL生长。在一些实施方案中,使用一定比率的抗CD3:CD28抗体结合于珠,以致与使用1:1比率观察到的扩繁相比观察到MIL扩繁的增加。在一些实施方案中,与使用1:1比率观察到的扩繁相比观察到增加约1-约3倍。在一些实施方案中,结合于珠的CD3:CD28抗体的比率在100:1-1:100的范围内及其间的所有整数值。在一些实施方案中,比抗CD3抗体更多的抗CD28抗体结合于颗粒,即CD3:CD28的比率小于1。在一些实施方案中,结合于珠的抗CD28抗体:抗CD3抗体的比率大于2:1。在一些实施方案中,使用1:100 CD3:CD28比率的抗体结合于珠。在一些实施方案中,使用1:75 CD3:CD28比率的抗体结合于珠。在一些实施方案中,使用1:50 CD3:CD28比率的抗体结合于珠。在一些实施方案中,使用1:30 CD3:CD28比率的抗体结合于珠。在一些实施方案中,使用1:10 CD3:CD28比率的抗体结合于珠。在一些实施方案中,使用1:3 CD3:CD28比率的抗体结合于珠。在一些实施方案中,使用3:1 CD3:CD28比率的抗体结合于珠。

[0124] 1:500-500:1及其间的任何整数值的颗粒:细胞的比率可用于刺激MIL。如本领域的普通技术人员可易于意识到的那样,颗粒:细胞的比率可取决于相对于靶细胞的颗粒大小。例如,小尺寸珠仅能结合几个细胞,而较大的珠能结合许多细胞。在某些实施方案中,细胞:颗粒的比率在1:100-100:1的范围内及其间的任何整数值,并且在进一步的实施方案中比率包含1:9-9:1及其间的任何整数值,也可用于刺激MIL。如以上提及的那样,导致MIL刺激的抗CD3和抗CD28偶联的颗粒:细胞的比率可以变化,然而某些数值包括但不限于 1:100、1:50、1:40、1:30、1:20、1:10、1:9、1:8、1:7、1:6、1:5、1:4、1:3、1:2、1:1、2:1、3:1、4:1、5:1、6:1、7:1、8:1、9:1、10:1和15:1。在一些实施方案中,比率为每个细胞至少1:1个颗粒。在一些实施方案中,使用1:1或更小的颗粒:细胞的比率。在一些实施方案中,颗粒:细胞比率为1:5。 在一些实施方案中,颗粒:细胞的比率可依刺激日而变化。例如,在一些实施方案中,颗粒:细胞的比率在第一天为1:1-10:1,并且之后每一天或每隔一天向细胞添加另外的颗粒长达10天,最终比率为1:1-1:10 (基于添加日的细胞计数)。在一些实施方案中,在刺激的第一天,颗粒:细胞的比率为1:1,并在刺激的第3和第5天调整为1:5。在一些实施方案中,在每天或每隔一天的基础上添加颗粒至刺激的第1天最终比率为1:1,和在刺激的第3和

第5天最终比率为1:5。在一些实施方案中，在刺激的第1天，颗粒:细胞的比率为2:1，和在刺激的第3和第5天调整为1:10。在一些实施方案中，在每天或每隔一天的基础上添加颗粒至刺激的第1天最终比率为1:1，和在刺激的第3和第5天最终比率为1:10。本领域的技术人员将会意识到，还可使用多种其他比率。例如，比率将依颗粒大小和细胞大小和类型而变化。

[0125] 在一些实施方案中，将MIL与药物包被的珠混合，随后分离珠和细胞，并然后培养细胞。在一些实施方案中，在培养之前，药物包被的珠和细胞不分离，而是一起培养。在一些实施方案中，珠和细胞首先通过施加力比如磁力进行浓缩，导致细胞表面标记物的连接增加，从而诱导细胞刺激。

[0126] 举例来说，细胞表面蛋白可通过使抗CD3和抗CD28连接的顺磁珠(3×28珠)接触MIL来连接。在一些实施方案中，将细胞和珠(例如1:1比率的DYNABEADS®M-450 CD3/CD28 T顺磁珠)在缓冲液中混合，优选地为PBS (不含二价阳离子比如钙和镁)。本领域的普通技术人员可易于意识到，可使用任何细胞浓度。例如，靶细胞可能在样本中非常稀少，并且仅包含样本的0.01%，或者整个样本(即100%)可包含目标靶细胞。因此，可使用任何细胞数目。在某些实施方案中，可以合乎需要的是显著减少其中颗粒和细胞混合在一起的体积(即增加细胞浓度)，以确保细胞与颗粒的最大接触。例如，在一些实施方案中，使用约20亿个细胞/ml的浓度。在一些实施方案中，使用大于1亿个细胞/ml。在一些实施方案中，使用1、1.5、2.0、2.5、3.0、3.5、4.0、4.5或5.0千万个细胞/ml的细胞浓度。在一些实施方案中，使用7.5、8.0、8.5、9.0、9.5千万或1亿个细胞/ml的细胞浓度。在一些实施方案中，可使用1.25或1.5亿个细胞/ml的浓度。使用高浓度可导致细胞产量增加、细胞激活和细胞扩繁。进一步地，使用高细胞浓度使得能够更有效地捕获可能弱表达目标靶抗原的细胞，比如CD28阴性细胞。这样的细胞群可具有治疗价值，并且在某些实施方案中将是期望获得的。

[0127] 在一些实施方案中，可将混合物培养几个小时(约3小时)-约14天或其间的任何小时整数值。在一些实施方案中，可将混合物培养21天。在一些实施方案中，将珠和MIL一起培养约8天。在一些实施方案中，将珠和MIL一起培养2-3天。也可期望几个循环的刺激，以致MIL的培养时间可为60天或者更长。适合于MIL培养的条件包括可含有增殖和生存力必需的因素的合适培养基(例如最小必需培养基或RPMI培养基1640或X-vivo 15 (Lonza))，包括血清(例如胎牛或人血清)、白细胞介素-2 (IL-2)、胰岛素、IFN-γ、IL-4、IL-7、GM-CSF、IL-10、IL-12、IL-15、TGFβ和TNF-α或技术人员已知的用于细胞生长的任何其他添加剂。其他用于细胞生长的添加剂包括但不限于表面活性剂、人血浆蛋白粉和还原剂比如N-乙酰半胱氨酸和2-巯基乙醇。培养基可包括RPMI 1640、AIM-V、DMEM、MEM、α-MEM、F-12、X-Vivo 15和X-Vivo 20、Optimizer，添加了氨基酸、丙酮酸钠和维生素，无血清或补充适量的血清(或血浆)或一组确定的激素，和/或一定量的足以用于MIL生长和扩繁的细胞因子。抗生素例如青霉素和链霉素仅包括在实验培养物中，而不包括在要输注入受试者体内的细胞培养物中。将靶细胞维持在支持生长必需的条件下，例如合适的温度(例如37℃)和气氛(例如空气+5% $CO_2$)。

[0128] 除了CD4和CD8标记物以外，其他表型标记物变化显著，但在很大程度上，在细胞扩繁过程期间可重现。因此，这样的重现性使得具有针对特定目的定制激活的MIL产物的能力。

[0129] 另外，可使用用于制备肿瘤浸润淋巴细胞的方法来制备MIL。例如，高剂量IL-2生

长条件可用于产生“年轻的”TIL，并且这些方法可适用于制备MIL（参见例如美国专利第8383099号，在此通过参照结合）。

[0130] 在一些实施方案中，MIL也可在低氧条件下被激活和/或扩繁。在低氧条件下生长MIL的一个实例可例如在WO2016037054中发现，其在此通过参照以其全部结合。

[0131] 在一些实施方案中，方法可包括：去除骨髓中的细胞、来自受试者的淋巴细胞和/或骨髓浸润淋巴细胞（“MIL”）；在低氧环境中温育细胞，从而产生激活的MIL；并将激活的MIL给予受试者。如本文描述的那样，细胞也可在存在抗CD3/抗CD28抗体和细胞因子的情况下被激活。如本文描述的那样，细胞因子也可用于激活MIL。编码CAR的核酸分子，比如本文描述的那些分子之一，可在MIL于低氧环境中温育之前或之后转染或感染至细胞中。

[0132] 低氧环境可包含少于约21%的氧，比如少于约20%、19%、18%、17%、16%、15%、14%、13%、12%、11%、10%、9%、8%、7%、6%、5%、4%或少于约3%的氧。例如，低氧环境可包含约0%的氧-约20%的氧，比如约0%的氧-约19%的氧、约0%的氧-约18%的氧、约0%的氧-约17%的氧、约0%的氧-约16%的氧、约0%的氧-约15%的氧、约0%的氧-约14%的氧、约0%的氧-约13%的氧、约0%的氧-约12%的氧、约0%的氧-约11%的氧、约0%的氧-约10%的氧、约0%的氧-约9%的氧、约0%的氧-约8%的氧、约0%的氧-约7%的氧、约0%的氧-约6%的氧、约0%的氧-约5%的氧、约0%的氧-约4%的氧或约0%的氧-约3%的氧。在一些实施方案中，低氧环境包含约1%-约7%的氧。在一些实施方案中，低氧环境为约1%-约2%的氧。在一些实施方案中，低氧环境为约0.5%-约1.5%的氧。在一些实施方案中，低氧环境为约0.5%-约2%的氧。低氧环境可包含约20%、19%、18%、17%、16%、15%、14%、13%、12%、11%、10%、9%、8%、7%、6%、5%、4%、3%、2%、1%或约0%的氧。在一些实施方案中，低氧环境包含约7%、6%、5%、4%、3%、2%或1%的氧。

[0133] 在低氧环境中温育MIL可包括将MIL例如在组织培养基中温育至少约1小时，比如至少约12小时、18小时、24小时、30小时、36小时、42小时、48小时、60小时、3天、4天、5天、6天、7天、8天、9天、10天、11天、12天、13天或者甚至至少约14天。温育可包括温育MIL约1小时-约30天，比如约1天-约20天、约1天-约14天或者约1天-约12天。在一些实施方案中，在低氧环境中温育MIL包括在低氧环境中温育MIL约2天-约5天。方法可包括在低氧环境中温育MIL约1天、2天、3天、4天、5天、6天、7天、8天、9天、10天、11天、12天、13天或14天。在一些实施方案中，方法包括在低氧环境中温育MIL约3天。在一些实施方案中，方法包括在低氧环境中温育MIL约2天-约4天。在一些实施方案中，方法包括在低氧环境中温育MIL约3天-约4天。

[0134] 在一些实施方案中，方法进一步包括在常氧环境中温育MIL，例如在低氧环境中温育MIL之后。

[0135] 常氧环境可包含至少约21%的氧。常氧环境可包含约5%的氧-约30%的氧，比如约10%的氧-约30%的氧、约15%的氧-约25%的氧、约18%的氧-约24%的氧、约19%的氧-约23%的氧或者约20%的氧-约22%的氧。在一些实施方案中，常氧环境包含约21%的氧。

[0136] 在常氧环境中温育MIL可包括例如在组织培养基中温育MIL至少约1小时，比如至少约12小时、18小时、24小时、30小时、36小时、42小时、48小时、60小时、3天、4天、5天、6天、7天、8天、9天、10天、11天、12天、13天或者甚至至少约14天。温育可包括温育MIL约1小时-约30天，比如约1天-约20天、约1天-约14天、约1天-约12天或者约2天-约12天。

[0137] 在一些实施方案中，在置于常氧环境中之后或在将其置于常氧环境中之前，细胞用编码本文描述的CAR的核酸分子转染或感染。

[0138] 在一些实施方案中，MIL通过自受试者提取骨髓样本并如本文描述的那样培养/温育细胞而获得。在一些实施方案中，将骨髓样本离心以除去红细胞。在一些实施方案中，骨髓样本不经受单采术(apheresis)或通过单采术获得。在一些实施方案中，骨髓样本不包含外周血淋巴细胞(“PBL”)或者骨髓样本实质上不含PBL。这些方法选择的细胞与已变为称为TIL的不同。因此，MIL不是TIL。

[0139] 在一些实施方案中，然后可将细胞接种于平板、烧瓶或袋中。在一些实施方案中，低氧条件可通过用95%氮气和5% $CO_2$气体混合物冲洗低氧室或细胞培养袋3分钟来实现。这可导致容器中存在例如1-2%或者更少的$O_2$气体。细胞然后可如本文描述的那样或如在WO2016037054的实施例中那样进行培养，其在此通过参照结合。

[0140] 在一些实施方案中，提供包含如本文描述的CAR的低氧MIL。在一些实施方案中，低氧MIL处于约0.5%-约5%氧气的环境中。在一些实施方案中，低氧MIL处于约1%-约2%氧气的环境中。在一些实施方案中，低氧MIL处于约1%-约3%氧气的环境中。 在一些实施方案中，低氧MIL处于约1%-约4%氧气的环境中。低氧MIL为已在低氧环境(比如本文描述的那些环境)中温育一段时间的MIL，比如本文描述的那些MIL。如本文描述的那样，低氧MIL也可在存在抗CD3/抗CD28珠或其他类似的激活试剂的情况下被激活。因此，包含CAR的低氧MIL也可为激活的低氧MIL。

[0141] VII. 治疗方法

在一些实施方案中，提供表达CAR的细胞(例如MIL)。细胞(或亲代细胞)可用包含编码CAR的核苷酸序列的载体转染。 载体可为慢病毒载体(LV)。例如，LV编码组合特异性抗体的抗原识别结构域与CD3ζ、CD28、4-1BB的胞内结构域或其任何组合的CAR。因此，在一些情况下，转导的MIL可引发CAR介导的T细胞反应。

[0142] 本文提供CAR将初级MIL的特异性重定向至肿瘤抗原的用途。因此，在一些实施方案中，提供用于在哺乳动物中刺激对靶细胞群或组织的MIL介导的免疫反应的方法，所述方法包括给予受试者表达CAR的MIL的步骤，其中CAR包含与预定靶标特异性地相互作用的结合部分、包含例如人CD3ζ的胞内结构域的ζ链部分和共刺激信号传导区。

[0143] 在一些实施方案中，提供细胞疗法，其中MIL被遗传修饰以表达CAR并把CAR-MIL输注给有需要的接受者。输注的细胞能够在接受者中杀伤肿瘤细胞(或其他靶标)。不像抗体疗法，CAR-MIL能够在体内复制，导致长期持续存在，这可导致持续的肿瘤控制。

[0144] 在一些实施方案中，CAR-MIL可经历强健的体内MIL扩繁，并可持续延长的时间量。

[0145] 可治疗的癌症包括没有血管化或尚未实质上血管化的肿瘤以及血管化的肿瘤。癌症可包含非实体肿瘤(比如血液肿瘤，例如白血病和淋巴瘤)或者可包含实体肿瘤。待用CAR治疗的癌症类型包括但不限于癌、母细胞瘤和肉瘤以及某些白血病或淋巴样恶性肿瘤、良性和恶性肿瘤以及恶性肿瘤，例如肉瘤、癌和黑素瘤。成人肿瘤/癌症和儿童肿瘤/癌症也包括在内。

[0146] 血液癌症为血液或骨髓的癌症。血液学(或血源性)癌症的实例包括白血病，包括急性白血病(比如急性淋巴细胞白血病、急性髓细胞白血病、急性髓性白血病和成髓细胞白血病、早幼粒细胞白血病、髓单核细胞白血病、单核细胞白血病和红白血病)、慢性白血病(比如慢性髓细胞(粒细胞)白血病、慢性髓性白血病和慢性淋巴细胞白血病)、真性红细胞增多症、淋巴瘤、霍奇金病，非霍奇金淋巴瘤(无痛性和高级形式)、多发性骨髓瘤、瓦尔登斯

特伦巨球蛋白血症、重链病、骨髓增生异常综合征、毛细胞白血病和骨髓发育不良。

[0147] 实体肿瘤为通常不含有囊肿或液体区域的异常组织块。实体肿瘤可为良性或恶性的。不同类型的实体肿瘤以形成它们的细胞类型命名(比如肉瘤、癌和淋巴瘤)。实体肿瘤比如肉瘤和癌的实例包括纤维肉瘤、粘液肉瘤、脂肪肉瘤、软骨肉瘤、骨肉瘤和其他肉瘤、滑膜瘤、间皮瘤、尤因氏瘤、平滑肌肉瘤、横纹肌肉瘤、结肠癌、淋巴样恶性肿瘤、胰腺癌、乳腺癌、肺癌、卵巢癌、前列腺癌、肝细胞癌、鳞状细胞癌、基底细胞癌、腺癌、汗腺癌、甲状腺髓样癌、甲状腺乳头状癌、嗜铬细胞瘤皮脂腺癌、乳头状癌、乳头状腺癌、髓样癌、支气管癌、肾细胞癌、肝细胞癌、胆管癌、绒毛膜癌、维尔姆斯瘤、宫颈癌、睾丸肿瘤、精原细胞瘤、膀胱癌、黑素瘤和CNS肿瘤(比如神经胶质瘤(比如脑干神经胶质瘤和混合型神经胶质瘤)、成胶质细胞瘤(也称为多形性成胶质细胞瘤)、星形细胞瘤、CNS淋巴瘤、生殖细胞瘤、成髓细胞瘤、神经鞘瘤颅咽管瘤、室管膜瘤、松果体瘤、成血管细胞瘤、听神经瘤、少突胶质细胞瘤、脑膜瘤(menangioma)、成神经细胞瘤、成视网膜细胞瘤和脑转移瘤)。

[0148] 在一些实施方案中,CAR的抗原结合部分被设计为治疗特定的癌症。例如,被设计为靶向CD19的CAR可用于治疗癌症和障碍,包括但不限于 pre-B ALL(儿科适应症)、成人ALL、套细胞淋巴瘤、弥漫性大B细胞淋巴瘤、同种异体骨髓移植后的挽救等。

[0149] 在一些实施方案中,CAR被设计为靶向CD22以治疗弥漫性大B细胞淋巴瘤。

[0150] 在一些实施方案中,癌症和障碍包括但不限于 pre-B ALL (儿科适应症)、成人ALL、套细胞淋巴瘤、弥漫性大B细胞淋巴瘤、同种异体骨髓移植后的挽救等,可使用靶向CD19、CD20、CD22和ROR1的CAR的组合治疗。

[0151] 在一些实施方案中,CAR可被设计为靶向间皮素以治疗间皮瘤、胰腺癌、卵巢癌等。

[0152] 在一些实施方案中,CAR可被设计为靶向CD33/IL3Ra以治疗急性髓性白血病等。

[0153] 在一些实施方案中,CAR可被设计为靶向c-Met以治疗三阴性乳腺癌、非小细胞肺癌等。

[0154] 在一些实施方案中,CAR可被设计为靶向PSMA以治疗前列腺癌等。

[0155] 在一些实施方案中,CAR可被设计为靶向糖脂F77以治疗前列腺癌等。

[0156] 在一些实施方案中,CAR可被设计为靶向EGFRvIII以治疗成胶质细胞瘤等。

[0157] 在一些实施方案中,CAR可被设计为靶向GD-2以治疗成神经细胞瘤、黑素瘤等。

[0158] 在一些实施方案中,CAR可被设计为靶向NY-ESO-1 TCR以治疗骨髓瘤、肉瘤、黑素瘤等。

[0159] 在一些实施方案中,CAR可被设计为靶向MAGE A3 TCR以治疗骨髓瘤、肉瘤、黑素瘤等。

[0160] 然而,实施方案不应解释为仅限于本文公开的抗原靶标和疾病。相反,实施方案应解释为包括与其中CAR可用于治疗疾病的疾病相关的任何抗原靶标。

[0161] CAR修饰的MIL也可用作用于在受试者比如人的离体免疫和/或体内疗法的疫苗类型。

[0162] 就离体免疫而言,在将细胞给予哺乳动物之前,体外发生以下中的至少一种:i)细胞的扩繁,ii) 将编码CAR的核酸引入到细胞中,和/或iii) 细胞的冷冻保存。在一些实施方案中,所有步骤在将细胞给予哺乳动物之前实施。

[0163] 离体过程为本领域熟知的,并在以下更充分地讨论。简而言之,自哺乳动物(比如

人)分离细胞并用本文公开的表达CAR的载体进行遗传修饰(即体外转导或转染)。可将CAR-MIL给予哺乳动物接受者以提供治疗益处。哺乳动物接受者可为人,并且CAR-MIL就接受者而言可为自体的。或者,细胞就接受者而言可为同种异体的、同源的或异种异体的。

[0164] 除了就离体免疫而言使用基于细胞的疫苗以外,本文也提供用于体内免疫以在患者中引发针对抗原的免疫反应的组合物和方法。

[0165] 通常,如本文描述的那样激活并扩繁的细胞可用于治疗和预防在免疫功能受损的个体中出现的疾病。在一些实施方案中,CAR修饰的MIL用于治疗CCL。在一些实施方案中,细胞用于治疗处于发展CCL风险的患者。因此,提供用于治疗或预防CCL的方法,所述方法包括给予有需要的受试者治疗有效量的CAR修饰的MIL。

[0166] CAR修饰的MIL可单独或者作为与稀释剂和/或与其他组分比如IL-2或其他细胞因子或细胞群组合的药物组合物给予。简而言之,药物组合物可包含与一种或多种药学上或生理学上可接受的载体、稀释剂或赋形剂组合的如本文描述的靶细胞群。这样的组合物可包含缓冲液比如中性缓冲盐水、磷酸盐缓冲盐水等;碳水化合物比如葡萄糖、甘露糖、蔗糖或葡聚糖、甘露醇;蛋白质、多肽或氨基酸比如甘氨酸;抗氧化剂;螯合剂比如EDTA或谷胱甘肽;佐剂(例如氢氧化铝)和防腐剂。在一些实施方案中,组合物被配制成用于静脉内给予。

[0167] 药物组合物可以适合于待治疗(或预防)的疾病的方式给予。给予的量和频率将根据患者的状况、患者疾病的类型和严重程度等因素来确定,尽管合适的剂量可通过临床试验来确定。

[0168] 当指明“免疫有效量”、“抗肿瘤有效量”、“肿瘤抑制有效量”或“治疗量”时,待给予的组合物的准确量可由医生考虑到患者(受试者)的年龄、体重、肿瘤大小、感染或转移的程度和状况的个体差异来确定。通常可以说,包含本文描述的MIL的药物组合物可以$10^4$-$10^9$个细胞/kg体重,优选地以$10^5$-$10^6$个细胞/kg体重的剂量给予,包括那些范围内的所有整数值。MIL组合物也可以这些剂量给予多次。细胞可通过使用在免疫疗法中通常已知的输注技术来给予(参见例如Rosenberg等人, New Eng. J. of Med. 319:1676, 1988)。用于特定患者的最佳剂量和治疗方案可由医学领域的技术人员通过监测患者的疾病体征并相应地调整治疗而易于确定。

[0169] 受试组合物的给予可以任何便利的方式实施,包括通过气雾吸入、注射、摄取、输液、植入或移植。本文描述的组合物可皮下、皮内、瘤内、结节内、髓内、肌内、通过静脉内(i.v.)注射或腹膜内给予患者。在一些实施方案中,MIL组合物通过皮内或皮下注射给予患者。在一些实施方案中,MIL组合物通过静脉内注射给予。MIL的组合物可例如直接注射到肿瘤、淋巴结或感染部位。

[0170] 在一些实施方案中,使用本文描述的方法或本领域已知的其中将MIL扩繁至治疗水平的其他方法激活并扩繁的细胞,连同(例如在之前、同时或之后)任何数目的以下相关治疗治疗模式给予患者:包括但不限于用药物比如抗病毒疗法、西多福韦和白细胞介素-2、阿糖胞苷(也称为ARA-C)的治疗或用于MS患者的那他珠单抗治疗或用于牛皮癣患者的依法珠单抗治疗或用于PML患者的其他治疗。在一些实施方案中,MIL可与化学疗法、放射;免疫抑制药物比如环孢菌素、硫唑嘌呤、甲氨蝶呤,霉酚酸酯和FK506;抗体或其他免疫清除药物比如CAM PATH、抗CD3抗体或其他抗体疗法、cytoxin、氟达拉滨、环孢菌素、FK506、雷帕霉素、麦考酚酸、类固醇、FR901228、细胞因子和照射组合使用。这些药物抑制钙依赖性磷酸酶

钙调磷酸酶(环孢菌素和FK506)或抑制对生长因子诱导的信号传导重要的p70S6激酶(雷帕霉素) (Liu等人, Cell 66:807-815, 1991; Henderson等人, Immun 73:316-321, 1991; Bierer等人, Curr. Opin. Immun 5:763-773, 1993)。在一些实施方案中,细胞组合物连同骨髓移植、使用化疗药物比如氟达拉滨的MIL消除疗法、外束放射疗法(XRT)、环磷酰胺或抗体比如OKT3或CAMPATH一起(例如在之前、同时或之后)给予患者。在一些实施方案中,细胞组合物在B细胞消除疗法,比如与CD20反应的药物例如利妥昔单抗之后给予。例如,在一些实施方案中,受试者可经历高剂量化学疗法的标准治疗,随后进行外周血干细胞移植。在一些实施方案中,在移植之后,受试者接受输注本文描述的扩繁的免疫细胞。在一些实施方案中,扩繁的细胞在手术之前或之后给予。

[0171] 待给予患者的治疗剂量将随着被治疗的病症的准确性质和治疗接受者而变化。可根据领域接受的实践实施用于给予人的剂量缩放。例如,对于成人患者,CAMPATH的剂量通常在1-约100 mg的范围内,通常每天给予为期1-30天之间的一段时间。在一些实施方案中,日剂量为每天1-10 mg,尽管在某些情况下可使用多达每天40 mg的较大剂量(描述于美国专利第6120766号中)。

[0172] VIII. 受试者

受试者可为包含MIL的任何生物体。例如,受试者可选自啮齿动物、犬、猫、猪、绵羊、牛、马和灵长类动物。受试者可为小鼠或人。

[0173] 受试者可能患有赘生物。赘生物可为良性赘生物、恶性赘生物或继发性赘生物。赘生物可能是癌症。赘生物可为淋巴瘤或白血病,比如慢性淋巴细胞性血病("CLL")或急性成淋巴细胞白血病("ALL")。受试者可能患有成胶质细胞瘤、成髓细胞瘤、乳腺癌、头颈癌、肾癌、卵巢癌、卡波西肉瘤、急性髓性白血病和B系恶性肿瘤。受试者可能患有多发性骨髓瘤。

[0174] 受试者可能患有急性髓性白血病、腺癌、骨肉瘤、成淋巴细胞白血病、淋巴瘤、B细胞淋巴瘤、B细胞性非霍奇金淋巴瘤、B系淋巴样恶性肿瘤、乳腺癌、卵巢癌、宫颈癌、结直肠癌、上皮癌、成胶质细胞瘤、神经胶质瘤、霍奇金淋巴瘤、无痛性B细胞淋巴瘤、白血病、淋巴瘤、肺癌、套细胞淋巴瘤、成髓细胞瘤、黑素瘤、成神经细胞瘤、前列腺癌、滤泡性淋巴瘤、肾细胞癌、横纹肌肉瘤。

## 实施例

[0175] 以下实施例为说明性的,但不是本文描述的方法和组合物的限制。在疗法中通常遇到的对本领域技术人员显而易见的各种条件和参数的其他合适的修改和适应性改变处于实施方案的精神和范围内。

[0176] 实施例1:MIL-CAR用于治疗B细胞淋巴瘤

MIL得自患有B细胞淋巴瘤的受试者。简而言之,如在WO2016037054 (其在此通过参照结合)中描述的那样,在自受试者获得骨髓样本后,在低氧条件下,于存在抗CD3/抗CD28珠和细胞因子的情况下温育细胞。将编码CAR的核酸分子转染到MIL中,所述CAR包含CD19的胞外结构域、CD19的跨膜结构域及CD3ζ和4-1BB的胞内结构域。然后使细胞在常氧条件下生长并使其扩繁。将激活并扩繁的MIL给予患有B细胞淋巴瘤的受试者。受试者的B细胞淋巴瘤得到缓解。总之,本文提供的实施方案和实施例证实表达CAR的细胞可有效地用于治疗癌症。

[0177] 实施例2:MIL-CAR用于治疗多发性骨髓瘤

MIL得自患有多发性骨髓瘤的受试者。简而言之，如在WO2016037054 (其在此通过参照结合)中描述的那样，在自受试者获得骨髓样本后，在低氧条件下，于存在抗CD3/抗CD28珠和细胞因子的情况下温育细胞。将编码CAR的核酸分子转染到MIL中，所述CAR包含CD38的胞外结构域、CD8的跨膜结构域及CD3ζ和4-1BB的胞内结构域。然后使细胞在常氧条件下生长并使其扩繁。将激活并扩繁的MIL给予患有多发性骨髓瘤的受试者。受试者的多发性骨髓瘤得到缓解。

[0178] 在该说明书中提及和/或在申请数据单(A;pplication Data Sheet)中列出的任何美国专利、美国专利申请公开、美国专利申请、外国专利、外国专利申请和包括CAS编号的非专利公开本文通过参照以其全部结合。