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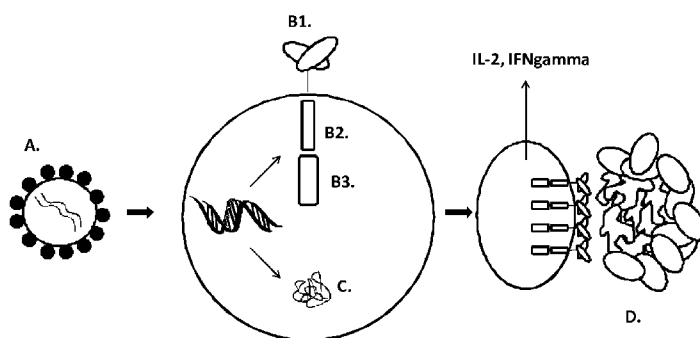
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Fig. 1.



A. Transducing particles; B. receptors with 3 parts. B1: Binder; B2: primary
signal domain; B3: co-stimulatory signal domain; C. Thymidine kinase; D.
Tumor necrotic center.

(57) Abstract: Featured herein are engineered human cells, which express an exogenous extracellular binding partner to an apoptot-
ic or necrotic cellular antigen and at least one exogenous intracellular signaling domain. These genetically-engineered cells can func-
tion as immune modulators that specifically recognize dying cells or tissues and upon binding, secrete Th1 cytokines including IL-2
and IFN-gamma, thereby achieving a localized pro-inflammatory response at the site of tumor tissues.

CELLULAR THERAPIES FOR TREATING AND PREVENTING CANCERS AND OTHER IMMUNE SYSTEM DISORDERS

Related Applications

5 This application claims the benefit of priority to United States Provisional Applications serial number 61/661,921, filed June 20, 2012; and serial number 61/663,043, filed June 22, 2013.

Background

10 The collection of immune cells is comprised of, among others, T cells, dendritic cells (DCs), B cells, NK cells, and neutrophils. Cytokines of the immune system are secreted proteins that mediate intercellular communications, thereby modulating a variety of immune functions including Th1 and Th2 immunity. During adoptive immune responses, T cell activation is initiated by activation of T cell receptors, mediated by
15 intracellular domains of the T cell receptor (TCR), which are typically associated isoforms of CD3. Additional ligand/receptor interactions provide “co-stimulatory” interactions that are required for effective T cell activation. One of these co-stimulatory interactions is binding of B7.1 (ligand) to CD28 (receptor). Another is binding of B7.2 (ligand) to CD28 (receptor) (Collins et al (2005) *Genome Biol.* 6:223). Another
20 ligand/receptor pair, which transmits an activating signal to T cells, is ICOS-L (ligand)/ICOS (receptor).

 Cancer can be classified as a disorder of the immune system. One particular mechanism for the cancer cells to evade immune surveillances is the employment of immunesuppressive cytokine networks, produced by cancer cells, and by associated tumor-
25 associated macrophages or stromal cells (see, e.g., Yu et al (2007) *Nature Rev. Immunol.* 7:41-51). For example, the T cell immunity against cancer cells is dampened by the secretion of interleukin-10 (IL-10) from the cancer cells or the associated macrophages. Natural ligand of TLR7/9, the DNA and HMGB1 complexes from the tumor necrosis, are sequestered by the TIM3 on cancer or stromal cells (Chiba et al (2012) *Nature*
30 *Immunology* 13: 832-842), resulting in reduced activation of dendritic cells by interferons. Together with other immune suppressive mechanism, it is not likely the case that any cancer cell, or cancer cell antigen, without more, can optimally activate any dendritic cell, and fail to elicit the T cell immunity.

To activate cellular immunity for cancer treatment, recombinant and pro-inflammatory cytokines are used as anti-cancer agents, but with only limited utility due to toxicity. Efficacy was found for treatment of renal carcinoma and melanoma (Atkins et al., (1999) *J. Clin. Oncol.* 17:2105-2116), but was also associated with toxicity (Siegel & Puri, (1991) *J. Clin. Oncol.* 9:694-704), such as vascular leak syndrome (Edwards et al., (1991) *J. Surg. Res.* 50:609-615; Lentsch et al., (1999) *Cancer Immunol. Immunother.* 47:243-248).

An improved therapy was to localize the therapeutic T cell activation to the cancerous tissues. For example, an antibody fusion linked to a cytokine (IL-12) had been used to deliver IL-12 to tumor-associated vasculature, where the antibody binds to fibronectin. However the compound was toxic to patients (Rudman et al., (2011) *Clin. Cancer Res.* 17:1998-2005). Antibody fused to IL-2 had been tested in clinical studies, resulting in limited efficacy yet significant adverse events (Osenga et al., (2006) *Clin Cancer Res.* 12:1750-1759; Shusterman et al., (2010) *J. Clin. Oncol.* 28:4969-4975). Adverse events in these targeted cytokine therapies resembled those with systemic interleukin-2 administration (Pasche and Neri (2012) *Drug Discov. Today* 17:583-590).

Chimeric antigen receptor (CAR) technology has advanced specific immune targeting to B cell lymphomas (Kalos et al. (2011) *Sci Transl Med.* 10:95ra73), but studies using CAR technology in clinical trials for solid tumors were obstructed by severe adverse effects including deadly cytokine storms or colitis (Pule et al., (2008) *Nature Med.* 14:1264-1270; Louis et al., (2011) *Blood* 118: 6050-6056; Morgan et al., (2010) *Mol. Ther.* 18:843-851; and Parkhurst (2011) *Mol. Ther.* 19:620-626).

There are tremendous medical needs for technologies that can activate the cellular immunities targeted to the cancerous tissues without systemic toxicities. In particular, there is an unmet need for new techniques for activated T - cell responses in the cancerous tissues for cancer treatment.

Although the immune system serves to kill infecting agents, including intracellular and extracellular microorganisms and viruses, the activation of immune system can also result in pathological responses. These pathological responses include various types of inflammation, asthma, allergies, and autoimmune disorders, such as multiple sclerosis, rheumatoid arthritis, Crohn's disease, psoriasis, and systemic lupus erythematosus.

Aberrant B cell activities are associated with multiple autoimmune diseases (Yanada 2008, Martin and Chan (2006) *Ann. Rev. Immunol.* 24:467-496). The “forbidden clone” theory (Burnett (1959) *Clonal Selection Theory of Acquired Immunity*, Cambridge Univ. Press), which posits that autoimmunity starts with a B cell clone that arises from the B cell pool and through interaction with T cells triggers a chain reaction leading to autoimmunity, continues to draw attention from researchers in the autoimmune research field. Recent success of B cell depletion therapies in multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) has further attracted interest of B cell targeting. In the germinal center of secondary lymph organs, it was shown that autoimmune cells proliferated in tight association with the newly defined follicular Th cells (Patakas et al (2011) *Immunol. Lett.* 138:38-46). In rheumatoid arthritis, ectopic germinal centers can be frequently found in the diseased synovium (Weyand and Goronzy (2003) *Ann. N.Y. Acad. Sci.* 987:140-149) consistent with the mechanism of action of the B cell depletion therapy in rheumatoid arthritis (Cohen et al (2006) *Arthritis Rheum.* 54:2793-2806). Townsend et al (2010) *Immunol. Rev.* 237:264-283, suggested that anti-CD20 treatment can accomplish therapeutic efficacy only if autoimmune B cell in the disease setting are diminished.

There are still medical needs for improved B cell depletion therapies, and new pharmaceutical agents of greater efficacy and safety to treat disorders induced by autoimmune B cells.

Summary

In one aspect, featured herein are engineered human cells, which express an exogenous extracellular binding partner to an apoptotic or necrotic cellular antigen and at least one exogenous intracellular signaling domain. These genetically-engineered cells can function as immune modulators that specifically recognize dying cells or tissues and upon binding, secrete Th1 cytokines including IL-2 and IFN-gamma, thereby achieving a localized pro-inflammatory response at the site of tumor tissues.

In certain embodiments, the engineered cell is a T lymphocyte, natural killer cell or natural killer T cell. In certain embodiments, the apoptotic or necrotic cellular antigen is selected from the group consisting of: DNA, histones, clarectulin, vitronectin, phosphatidyl serine, ribonucleoprotein (RNP) complexes, interleukin 6, myosin, disialoganglioside GD2, ERBB2, annexin, alpha-D-mannose and beta-D-galactose-specific plasma membrane glycoproteins, the GlcNAc carbohydrate and complexes of any of the above.

In certain embodiments, the binding partner is selected from the group consisting of: antibodies, antibody fragments, binders, natural ligands and receptors. In other embodiments, the binding partner is a human, humanized or de-immunized protein.

5 In certain embodiments, the signaling domain is selected from the group consisting of: FC-gamma-RI, CD28, CD3-zeta, Janus kinase, SYK-PTK, CD137 (4-1BB), CD3-epsilon, ICOS and CD134 (OX-40).

In certain embodiments, the engineered cell additionally expresses a suicide protein, such as a thymidine kinase, cytosine deamidase, pro-caspase 2 or Fas death receptor.

10 In certain embodiments, cells engineered to express an exogenous extracellular binding partner to an apoptotic or necrotic cellular antigen additionally expresses a binding partner to another antigen, for example a cancer cell antigen or a B-lymphocyte antigen.

The approach to imposing a change in cytokine profile, especially in the vicinity of tumors as described herein, is particularly desirable, since tumors, tumor-associated macrophages and T regulatory cells (Tregs), can suppress cytotoxic T cell response against
15 tumors and maintain an immune environment favorable to tumor growth.

In another aspect, featured herein are engineered cells, which express an exogenous extracellular binding partner to a cellular antigen, and at least one exogenous intracellular signaling domain and a suicide protein. In certain embodiments, the engineered cell binds to a B cell antigen and suppresses B cell function. For example, the engineered cell can
20 bind to a B cell antigen selected from the group consisting of CD19, CD20, CD21, CD22, CD37, CD72, CD79a, CD79b, CD138, Cd27, CD38 and CD78. In certain embodiments, the binding partner is a human, humanized or de-immunized protein.

In certain embodiments, the suicide protein is selected from the group consisting of: thymidine kinase, cytosine deamidase, pro-caspase 2 or a Fas death receptor. In certain
25 embodiments, the engineered cell additionally expresses an extracellular binding partner to another antigen. In certain embodiments, the engineered cell is a human cell, such as a human T lymphocyte, natural killer cell or natural killer T cell.

Therapeutic methods using an effective amount of the engineered cells disclosed

herein to reversibly remove patient's autoimmune B cells, are expected to produce a sustained clinical remission without the detrimental effect of the induction therapy necessary for hematopoietic stem cell transplantation.

In further aspects are featured DNA constructs and vectors containing the constructs
5 and uses thereof for engineering cells.

In still further aspects are featured methods for treating immune system disorders in a subject comprising administering to the subject an effective amount of engineered cells as described herein.

Further features and advantages will become apparent from the following Detailed
10 Description and Claims.

Description of the Figures

Figure 1 is a schematic showing engineered cells described herein. (A) shows a transducing particle, which includes DNA that when expressed produces (B) a chimeric
15 antigen receptor comprised of: B1- a binding composition, B2- a primary signal domain, and B3- a co-stimulatory signal domain and (C) thymidine kinase. When expressed, the binding composition, B1, can bind to an apoptotic or necrotic antigen (e.g. present in a tumor necrotic center). The event of binding transmits a signal to the T cell's interior, which provokes the T cell to secrete cytokines, such as interleukin-2 (IL-2) and
20 interferon-gamma (IFN-gamma). If the cells are contacted with ganciclovir, the expression of the thymidine kinase enzyme in the cell mediates killing.

Figure 2 illustrates the design of two vectors encoding chimeric antigen receptors (CARs). A. consists of a CD8secretion signal sequence (ss) fused to DNA encoding a
25 single chain variable domain (ScFv), a CD8 hinge region (CD8 hinge), a CD8 transmembrane region (CD8 TM), a 4-1BB intracellular domain (IC) and a CD3zeta intracellular (IC) domain. B. shows the same construct as in A plus a gene encoding the herpes simplex virus thymidine kinase (HSV-TK).

Figure 3 is a plasmid map for the lentiviral vector 3T-101. Lentiviral vector 3T-101. MCS: Multi cloning site to be used for subcloning of CAR fusion gene fragment.

Figure 4 is a plasmid map for the lentiviral vector 3T-102. HSV-TK: Herpes simplex virus type I thymidine kinase. IRES: internal ribosomal entry site.

Figure 5 provides the results of a flow cytometry analysis of CAR expression by 293FT cells. 3T-101 vector control and CAR NHS expression construct expressing CAR protein in 293FT cells.

Figure 6 provides the results of a flow cytometry analysis of CAR expression by transduced human T cells. 3T-102 vector control and CAR NHS expression construct expressing CAR protein in human T cells.

Detailed Description

Definitions. The following terms and phrases as used herein shall have the following meanings.

“Antigen” refers to a substance that causes a mammalian immune system to generate antibodies. Certain antigens may be characteristic of various cells. For example, “apoptotic or necrotic antigens” refer to antigens that are produced by apoptosis or necrosis of a cell. Apoptotically dying cells (cells undergoing programmed death) activate a set of degradative enzymes, the caspases, that mediate the controlled disassembly and degradation of a cell by nearby phagocytes. Necrotic cells, which are undergoing unexpected or accidental cell death, undergo a less orderly process than apoptosis. As necrotic cells begin to die, they swell, holes appear in the plasma membrane and intracellular materials spill out into the surrounding environment. As the cell dies, its ability to maintain the integrity of the plasma membrane and to pump ions is lost. Unregulated calcium ions induce a generation of toxic chemicals and activate enzymes that lead to the degradation of cellular molecules. As the cell is disassembled, various breakdown products are produced and released into the area, including free fatty acid (FFAs) derivatives of the cell’s phospholipid membrane, such as arachidonic acid. FFAs are substrates for cyclooxygenases (e.g. COX1 and COX2), which produce eicosanoids, such as prostaglandins, which mediate inflammatory responses. Histological features of necrotic tumors have been described

(see, e.g., Miettinen (2010) *Modern Soft Tissue Pathology: Tumors and Non-Neoplastic Conditions*, 3rd ed., Cambridge Univ. Press, Cambridge, UK (1116 pages); Fletcher (2007) *Diagnostic Histopathology of Tumors*, Churchill Livingstone, Philadelphia, PA (1992 pages)). Reagents for detecting blebs, blebbing, or apoptotic cells are available (see, e.g.,
 5 US2011/0257434 of Ziv and Shirvan, and US2011/0110861 of Lahoud et al, which are hereby incorporated by reference in their entirety).

Examples of “apoptotic or necrotic cellular antigens” include naked DNA, histones, complexes of DNA and histones, claretin, vitronectin, phosphatidyl serine, ribonucleoprotein (RNP) complexes, interleukin 6, myosin, disialoganglioside GD2,
 10 ERBB2, annexin, alpha-D-mannose and beta-D-galactose-specific plasma membrane glycoproteins, the GlcNAc carbohydrate, a marker for phagocytosis.

In addition, specific cell types may have certain cell type specific apoptotic or necrotic markers. For example, beta-glucuronidase and serum immunoreactive prolyl 4-hydroxylase for hepatic cell death; cardiac troponin, cardiac troponin I and creatine kinase
 15 MB for myocardial cell death.

“B cell antigen” or “B-lymphocyte antigen” refers to an antigen that is expressed by a B-lymphocyte. Examples include: CD19, CD20, CD21, CD22, CD37, CD72, CD79a, CD79b, CD138, Cd27, CD38 and CD78. Other B cell antigens include, e.g., PD1/CD279, GCET-1, hFCRL1/CD307a, FCRL2/CD307b, CXCR5/CD185, B7-DC/CD273,
 20 MRC/CD200, CD130, CXCR4/CD184, Siglec-5/14, or CD150. Subgroup of mAbs in PC-MZL includes BTLA/CD272, BLIMP-1, hCD38, ZFYVE19, DAPK3, OGFOD1, C6orf130, MDS032, PAX5 (Fanoni et al (2011) *Immunol. Lett.* 134:157-160; Marina et al (2010) *Cancer Res.* 70:1344-1355; Gibson et al (2006) *Am. J. Clin. Pathol.* 126:916-924; US2012/0114556 of Goldenberg and Hanson, and US2012/0121643 of Dubensky which
 25 are incorporated herein in their entirety).

“Cancer cell antigen” refers to an antigen that is expressed by cancerous or tumor cells. Examples include products of mutated oncogenes and tumor suppressor genes, products of other mutated genes, overexpressed or aberrantly expressed cellular proteins, tumor antigens produced by oncogenic viruses, oncofetal antigens, altered cell surface
 30 glycolipids or glycoproteins and cell type specific differentiation antigens. A tumor antigen may for example comprise an oligosaccharide, lipid, nucleic acid, or any combination

thereof. Examples include carboxyanhydrase IX (Vullo (2003) *Bioorg. Med. Chem. Lett.* 13:1005-1009), GD2 ganglioside (Matthay et al (2012) *Clin. Cancer Res.* 18:2740-2753); SS-A/Ro antigen, for example, Ro52 and Ro60 (Saegusa et al (2002) *Free Radical Biol. Med.* 32:1006-1016); ED-B variant of fibronectin (Rudman et al (2011) *Clin. Cancer Res.* 17:1998-2005), mesothelin, prostate stem cell antigen (PSCA), WT-1, EphA2, carcinoembryonic antigen (CEA), HER-2/Neu/ErbB2, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, NY-ESO-1, NY-CO-8; NY-CO-9; NY-CO-13; NY-CO-16; NY-CO-20; NY-CO-38; NY-CO-45, K-RAS; H-RAS; N-RAS; TRP-1; TRP-2, MART-1; MUC-1; MUC-2; GAGE/PAGE family (see, US20120121643 of Dubensky).

10 “B cell disorder” refers to a disease or condition that is caused or contributed to by aberrant B cells or aberrant B cell function. Examples include: defects of B cell development/immunoglobulin production (immunodeficiencies) and excessive/uncontrolled proliferation (lymphomas, leukemias) as well as diseases or disorders, which have an autoimmune component, such as multiple sclerosis, systemic lupus erythematosus, type II
15 diabetes, lupus nephritis, dermatomyositis, psoriasis, Crohn’s disease and immune thrombocytopenic purpura.

 “Binding partner” refers to molecules that can bind to an antigen. Examples include antibodies, antibody fragments, binders, ligand and receptors.

 “Cancer” refers to a cancer, a tumor, a metastasis, angiogenesis of a tumor, and
20 precancerous disorders such as dysplasia, including hematological cancers such as multiple myelomas, lymphomas, lymphoid neoplasms (acute lymphocytic leukemia (ALL); chronic lymphocytic leukemia (CLL); hairy cell leukemia (HCL)), and myeloid neoplasms (acute myeloid leukemia (AML); acute promyelocytic leukemia (APL); chronic myeloid leukemia (CML) and myelodysplastic syndromes (MDS)) (see, e.g.,
25 Hematological Cancers in Brody (2012) Clinical Trials, Academic Press/Elsevier, San Diego, CA, pp. 279-325).

 “Engineered cell” refers to a cell, which has been genetically engineered to encode and express one or more exogenous protein, i.e. a protein, which was not previously encoded or expressed by the cell.

30 “Immune system disorder” “refers to a disease or disorder, which is associated with a pathological immune response in a subject. Examples include allergies, asthma, various

types of inflammation, cancers (solid and non-solid) and autoimmune disorders such as multiple sclerosis, rheumatoid arthritis, Crohn's disease, psoriasis and systemic lupus erythemosis.

“Lymphocytes” encompass two major groups of cells, T cells and B cells. T cells take the form of CD8⁺ T cells and CD4⁺ T cells. CD8⁺ T cells use perforin and granzyme to kill other cells. CD4⁺ T cells have the function of optimizing function of other immune cells, such as CD8⁺ T cells and dendritic cells. T cells express T cell receptor (TCR), which comprises the alpha chain and beta chain. TCR recognizes antigens presented on the surface of other cells, where these antigens take the form of peptides presented by major histocompatibility complex (MHC), that is, the membrane-bound MHC that is expressed by the other cells. B cells express the B cell receptor (BCR), which resembles a typical antibody, in that it has two light chains and two heavy chains. BCR has an antibody-like structure. BCR binds to antigens in a manner similar to that when an antibody binds to an antigen.

“Signaling domain” refers to intracellular moieties of membrane receptors, or cytoplasmic portions of oligomeric receptor complexes that mediate activation, proliferation, or differentiation signals of external stimuli. Typically, signaling domains function as docking sites for signaling kinases or contain kinase domain themselves. Examples of signaling domains include FC-gamma-RI, CD28, CD3-zeta, Janus kinase, SYK-PTK, CD137 (4-1BB), CD3-epsilon, ICOS and CD134 (OX-40).

“Suicide protein” refers to a protein that when expressed by a cell can interact with an exogenous molecule in a manner that results in cell death. Exemplary suicide proteins include thymidine kinase (which interacts with ganciclovir), cytosine deamidase (which interacts with 5-fluorocytosine), pro-caspase 2 (which interacts with doxycycline) and a Fas death receptor (which interacts with AP1903).

Engineering mammalian cells Featured herein are engineered human cells (e.g. T lymphocytes, natural killer cells or natural killer T cells), which express an exogenous chimeric antigen receptor comprised of an extracellular binding partner to an apoptotic or necrotic cellular antigen and at least one intracellular signaling domain.

Figure 1 is a schematic showing the engineered cells. (A) shows a transducing particle, which includes DNA that when expressed produces (B) a chimeric antigen

receptor comprised of: B1- a binding partner to an apoptotic or necrotic cellular antigen B2- a primary signal domain, and B3- a co-stimulatory signal domain and (C) thymidine kinase. When expressed, the binding composition, B1, can bind to an apoptotic or necrotic antigen (e.g. present in a tumor necrotic center). The event of binding transmits a signal to the

5 T cell's interior, which provokes the T cell to secrete cytokines, such as interleukin-2 (IL-2) and interferon-gamma (IFN-gamma) as well as release of granzyme or perforin. If the cells are contacted with ganciclovir, the expression of the thymidine kinase enzyme in the cell mediates killing. Since the engineered CAR lymphocytes target dying cells, release of granzyme or perforin is not expected to contribute to the death of living cells, eliminating

10 toxicity induced by therapeutic T cells. However, the expressed cytokines, for example, a Th1-type cytokine such as IFN-gamma, provoke a localized Th1-type response that is not systemic, but that is localized in the vicinity of the tumor.

The methods and reagents described herein are not only useful for mediating immune attack against solid tumors, but also for mediating immune attack against

15 hematological cancers of high cellular density, which are also known as "liquid tumors" or "liquid cancers."

The exogenous CAR may be comprised of human or humanized sequences. (see, e.g., US2011/0293632 to Presta and US2009/0181015 to Presta et al, which are hereby incorporated by reference in their entirety).

20

Viral vectors, including lentivirus-based vectors, adenovirus-based vectors, and retrovirus-based vectors can be used for the stable integration of genes into the mammalian genome (see, e.g., Nowrouzi et al (2001) *Viruses*. 3:429-455). In addition to using viral vectors, homologous recombination can be used to incorporate nucleic acids into the

25 mammalian genome. See, e.g., 2009/0286320 of O'Gorman et al, US2010/0304489 of Geijsen et al, US20090191171 of Ma, and US 2009/0010948 of Huang et al, which are incorporated herein by reference. A schematic diagram of homologous recombination is available (see, Fig. 3 of US2012/0121643 of Dubensky et al). Homologous recombination can be used to introduce loxP sites into the genome, where the introduced loxP sites are

30 subsequently used for site-specific recombination. Other methods for introducing exogenous gene expression into engineered cells include sleeping beauty transposon system (Maiti et al (2013) *J. Immunother.* 2013 Feb;36:112-123), use of non-viral RNA (Riet et al.

(2013) *Methods Mol Biol.* 969:187-201, and TALEN-introduced expression (Sakuma et al. (2013) *Genes Cells.*18:315-326).

Lentivirus-based vectors may be mixed with unfractionated peripheral blood
5 mononuclear cells (PBMCs), or with a preparation where the engineered lymphocytes constitute at least 10% of the cells present, or at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or at least 98% of the cells present.

The extracellular binding partner to an apoptotic or necrotic cellular antigen can include naked DNA, histones, complexes of DNA and histones, clarectulin, vitronectin,
10 phosphatidyl serine, ribonucleoprotein (RNP) complexes, interleukin 6, myosin, disialoganglioside GD2, ERBB2, annexin, alpha-D-mannose and beta-D-galactose-specific plasma membrane glycoproteins, the GlcNac carbohydrate, a marker for phagocytosis.

The signaling domain can comprises FC-gamma-RI; CD28; TCR-zeta; SYK-PTK;
15 CD137 (4-1BB); CD3-epsilon, ICOS, CD134 (OX-40) (see, e.g., Milone et al (2009) *Mol. Therapy* 17:1453-1464; Ghandhi and Jones (2011) *Immunotherapy* 3:1441-1443; US20120121504 of Rader, which are incorporated herein by reference). The skilled artisan can consult Journal of Immunology, Nature Immunology, or a related journal, and acquire GenBank numbers that identify the polypeptide sequence and nucleic acid sequence.
20 Engineered lymphocytes may contain multiple signaling domains, for example, endodomains from both 4-1BB and CD3-zeta.

Cells may be engineered to contain a nucleic acid encoding a protein that mediates killing of the cell harboring the gene (a suicide protein), for example a nucleic acid encoding herpes simplex virus-thymidine kinase (TK) gene, or *Escherichia coli* cytosine
25 deamidase (CD) gene (see, e.g., GenBank Acc. No. NC_010468). Where a T cell is engineered with thymidine kinase gene, the cell can be killed by administering ganciclovir (Matthews and Boehme (1988) *Rev. Infect. Dis.* 10 (Suppl. 3) S490-S494, of US2012/0142071 of Black, which is incorporated herein in its entirety). Where a T cell is engineered with cytosine deamidase, the cell can be killed by administering 5-
30 fluorocytosine. Also, a cell can be engineered to express an apoptotic protein, pro-caspase-2, where the cell can be killed by administering doxycycline (Knott et al (2005) *Cancer Biol. Ther.* 4:532-536) or a Fas death receptor, where death of the CAR-engineered T cell is

induced by administering AP1903 (Thomis et al (2001) *Blood*. 97:1249-1257; US2005/0271647, which are hereby incorporated in their entirety).

Exogenously introduced proteins may be engineered to reduce or avoid the presence of consensus sequences for MHC binding. These consensus sequences are disclosed for example in US patent application 2009/0012004 by Sette et al.

Engineered lymphocytes may exhibit altered proliferation (expansion), persistence (lifetime inside human subject or experimental animal), affinity maturation (see, e.g., US2012/0039870 of Dolk et al, which is incorporated by reference), CD4⁺ T cell response, CD8⁺ T cell response, Th1-type response, Th2-type response, effector T cell response, and/or central memory response (see, e.g., US2011/0091481 of Burnette, and US20080293070 and Sekaly et al, which are incorporated by reference).

Therapeutic Methods Lymphocytes may be collected from a patient, engineered to express chimeric antigen receptors (CARs), then infused back into the same patient.

Where a population of engineered cells is administered, the infused dose can be, for example, about 1×10^6 T cells/m², 2×10^6 T cells/m², 5×10^6 T cells/m², 1×10^7 T cells/m², 2×10^7 T cells/m², 5×10^7 T cells/m², 1×10^8 T cells/m², 2×10^8 T cells/m², 5×10^8 T cells/m², and the like. The formulation may include a mixed T cell population, for example, about 0%CD4⁺ T cells and about 100% CD8⁺ T cells, about 10% CD4⁺ T cells and about 90% CD8⁺ T cells, about 20% CD4⁺ T cells and about 80% CD8⁺ T cells, about 30% CD4⁺ T cells and about 70% CD8⁺ T cells, about 40% CD4⁺ T cells and about 60% CD8⁺ T cells, about 50% CD4⁺ T cells and about 50% CD8⁺ T cells, about 60% CD4⁺ T cells and about 40% CD8⁺ T cells, about 70% CD4⁺ T cells and about 30% CD8⁺ T cells, about 80% CD4⁺ T cells and about 20% CD8⁺ T cells, about 90% CD4⁺ T cells and about 10% CD8⁺ T cells, and about 100% CD4⁺ T cells and about 0% CD8⁺ T cells.

Engineered cells, as described herein, may be administered in combination with one or more anti-cancer drugs, including antibodies, such as Rituximab, Trastuzumab, Alemtuzumab, Bevacizumab, Cetuximab, Denosumab, Gemtuzumab, Ipilimumab,

Ofatumumab, Panitumumab, Tositumomab, etc.; small molecules, such as imatinib, gefitinib, erlotinib, bortezomib, tofacitinib, crizotinib, apatinib, fludarabine, cyclophosphamide, cladribine, cytarabine, anthracycline, clofarabine, laromustine, decitabine, azacytidine, all-trans-retinoic acid, arsenic trioxide, lenalidomide, 5-azadeoxycytidine; proteins, including cytokines, such as interleukin 2, interleukin 12, interferon alpha, interferon gamma, TNF-alpha, etc.

Engineered cells may also be administered in conjunction with a reagent that modulates T cell activity, such as a toll-like receptor (TLR) such as PF-3512676, also known as CPG7909, which is a TLR9 agonist; polyinosinic:polycytidylic acid (Poly(I:C)), which is a TLR3 agonist; glucopyranosyl lipid A (GLA), which is a TLR4 agonist; imiquimod, which is a TLR7 agonist. *Listeria monocytogenes* peptidoglycan, which is a TLR2 agonist; *Listeria monocytogenes* flagellin, which is a TLR5 agonist; bacterial nucleic acids, which are TLR9 agonists. Patients, to whom engineered cells have been administered may be measured for clinical endpoints, such as, event-free survival (EFS), progression-free survival (PFS), overall survival (OS), and relapse-free survival (RFS). Other endpoints include, for example, minimal residual disease (MRD) (see, e.g., Gabert et al (2003) *Leukemia* 17:2318-2357), which may be used to predict overall survival, for example, in studies of leukemia or lymphoma. Polymerase chain reaction (PCR) or flow cytometry may be used to detect residual hematological cancer cells in the bloodstream, and provide data that represents MRD (see, e.g., Hoshino (2004) *Tohoku J. Exp. Med.* 203:155-164; Kenkre and Smith (2008) *Curr. Oncol. Rep.* 10:393-403).

Engineered cells may be combined with a therapeutic agent that reverses the acidosis of the tumor environment.

Engineered cells may also be administered in conjunction with therapeutic vaccines which may benefit from the changes in the tumor microenvironment induced by the engineered cells. Examples of the therapeutic vaccines in combination therapies include Sipuleucel-T, Oncophage, CancerVax etc.

Reagents and methods for determining, assessing, monitoring, and diagnosing immune response may also be used to evaluate the engineered cells, including:

i. Methods for measuring cellular parameters. Effector T cells; central memory T cells (TCM); effector memory T cells (TEM), and constituents thereof may be measured, as well as the biological functions of these cells including cytotoxic function, expression of markers, affinity for antigen, number of cells in a biological compartment such as serum, preferred location in the body such as in lymph node or spleen, and rate of response when exposed or re-exposed to antigen.

ii. Methods for measuring antibodies. The affinity maturation of antibodies may be measured (see, e.g., McHeyzer-Williams and McHeyzer-Williams (2005) *Ann. Rev. Immunol.* 23:487-513), antibody titer or isotype, including IgG (IgG1; IgG2; IgG3; IgG4); IgA (IgA1; IgA2); IgM; IgD; IgE; isotype switching of antibodies, for example, decreases in IgM and increases in IgG (see, e.g., Hasbold et al (2004) *Nature Immunol.* 5:55-63; Ryffel et al (1997) *J. Immunol.* 158:2126-2133; Lund et al (2002) *J. Immunol.* 169:5236-5243; Palladino et al (1995) *J. Virol.* 69:2075-2081; Karrer et al (2000) *J. Immunol.* 164:768-778); isotype switching that is a function of Th1-type or Th2-type response (Delale et al (2005) *J. Immunol.* 175:6723-6732; McKenzie, et al (1999) *J. Exp. Med.* 189:1565-1572; Fayette et al (1997) *J. Exp. Med.* 185:1909-1918).

iii. Parameters of B cells. Naive B cells (high in membrane IgD and low in CD27), memory B cells (low in IgD and high in CD27), and constituents of these cells (see, e.g., Fecteau and Neron (2003) *J. Immunol.* 171:4621-4629) may be measured as well as the formation of memory B cells within germinal centers (see, e.g., Ohkubo et al (2005) *J. Immunol.* 174:7703-7710). Terminally differentiated B cells, for example, cell's ability to respond to CXCL12 may be measured (see, e.g., Roy et al (2002) *J. Immunol.* 169:1676-1682), as well as the commitment of antibody-secreting cells (ASCs) (see, e.g., Hasbold, et al (2004) *Nature Immunol.* 5:55-63).

iv. Parameters of T cells. The affinity of a cytotoxic T cell for a target cell (see, e.g., Montoya and Del Val (1999) *J. Immunol.* 163:1914-1922) can be measured. In addition, markers, for example, effector memory T cells (TEM) can be identified as CD62L^{LOW} and CCR7^{LOW}, where these cells show immediate effector function with antigen re-encounter. Central memory T cells (TCM) can be identified by relatively high expression of CD62L and CCR7, where the cells show relatively slow activation kinetics. Other available markers include, e.g., CCL4, CCL5, XCL1, granulysin, granzyme A,

granzyme B, and so on (see, e.g., Chtanova et al (2005) *J. Immunol.* 175:7837-7847; Kondrack, et al (2003) *J. Exp. Med.* 198:1797-1806; Huster, et al (2004) *Proc. Natl. Acad. Sci. USA* 101:5610-5615; Ahmadzadeh, et al (2001) *J. Immunol.* 166:926-935; Goldrath et al (2004) *Proc. Natl. Acad. Sci. USA* 101:16885-16890; Wherry et al (2003) *Nature Immunol.* 4:225-234; Sallusto et al (2004) *Ann. Rev. Immunol.* 22:745-763). Different types of immune cells, as well as different stages of maturation of a particular cell, or different stages of activation of a cell, can be distinguished by titrating with a reagent specific to any given marker (see, e.g., Ahmadzah, et al (2001) *J. Immunol.* 166:926-935). Other parameters for T cells include cytokine expression profile, cytokine secretion profile, and cytotoxicity profile (see, e.g., Culver (1991) *Proc. Natl. Acad. Sci. USA* 88:3155-3159).

v. Parameters of antigen presenting cells (APCs), including dendritic cells (DCs). The amount (mmoles) of peptide presented (or bound) per mmole MHC Class I can be measured. Moreover, the amount of peptide presented or bound per mmol of MHC Class II and the amino acid sequence of the bound peptides (see, e.g., Velazquez et al (2001) *J. Immunol.* 166:5488-5494). In addition, the relative ability of the APC to present epitopes derived from peptides versus epitopes derived from proteins can be measured, as well as the ability to present epitopes acquired from low levels of peptides versus high levels of peptides and, in other aspects, the identity of the APC suitable for presentation (see, e.g., Constant, et al (1995) *J. Immunol.* 154:4915-4923).

Engineered cells may exhibit an increase in: cytokine expression; cytotoxic T cell response; T cell proliferation; Elispot assay activity; tetramer assay activity; intracellular staining (ICS) activity; T cell survival; survival against a tumor, infective agent, or immune disorder; or any combination of the above, of at least 10%, at least 20%, at least 50%, at least 100% (2-fold), at least 5-fold, at least 10-fold, and the like, as compared with the equivalent cell that has not been engineered to express a CAR.

Methods for determining binding affinities, binding specificities, and affinity maturation are known (see, e.g., Chen, et al (2004) *J. Immunol.* 173:5021-5027; Rees, et al (1999) *Proc. Natl. Acad. Sci. USA* 96:9781-9786; Busch and Pamer (1999). *J. Exp. Med.* 189:701-709; Ploss, et al (2005) *J. Immunol.* 175:5998-6005; Brams, et al (1998) *J. Immunol.* 160:2051-2058; and Choi, et al (2003) *J. Immunol.* 171:5116-5123).

Methods for using animals in the study of cancer, metastasis, and angiogenesis, and

for using animal tumor data for extrapolating human treatments are known (see, e.g., Hirst and Balmain (2004) *Eur J Cancer* 40:1974-1980; Griswold, et al (1991) *Cancer Metastasis Rev.* 10:255-261; Hoffman (1999) *Invest. New Drugs* 17:343-359; Boone, et al (1990) *Cancer Res.* 50:2-9; Moulder, et al (1988) *Int. J. Radiat. Oncol. Biol. Phys.* 14:913-927; Tuveson and Jacks (2002) *Curr. Opin. Genet. Dev.* 12:105-110; Jackson-Grusby (2002) *Oncogene* 21:5504-5514; Teicher, B. A. (2001) *Tumor Models in Cancer Research*, Humana Press, Totowa, N.J.; Hasan, et al (2004) *Angiogenesis* 7:1-16; Radovanovic, et al (2004) *Cancer Treat. Res.* 117:97-114; Crnic and Christofori (2004) *Int. J. Dev. Biol.* 48:573-581; U.S. Dept. Health and Human Services, Food and Drug Administration (2002) Guidance for Industry. Estimating the safe starting dose in clinical trials for therapeutics in adult healthy volunteers).

Standard methods of biochemistry and molecular biology are described in the art (e.g., Maniatis, et al (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y.; Sambrook and Russell (2001) *Molecular Cloning*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Wu (1993) *Recombinant DNA*, Vol. 217, Academic Press, San Diego, Calif.; Innis, et al (eds.) (1990) *PCR Protocols: A Guide to Methods and Applications*, Academic Press, N.Y. Standard methods are also found in Ausubel, et al (2001) *Curr. Protocols in Mol. Biol.*, Vols.1-4, John Wiley and Sons, Inc. New York, N.Y., which describes cloning in bacterial cells and DNA mutagenesis (Vol. 1), cloning in mammalian cells and yeast (Vol. 2), glycoconjugates and protein expression (Vol. 3), and bioinformatics (Vol. 4)). Methods for producing fusion proteins are described (see, e.g., Invitrogen (2005) *Catalogue*, Carlsbad, Calif.; Amersham Pharmacia Biotech. (2005) *Catalogue*, Piscataway, N.J.; Liu, et al (2001) *Curr. Protein Pept. Sci.* 2:107-121; Graddis, et al (2002) *Curr. Pharm. Biotechnol.* 3:285-297).

Methods for preparing recombinant antibodies are also well-described in the art (e.g., US2012/0148597 of Hanson et al, US2012/0134994 of Kim et al, US2012/0076802 of Lanzavecchia et al, US2011/0318350 of Jaspers et al, which are hereby incorporated in their entirety, for example, for their disclosure of linkers, promoters, enzymes, plasmids, vectors, cloning strategies, heavy chain constant regions, light chain constant regions, variable regions, and regulatory sequences, for example, sequences that regulate transcription, translation, membrane insertion, subcellular location, and so on.

Methods for purifying proteins, such as by immunoprecipitation, column chromatography, electrophoresis, isoelectric focusing, centrifugation, and crystallization, software packages for determining antigenic fragments, leader sequences, MHC-binding peptides, and so on, are described, as cited in US2012/0121643 of Dubensky et al.

5 Elispot assays and intracellular cytokine staining (ICS) for characterizing immune cells are available (see, e.g., Lalvani, et al (1997) *J. Exp. Med.* 186:859-865; Waldrop, et al (1997) *J. Clin. Invest.* 99:1739-1750; Hudgens, et al (2004) *J. Immunol. Methods* 288:19-34; Goulder, et al (2001) *J. Virol.* 75:1339-1347; Goulder, et al (2000) *J. Exp. Med.* 192:1819-1831; Anthony and Lehman (2003) *Methods* 29:260-269; Badovinac and Harty
10 (2000) *J. Immunol. Methods* 238:107-117).

The invention, now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

15 **Example 1: Construction of plasmid vectors**

Lentiviral vector plasmids were derived from pLVX-EF1alpha-IRES-Puro (Clontech). The first step of the vector modification was a PCR amplification of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) element (about 700 bps) using the primers 5'-
20 AGCTGGATCCAATCAACCTCTGGATTACAAAATTTGTG (SEQ ID No. 1) and 5'-TAAAGGTACCTGAGGTGTGACTGGA (SEQ ID No. 2). The PCR fragment was digested with the restriction enzymes BamHI and Acc65I. At the same time, the pLVX-EF1alpha-IRES-Puro plasmid DNA was digested with the restriction enzymes BamHI and Acc65I, followed by subcloning of the WPRE fragment into the plasmid
25 vector. The resulting vector was named as 3T-101 plasmid (Fig 3). In the second step, the gene encoding the thymidine kinase from the type I Herpes Simplex Virus was de novo synthesized, along with the IRES sequence. The resulting fragment was subcloned into the XbaI/BamH site of 3T-101. The resulting vector is named as 3T-102 (Fig 4).

30 ScFvs were made with the VL and VH chains based on the sequence of the NHS76 monoclonal antibody (Sharifi et al. (2001) *Hybridoma & Hybridomics* 20: 305-312;

Williams et al. (2000) WO 00/01826), the KSUG monoclonal antibody (Song et al., 1998, Genbank accession: U38588.1, U38589.1), or the 3H9 monoclonal antibody (Shlomchik et al., 1987, Genbank accession: M18237.1; M18234.1), anti CD19 monoclonal antibody FMC63 (Nicholson et al. (1997) *Mol. Immunol.* 34: 1157-1165, Genbank accession: Y14283; Y14284) and anti CD19 monoclonal antibody anti-B4 (Nadler et al. (1983), *J. Immunol.* 131:244 – 250; Super et al. WO 2007/076950). VH and VL chains were linked by 4 GS linkers to ensure proper folding. The ScFvs may be linked to a CD8-alpha hinge region, which is linked to a CD8 alpha transmembrane domain with two extra protruding amino acids at the cytoplasmic side (Norment et al. (1989) *J. Immunol.* 142:3312-3319).

CD8-beta hinge polypeptides and nucleic acids encoding said polypeptides are provided as GenBank Acc. No. BC100914.1. The intracellular domains were derived from 4-1BB (Schwarz et al. (1995) *Blood* 85:1043-1052, Genbank accession: L12964), and CD3 zeta intracellular domain was deduced from the NCBI Reference Sequence NM_198053.2. The full-length intracellular domains were fused in the order of 4-1BB/CD3 zeta or CD3zeta/4-1BB, respectively. For reference, gene bank accession numbers used for gene designs are NM_001768 (CD8 alpha), and HQ685985 (HSV thymidine kinase). The nucleotide sequence for GS linker is: GCGGGTGGTGG CTCTGGCGGTGGTGGCT CTGGCGGAGGTGGCTCTGGCGGT TCG (SEQ ID NO. 3). Exemplary constructs for fusions proteins are illustrated in Figure 2.

DNA fragments for the fusion genes were synthesized de novo, and confirmed by sequencing, and subcloned as SpeI-BamHI fragment into the plasmid vectors 3T-101 and 3T-102, according to the standard molecular biology methods. As examples, anti necrotic CAR NHS, anti CD19 CAR B4, or CAR FMC were constructed.

Example 2: 293FT cell transfection and flow cytometry analysis

Lentiviral vectors containing the fusion genes were transfected into 293FT cells using Lipofectamin as recommended by the manufacturer (Invitrogen). The presence of the fusion proteins on the surface of transfected 293FT cells were analyzed by flow cytometry.

1×10^6 transfected 293FT cells were washed with ice cold flow cytometry buffer (PBS, 0.4% BSA and 0.1% NaN_3), then resuspended in 1ml of flow cytometry buffer followed by the addition of goat IgG (Goat Gamma Globulin, Jackson Immuno Research, Catalog# 005-000-002) to the final concentration of 1 $\mu\text{g/ml}$. The solution was incubated in ice for 5 minutes, before adding 8 μg of biotin labelled polyclonal goat-anti-mouse F(ab)2

fragment (Jackson ImmunoResearch, Cat # 115-066-072). As a reference, 8 µg of biotin labelled polyclonal goat antibody F(ab')₂ fragment (Biotin-SP-ChromPure Goat IgG, F(ab')₂ fragment, Jackson ImmunoResearch, Cat # 005-060-006) was incubated in ice for 25 minutes and the cells were washed once using flow cytometry buffer. The washed cells
5 were labelled with 5 µl R-Phycoerythrin Streptavidin (Jackson ImmunoResearch, 016-110-084) in 0.5ml flow cytometry buffer and incubated in the dark on ice for 20 minutes. The labeled cells were washed three times using flow cytometry buffer and expression was analyzed by flow cytometry. Figure 5 shows the results of the flow cytometry analysis for the transient expression of CAR NHS in 293FT cells. Note CAR NHS denotes a chimeric
10 antibody receptor in which the ScFv was derived from the NHS76 monoclonal antibody. Figure 6 provides the results of a flow cytometry analysis of CAR expression by transduced human T cells. 3T-102 vector control and CAR NHS expression construct expressing CAR protein in human T cells.

Example 3: Production of lentivirus particles

15 Lentiviral vectors were co-transfected into 293FT cells, which were cultured in DMEM medium in the presence of 10% fetal bovine serum. The other packaging lentiviral vectors in addition the CAR vectors included pMDLg/pRRE, pRSV/Rev and pMD.G (Dull *et al.*, (1998) *J. Virol.* 72: 8463-8471). The lentiviral particles were harvested 72 hours after the transfection, stored in -80°C and fast thawed when needed. The entire procedure was
20 performed under aseptic conditions.

Example 4: Preparation of CAR-engineered T cells

T cell isolation: Peripheral blood mononuclear cells (PBMC) were purchased from Cellular Technologies Ltd. PBMC were thawed and cultured for 2 hours in T25 flasks to eliminate adhesive cells. The non-adhesive cells were collected and non-adhesion primary
25 Pan T cells were enriched using negative selection (Miltenyi). Cell culture medium RPMI-1640, 10mM HEPES buffer, penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% FBS were used throughout the process.

T cell activation: CD3/CD28 Dynabeads (Life Technologies) were washed once with RPMI-1640 medium and mixed with lymphocytes in a ratio of 1:1 and incubated at
30 37°C. For in vitro testing, T cells were activated with artificially induced apoptotic cells, or B lymphoma cells.

Induced apoptosis and monitoring: Jurkat cells (clone E6-1 from ATCC) were harvested from culture, resuspended at a density of 10^6 cells per ml in RPMI medium 1640 (Mediatech, Herndon, VA) containing 10% FBS and 2.0 μ M camptothecin (Sigma Chemical Co., St. Louis, MO) for 3 to 6 h, or with 1.0 μ M staurosporine (Sigma) and treated for 16 h at 37°C to induce apoptosis. Apoptosis was assessed by light microscopy and cells were harvested after at least 25% of cells in the population exhibited surface blebs. 5×10^5 cells were divided into aliquots, placed into tubes, and washed with 4.0 ml of ice-cold Hanks' balanced salt solution (Mediatech) containing 1.0 mM CaCl_2 , 3% FBS, and 0.02% NaN_3 . Washed cells were incubated and stained with FITC-conjugated annexin V (BD Biosciences, San Diego). Before analysis, cells were further stained with 5 μ g/ml of propidium iodide (PI).

Transduction of T cells: T cells were transduced when co-cultured with lentivirus in a ratio of 1:5. The fusion genes were reverse-transcribed and integrated into the host cell genome. T cell surface CAR was detected using flow cytometry. Figure 6 is an example of flow cytometry characterization of T cells transduced with lentiviral particles.

Cytokine Analysis: In the absence of other cytokines, transduced T cells were co-cultured with apoptotic cells to activate the T cells with receptors for complexed or non-complexed DNA. Secreted cytokines were detected using TH1/TH2 cytokine cytometric bead array kit (BD Biosciences, San Diego, CA).

Example 5: Immune competent tumor mouse models

Tumor-bearing mice can be obtained by injecting 10^6 F9 murine teratocarcinoma cells subcutaneously into 10- to 12-week-old female 129SvEv mice or 10^6 C51 murine colon adenocarcinoma cells in 10- to 12-week-old female BALB/c mice.

4 to 5 days after tumor cell implantation, when tumors are clearly palpable, mice can be grouped (n greater or equal to 4) and injected intravenously into the lateral tail vein with saline, or transduced CAR T cells.

Mice can be monitored daily and tumor growth measured with a caliper using the following formula: volume = length $\times$ width² $\times$ 0.5. Animals can be sacrificed when a tumor reaches a volume of $>2,000 \text{ mm}^3$. Tumor sizes can be expressed as mean $\pm$ SE.

Example 6: Metastatic tumor models

Liver metastases. Male 129SvEv mice can be injected intravenously with 5×10^5 mutant F9 murine teratocarcinoma cells. Three days after tumor cell implantation, mice can be divided into three groups (n greater or equal to 5) and injected intravenously with CAR T cells. Mice can be sacrificed after 3 weeks, the livers excised, pictures taken, and metastatic foci per liver counted.

Lung metastases. Female BALB/c mice can be injected intravenously with 10^5 C51 murine colon adenocarcinoma cells. Three days after tumor cell implantation, mice can be divided into three groups (n greater or equal to 5) and injected intravenously with CAR T cells. Injections can be repeated thrice every second day. Mice can be sacrificed after 3 weeks; the lungs removed, fixed in saline containing 3% formaldehyde, and examined with a Zeiss stereomicroscope. Results can be expressed as numbers of metastatic foci per lung.

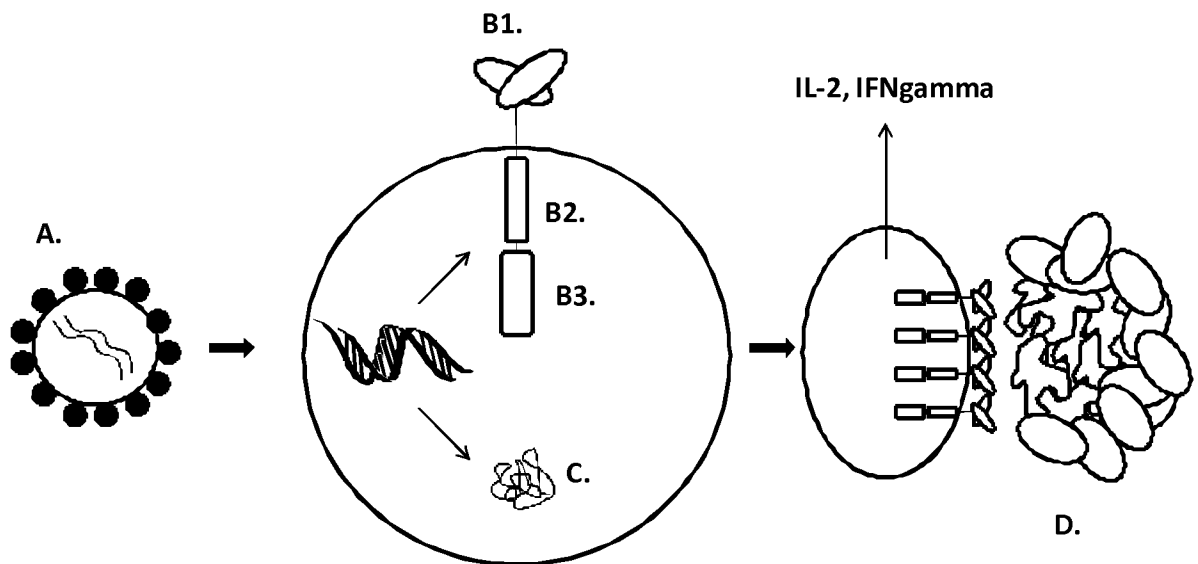
Claims

1. An engineered human cell, which expresses an exogenous extracellular binding partner to an antigen on a necrotic or apoptotic cell and at least one exogenous intracellular signaling domain.
2. An engineered cell of claim 1, wherein the antigen is selected from the group consisting of: DNA, histones, clarectulin, vitronectin, phosphatidyl serine, ribonucleoprotein (RNP) complexes, interleukin 6, myosin, disialoganglioside GD2, ERBB2, annexin, alpha-D-mannose and beta-D-galactose-specific plasma membrane glycoproteins, the GlcNAc carbohydrate and complexes of any of the above.
3. An engineered cell of claim 1, wherein the binding partner is selected from the group consisting of: antibodies, binders, natural ligands and receptors.
4. An engineered cell of claim 1, wherein the binding partner is a human, humanized or de-immunized protein.
5. An engineered cell of claim 1, which additionally expresses a suicide protein.
6. An engineered cell of claim 5, wherein the suicide protein is selected from the group consisting of: thymidine kinase, cytosine deamidase, pro-caspase 2 or the Fas death receptor.
7. An engineered cell of claim 1, wherein the signaling domain is selected from the group consisting of: FC-gamma-RI, CD28, CD3-zeta, Janus kinase, SYK-PTK, CD137 (4-1BB), CD3-epsilon, ICOS and CD134 (OX-40).
8. An engineered host cell of claim 1, which additionally expresses a binding partner to another antigen.
9. An engineered cell of claim 1, which is a T lymphocyte, natural killer cell or natural killer T cell.
10. An engineered cell of claim 1, which additionally comprises a co-stimulatory signaling

domain.

11. An engineered cell, which expresses an exogenous extracellular binding partner to a cellular antigen, at least one exogenous intracellular signaling domain and a suicide protein.
12. An engineered host cell of claim 11, wherein the antigen is specific to B cells.
13. An engineered host cell of claim 12, which is selected from the group consisting of CD19, CD20, CD21, CD22, CD37, CD72, CD79a, CD79b, CD138, Cd27, CD38 and CD78.
14. An engineered host cell of claim 11, wherein the binding partner is a human, humanized or de-immunized protein.
15. An engineered host cell of claim 11, wherein the suicide protein is selected from the group consisting of: thymidine kinase, cytosine deamidase, pro-caspase 2 or the Fas death receptor.
16. An engineered host cell of claim 11, which additionally expresses an extracellular binding partner to another antigen.
17. An engineered cell of claim 11, which is human.
18. An engineered cell of claim 11, which is a T lymphocyte, natural killer cell or natural killer T cell.
19. An engineered cell of claim 11, which additionally comprises a co-stimulatory signaling domain.

Fig. 1.



A. Transducing particles; B. receptors with 3 parts. B1: Binder; B2: primary signal domain; B3: co-stimulatory signal domain; C. Thymidine kinase; D. Tumor necrotic center.

Fig 2. CAR protein design

A. CAR protein design 1. B. CAR protein fused to HSV-TK 2. CD8 SS:CD8 secretion signal peptide, ScFv: single chain variable domain, CD8 hinge: CD8 hinge region, CD8 TM: CD8 transmembrane region, 4-1BB IC: 4-1BB intracellular domain, CD3 zeta IC: CD3 zeta intracellular domain. HSV-TK: Herpes simplex virus thymidine kinase

A.

CD8 SS	ScFv	CD8 hinge	CD8 TM	4-1BB IC	CD3zeta IC
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B.

CD8 SS	ScFv	CD8 hinge	CD8 TM	4-1BB IC	CD3zeta IC	HSV-TK
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Fig. 3. Lentiviral vector 3T-101. MCS: Multi cloning site, which were used for subcloning of CAR fusion gene fragment.

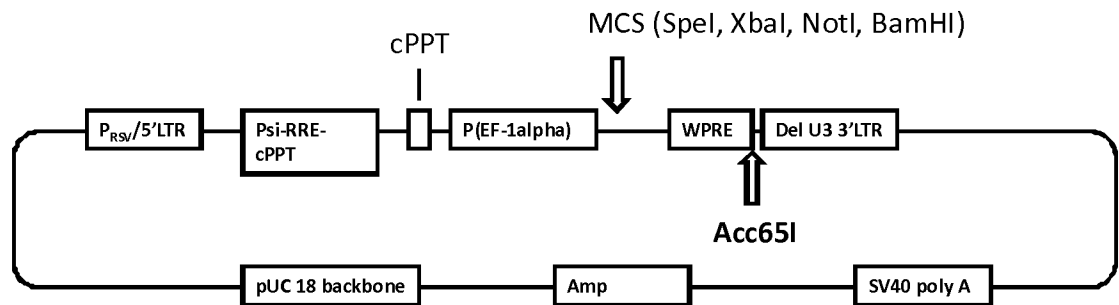


Fig. 4. Plasmid map for lenti viral vector 3T-102. HSV-TK: Herpes simplex virus type I thymidine kinase. IRES: internal ribosomal entry site.

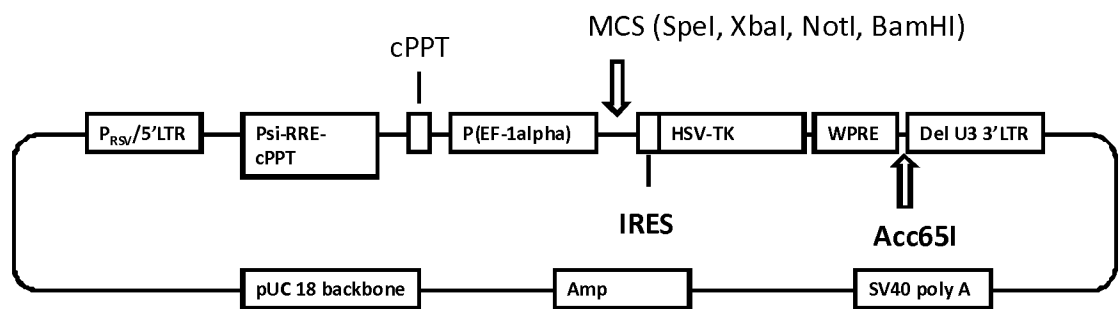


Fig. 5. Flow cytometry analysis of CAR expression in 293FT cells. 3T-101 vector control and CAR NHS expression construct were transfected into 293FT cells and characterized by flow cytometry.

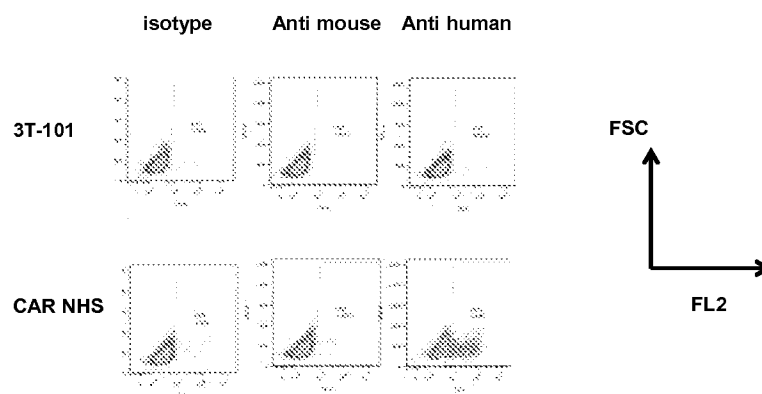
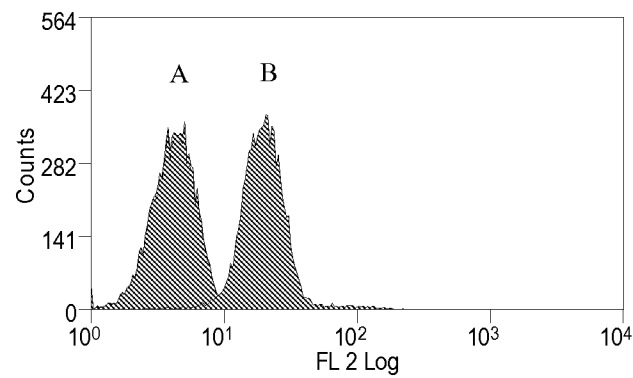


Fig. 6. Flow cytometry analysis of CAR expression in human T cells. 3T-102 vector control (peak A in graph) and CAR NHS expression construct (peak B in graph) were packaged into pseudotyped lentivirus. Virus particles were used to transduce human T cells. After 5 days of incubation of transduced T cells, expression of the chimeric antigen receptors were characterized by flow cytometry.



A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 1/00, 5/0783, 15/09 (2013.01)

USPC - 435/372.3, 334, 343.1

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(8): C12N 1/00; 5/00, 5/0783, 5/10, 5/12, 5/16, 5/22, 5/24, 5/26, 5/28; 15/00, 15/09 (2013.01)

USPC: 435/326, 328, 334, 343.1, 344, 366, 372.2, 372.3

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)

MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C, B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; ProQuest; PubMed; engineer*, 'genetically modified,' 'natural killer,' 'T-lymphocytes,' 'antibod*', 'receptor*', 'FC-gamma-RI,' 'CD28,' 'CD3-zeta,' 'Janus kinase,' 'SYK-PTK,' 'CD137,' '4-1BB,' 'CD3-epsilon,' 'ICOS,' 'CD134,' 'OX-40,' 'thymidine kinase,' 'cytosine deamidase,' 'pro-caspase 2'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMAI, C et al. Genetic Modification Of Primary Natural Killer Cells Overcomes Inhibitory Signals And Induces Specific Killing Of Leukemic Cells. Blood. 01 July 2005, Vol. 106, pp 376-383;	1, 3, 4, 7-10
Y	page 376, column 2, paragraph 2 to page 377, column 1, paragraph 1; page 377, column 2, paragraph 1; figures 3, 5; page 379, column 1, paragraph 2 to column 2, paragraph 1; page 382, column 1, paragraph 2 to column 2, paragraph 2.	2, 5, 6, 11-19
Y	MARKTEL, S et al. Immunologic Potential Of Donor Lymphocytes Expressing A Suicide Gene For Early Immune Reconstitution After Hematopoietic T-Cell-Depleted Stem Cell Transplantation. Blood. 15 February 2003, Vol. 101, No. 4; pp 1290-1298; abstract.	5, 6, 11-19
Y	US 8173424 B2 (MARKS, JD et al.), May 8, 2012; abstract; column 1, lines 62-67 to column 2, lines 1-15; column 2, lines 46-67; column 3, lines 25-54	2
A	DALDRUP-LINK, HE et al. In Vivo Tracking Of Genetically Engineered, Anti-HER2/Neu Directed Natural Killer Cells To HER2/Neu Positive Mammary Tumors With Magnetic Resonance Imaging. European Radiology. Vol. 15, No. 1; pp 4-13, 2005 (accessed 12 November 2013 at URL< http://escholarship.org/uc/item/3k79h4tx >).	1, 11

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


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Date of the actual completion of the international search

12 November 2013 (12.11.2013)

Date of mailing of the international search report

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