

TITLE OF INVENTION: CHIMERIC ANTIGEN RECEPTORS THAT BIND TO SSEA4 AND USES THEREOF

BACKGROUND

Targeted cancer immunotherapy, as compared to chemotherapy, holds the
5 promise of not only better efficacy, both short-term and long-term, but also fewer side
effects.

For example, anti-cancer vaccines targeting a tumor-specific carbohydrate
antigen, e.g., Globo H, stage-specific embryonic antigen 3 (“SSEA3”), and stage-
specific embryonic antigen 4 (“SSEA4”) have been developed to stimulate a patient’s
10 own immune system to develop antibodies against these antigens, which leads to
antibody-dependent cellular cytotoxicity, antibody-dependent phagocytosis,
complement-dependent cell lysis, as well as direct cytostatic and/or cytotoxic effects.

Such an approach often loses effectiveness over time as a result of an
inhibitory environment in the tumor. The inhibitory environment blocks one or all of
15 antibodies, NK cells, macrophages, and complement from entering the tumor.

Recently, chimeric antigen receptors (“CARs”) have been developed to
obviate the drawbacks mentioned above. A CAR contains (i) an extracellular domain
that binds to the tumor antigen and (ii) one or more intracellular domains that provide
both primary and co-stimulatory signals to the T cells. T cells can be engineered *in*
20 *vitro* to express CAR having an extracellular domain of choice.

The CAR approach has proven to be effective, yet not without serious side
effects. For example, activation of a large number of T cells expressing CAR causes
cytokine release syndrome. This syndrome, characterized by high fever, hypotension,
and hypoxia, can result in multi-organ failure and even death.

25 There is a need to develop CAR-based tumor therapies that are safer and more
effective than those currently in use.

SUMMARY

To meet the need discussed above, an isolated nucleic acid is disclosed that
contains a nucleotide sequence that encodes the polypeptide of SEQ ID NO: 3. The
30 polypeptide of SEQ ID NO: 3 specifically binds to stage-specific embryonic antigen 4
(SSEA4).

Also disclosed is a recombinant cell comprising the isolated nucleic acid described above, where the recombinant cell expresses the polypeptide of SEQ ID NO: 2.

Further, a viral vector containing the above isolated nucleic acid is within the scope of the invention. The viral vector is a lentiviral vector, a gamma-retroviral vector, or an adeno-associated viral vector.

Moreover, an isolated polypeptide including the sequence of SEQ ID NO: 3, Again, the isolated polypeptide specifically binds to SSEA4.

Provided as well is a chimeric antigen receptor (CAR) that includes a single chain Fv (scFv) having the sequence of SEQ ID NO: 3 and specifically binding to SSEA4, and a first endodomain from CD3 ζ or Fc ϵ RI γ .

Finally, a method is disclosed for treating a tumor in a subject, the method including the steps of (i) obtaining T cells from a subject having a tumor; (ii) transducing the T cells *in vitro* with a vector that contains a nucleic acid encoding a CAR including a scFv that specifically recognizes SSEA-4, whereby the transduced T cells express the CAR; (iii) expanding the transduced T cells *in vitro*; and (iv) infusing the expanded transduced T cells into the subject having a tumor, whereby an anti-tumor T cell response is raised. The scFv has the amino acid sequence of SEQ ID NO: 3 and cells in the tumor express SSEA4.

The details of one or more embodiments of the invention are set forth in the description and drawings below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The description below refers to the accompanying drawings, of which:

Fig. 1 is a bar graph of percent lysis of target cells by the indicated effector T cells at different effector to target ratios;

Fig. 2A is a bar graph showing the amount of IL-2 released by the indicated effector T cells after coculturing them with target MCF-7 cells at different effector to target ratios; and

Fig. 2B is a bar graph showing the amount of IFN- γ released by the indicated effector T cells after coculturing them with target MCF-7 cells at different effector to target ratios.

DETAILED DESCRIPTION

As mentioned above, to meet the need to develop CAR-based tumor therapies, an isolated nucleic acid is provided that includes a nucleotide sequence encoding the polypeptide of SEQ ID NO: 3. The polypeptide of SEQ ID NO: 3 is an scFv that specifically binds to SSEA4. In a particular example, the isolated nucleic acid has the nucleotide sequence of SEQ ID NO: 1.

Also within the scope of the invention is a recombinant cell that contains the isolated nucleic acid having the nucleotide sequence of SEQ ID NO: 1. The recombinant cell expresses the polypeptide of SEQ ID NO: 2, i.e., a CAR construct that includes the scFv of SEQ ID NO: 3. The recombinant cell can be a T cell, e.g., a CD4⁺ or CD8⁺ T cell. Other cells that can be used include NK, iNKT, monocytes, macrophages, microglia, dendritic cells, and neutrophils.

The isolated nucleic acid that includes a nucleotide sequence encoding the polypeptide of SEQ ID NO: 3, e.g., a CAR construct, can be contained within a viral vector.

Exemplary viral vectors include a lentiviral vector, a gamma-retroviral vector, and an adeno-associated viral vector. Viral vectors based on lentivirus or gamma retroviruses are set forth in Dai et al. 2016, J. Natl. Cancer Inst. 108:1-14 (“Dai et al.”); Jin et al. 2016, EMBO Mol. Med. 8:702-711; Liechtenstein et al. 2013, Cancers 5:815-837; and Schonfeld et al. 2015, Mol. Therapy 23:330-338. Such viral vectors are used for integrating the CAR-encoding nucleic acid into T cell genomic DNA to produce stable expression of the CAR.

In a particular example, the viral vector is a lentiviral vector that includes the nucleotide sequence of SEQ ID NO: 1.

Alternatively, the CAR construct can be included in a vector that contains sequences to facilitate transposon-mediated genomic integration into T cells of the CAR-encoding nucleic acid, e.g., SEQ ID NO: 1. Examples of these expression vectors are the so-called “PiggyBac” and “Sleeping Beauty” expression vectors. See Nakazawa et al. 2011, Mol. Ther. 19:2133-2143 and Maiti et al. 2013, J. Immunotherapy 36:112-123.

In yet another alternative, a vector containing the CAR construct also contains genomic nucleic acid sequences flanking the CAR construct that allow for clustered regularly interspaced short palindromic repeat (CRISPR)-mediated insertion of the

CAR construct into the genome of the T cells. Examples of CRISPR constructs for inserting the CAR into the genome can be found, e.g., in Miura et al. 2018, Nature Protocols 13:195-215 and He et al. 2016, Nucl. Acids Res. 44:1-14.

Further disclosed is an isolated polypeptide containing the sequence of SEQ ID NO: 3. The isolated polypeptide, an scFv, specifically binds to SSEA-4.

Additionally provided is a CAR that includes an scFv that specifically binds to stage-specific embryonic antigen 4. The scFv can have the sequence of SEQ ID NO: 3. The CAR further includes a first endodomain from CD3 ζ or Fc ϵ RI γ . In an exemplary CAR, the first endodomain is from CD3 ζ

The CAR can also contain a second endodomain. The second endodomain can be, but is not limited to, an endodomain from CD28, CD137, CD4, OX40, and ICOS. If a second endodomain is present in the CAR, the scFv is fused to the second endodomain and the second endodomain is fused to the first endodomain. A particular example of a CAR has a second endodomain from CD137. In another specific example, the CAR has the amino acid sequence of SEQ ID NO: 4.

As mentioned above, a tumor-treating method is provided including, among others, the step of obtaining T cells from a subject having a tumor and the step of transducing the T cells *in vitro* with a vector that contains a nucleic acid encoding a CAR including a scFv that specifically recognizes SSEA4.

Procedures for obtaining T cells are known in the art. See, e.g., Kaiser et al. 2015, Cancer Gene Therapy 22:72-78 ("Kaiser et al."). The T cells can be CD4⁺, CD8⁺, or NK cells. In an exemplary method, CD8⁺ cells are obtained from the subject.

The T cells are transduced *in vitro* with the CAR vector described above. Transduction of T cells can be performed by electroporation, lipofection, lentiviral infection, gamma retrovirus infection, or adeno-associated virus infection, depending upon the type of CAR vector employed.

More specifically, if the CAR vector is a PiggyBac, Sleeping Beauty, or CRISPR-based expression vector, it can be transduced into the T cells via electroporation or lipofection. A CRISPR-base expression vector is co-transfected with a vector that expresses a guide RNA complementary to a sequence adjacent to a protospacer adjacent motif at an intended genomic insertion site in the T cells.

If the CAR vector is viral-based, virus particles are prepared and used to infect T cells.

The tumor treatment method also includes the step of expanding the transduced T cells *in vitro* and the step of infusing the expanded transduced T cells into the subject having a tumor.

Transduced T cells are expanded *in vitro*, using methods known in the art. See
5 Kaiser et al. The expanded T cells are then infused in one batch or in two or more batches into the subject having a tumor.

In a specific alternative of the tumor-treating method, the method further includes a preconditioning step that is performed prior to the just-mentioned infusion step. The preconditioning step is accomplished by treating the subject with a drug
10 that induces lymphodepletion. Examples of these drugs include cyclophosphamide and fludarabine. Additional drug examples can be found in Dai et al. and Han et al. 2013, J. Hematol. Oncol. 6:47-53.

In the tumor-treating method, the transduced T cells can further express the polypeptide of SEQ ID NO: 5, i.e., an epidermal growth factor receptor t domain III-
15 IV (EGFRt), in addition to the CAR. In this way, the infused expanded T cells can be deleted *in vivo* with an anti-epidermal growth factor receptor antibody that binds to EGFRt. For example, cetuximab is administered to the subject to kill infused T cells *in vivo*. An exemplary nucleic acid that encodes the CAR together with EGFRt has the nucleic acid sequence of SEQ ID NO: 1.

20 The method set forth above can be used for treating a tumor that contains cells expressing SSEA4. The tumors that can be treated include, but are not limited to breast, colon, gastrointestinal, kidney, lung, liver, ovarian, pancreatic, rectal, stomach, testicular, thymic, cervical, prostate, bladder, skin, nasopharyngeal, esophageal, oral, head and neck, bone, cartilage, muscle, lymph node, bone marrow, and brain tumors.

25 Without further elaboration, it is believed that one skilled in the art can, based on the disclosure herein, utilize the present disclosure to its fullest extent. The following specific examples are, therefore, to be construed as merely descriptive, and not limitative of the remainder of the disclosure in any way whatsoever. All publications and patent documents cited herein are incorporated by
30 reference in their entirety.

EXAMPLES

Example 1: Production of a lentivirus containing an anti-SSEA4 CAR construct

Construction of lentiviral vector encoding an anti-SSEA4 CAR

A lentiviral construct was prepared in *E. coli* using standard recombinant
 5 DNA techniques and verified by DNA sequencing. More specifically, a nucleic acid
 encoding an scFv having the sequence of SEQ ID NO: 3 was cloned into a lentiviral
 plasmid vector downstream of an EF-1 alpha promoter and a signal peptide encoding
 sequence and upstream of a CD8 hinge-encoding sequence to create a CAR cassette.
 The CAR cassette also encodes a CD8 transmembrane domain, a CD137 intracellular
 10 signaling domain, a CD3 ζ endodomain, a *Thosea asigna* self-cleaving peptide T2A,
 and an EGFRt domain III-IV. The CAR cassette has the nucleic acid sequence of
 SEQ ID NO: 1. The lentiviral plasmid vector contains additional sequences to
 facilitate production of lentivirus particles.

Lentivirus packaging and production

15 Packaging and production of lentiviruses was performed using established
 techniques. Packaging cells, i.e., 293T cells, were plated at 5×10^6 cells in 10 mL of
 a complete culture medium in a 10 cm culture dish. The cells were incubated
 overnight at 37°C in 5% CO₂. A transfection complex was prepared by combining in
 PBS a transfection reagent, the lentiviral vector described above, a packaging vector,
 20 and an envelope vector. The transfection complex was added to the culture dish
 containing the packaging cells and the cells incubated for 6 to 8 h at 37°C in 5% CO₂.
 The medium was replaced and the cells incubated for 24 h. The culture medium was
 collected and replaced with fresh medium. This 24 h incubation and medium
 collection was repeated twice. All of the collected medium was combined and passed
 25 through a 0.45 μ m filter. The filtrate was centrifuged at 50,000 x g for 2 h to pellet
 the lentivirus particles. Lentiviral stocks were suspended in PBS and stored at -80°C.

Lentivirus titration

Lentivirus titers were determined by measuring the amount of lentiviral DNA
 integrated into the genome of infected cells. 293T cells were plated in 24-well plates
 30 at a density of 50,000 cells/well and incubated overnight. Concentrated lentivirus
 stocks were added to each well together with polybrene to a concentration of
 6 μ g/mL. The plate was centrifuged briefly and then placed in an incubator at 37°C

with 5% CO₂ for 72 hours. Genomic DNA from the lentivirus-transduced cells was extracted with a commercial kit.

Real-time quantitative PCR (RT-QPCR) was used to determine the copy number of lentiviral DNA present in the extracted genomic DNA. The albumin gene was also measured to normalize the results. The primers and probes used for RT-QPCR are shown in Table 1 below.

Table 1. RT-QPCR primers and probes

Primer	Sequence (fluorescent labels)	SEQ ID NO.
LTR F ^a	TGACAGCCGCCTAGCATTTTC	6
LTR R ^a	GCTCGATATCAGCAGTTCTTGAAG	7
LTR Probe ^a	CACGTGGCCCGAGAGCTGCATC (5'-FAM-BHQ1-3')	8
ALB F ^b	GCTGTCATCTCTTGTGGGCTGT	9
ALB R ^b	ACTCATGGGAGCTGCTGGTTC	10
ALB Probe ^b	CCTGTCATGCCCACACAAATCTCTCC (5'-FAM-BHQ1-3')	11

^a LTR = long terminal repeat. These primers specifically amplify lentiviral sequences.

^b ALB = albumin. These primers specifically amplify the albumin gene

Standard curves were constructed by amplifying known amounts of plasmid DNAs carrying the albumin or LTR gene sequences using the RT-QPCR primers described above. The copy number of lentiviral DNA in the genomic DNA was calculated as the ratio of the quantity of LTR sequences divided by the quantity of albumin sequences.

The lentivirus titer was then calculated using the following formula:

$$\text{Lentivirus titer} = \frac{\text{number of cells plated} \times \text{number of copies of lentivirus per cell}}{\text{volume of lentivirus stock added}}$$

An exemplary lentivirus preparation contained 2.6×10^8 transducing units/mL

Example 2: Preparation of anti-SSEA4 CAR T cells

T cells expressing anti-SSEA4 CAR were produced using established techniques. First, peripheral blood mononuclear cells (PBMC) were isolated from whole blood with standard blood separation tubes and the cells re-suspended in complete culture medium. T cells were isolated from the PBMC using a standard magnetic bead separation technique.

The isolated T cells were dispensed into a tissue culture plate and growth media supplemented with 200 IU/mL IL2, 10 ng/mL IL7, 5 ng/mL IL15, and 5 ng/mL IL21 was added such that the cell density was 0.5×10^6 to 1×10^6 cells/mL. The plate was incubated at 37 °C in 5% CO₂ for 3 days. A lentivirus preparation produced as described above in Example 1 was added to the T cells, and polybrene was also added to a final concentration of 6 µg/ml. The plate was centrifuged at 800 x g for 1 hour at room temperature, and then incubated for 5 days at 37 °C in 5% CO₂. During the 5 day incubation, the T cells were maintained at a cell density of 0.5×10^6 cells/mL. The percentage of T cells expressing the anti-SSEA4 CAR was determined by fluorescence-activated cell sorting using an antibody against the EGFR domain III-IV.

In an exemplary preparation, 45.7% of T cells expressed the anti-SSEA4 CAR.

Example 3: Lysis of MCF-7 target cells by anti-SSEA4 CAR T effector cells

The ability of anti-SSEA4 CAR T cells to lyse target cells was assessed by a co-culture assay. MCF-7 breast cancer cells, which express SSEA-4, were used as the target cells. 100 µL of MCF-7 target cells at 5×10^5 cells/mL were transferred into each well of a 96-well plate and cultured overnight at 37°C in 5% CO₂. Effector cells, i.e., anti-SSEA4 CAR T cells, untransduced T cells, and T cells transduced with a negative control lentivirus, were each suspended in serum-free RPMI1640 medium. The culture medium from the 96 well plate was removed and the target cells washed once with PBS. T cells were added into separate wells at effector to target (E/T) ratios of 1:1, 2:1, 5:1 and 10:1. The final volume of medium in each well was adjusted to 100 µL/well using RPMI1640. The co-culture was incubated for 6 h at 37°C in 5% CO₂.

A commercial kit (CytoTox 96® non-radioactive cytotoxicity assay; Promega, WI USA) was used to measure lysis of the target MCF-7 cells by determining the level of lactate dehydrogenase (LDH) released from these cells upon lysis. After co-culturing, the 96-well plate was centrifuged at 1200 x g for 5 min. at room temperature, and 50µL of supernatant from each well was transferred to a new 96 well plate. The LDH level in each supernatant was determined as directed by the manufacturer. Certain wells containing only target cells were treated with a lysis buffer before the centrifugation step. The supernatants from these wells were used to

determine the maximum amount of LDH released by the MCF-7 cells. The results are presented in Fig. 1.

The data shows that anti-SSEA4 CAR-T cells lysed significantly more target MCF-7 cells at all E/T ratios, as compared to untransduced T cells and empty
5 lentivirus transduced T cells.

Example 4: Cytokine release by anti-SSEA4 CAR-T cells

The CAR-T cells described above were co-cultured with target cell line MCF7 in 96-well plates at different E/T ratio for 24 hours in RPMI1640 medium supplemented with 10% FBS in 5% CO₂ at 37°C. Culture media was harvested to
10 measure cytokine release by the CAR-T cells. Briefly, the 96-well plate was centrifuged at 1200 x g for 5 min. at room temperature, after which 50 µL of supernatant from each well was transferred into a new 96 well plate. The concentration of cytokines IL-2 and IFN-γ was determined using a commercial ELISA kit according to the manufacturer's instructions. The results are shown in
15 Figs 2A and 2B. The data shows that SSEA4-specific CAR-T cells robustly secreted both IL-2 and IFN-γ after engaging target tumor cells and this secretion level was significantly greater than either untransduced T cells or T cells transduced with a lentivirus lacking the CAR construct.

OTHER EMBODIMENTS

20 All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

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

What is claimed is:

1. An isolated polypeptide comprising the sequence of SEQ ID NO: 3, wherein the isolated polypeptide specifically binds to stage-specific embryonic antigen 4.
2. A chimeric antigen receptor, comprising a single chain Fv (scFv) that specifically binds to stage-specific embryonic antigen 4, and a first endodomain from CD3 ζ or Fc ϵ RI γ , wherein the scFv has the sequence of SEQ ID NO: 3.
3. The chimeric antigen receptor of claim 8, further comprising a second endodomain from CD28, CD137, CD4, OX40, or ICOS, wherein the scFv is fused to the second endodomain and the second endodomain is fused to the first endodomain.
4. The chimeric antigen receptor of claim 9, wherein the chimeric antigen receptor has the sequence of SEQ ID NO: 4.
5. The chimeric antigen receptor of claim 3, further comprising an epidermal growth factor receptor t domain III-IV at its C-terminus.
6. The chimeric antigen receptor of claim 5, wherein the chimeric antigen receptor has the sequence of SEQ ID NO: 2.