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Compositions and methods for improving immunotherapy

Abstract

This disclosure provides compositions and methods for improving immunotherapy, specifically against diseases like HIV or lymphoma that manifest within B cell follicles.

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Background/Summary

CROSS REFERENCE TO RELATED APPLICATIONS (1) This application claims the benefit of priority under 35 U.S.C. § 119(e) to U.S. Application No. 62/514,438 filed Jun. 2, 2017.

TECHNICAL FIELD

(1) This disclosure generally relates to compositions and methods for improving immunotherapy.

BACKGROUND

(2) An estimated 37 million people are currently infected with HIV-1 including more than 1 million in the United States. Last year, approximately 2 million people became infected with HIV and 1.2 million people died of AIDS. No effective vaccine has yet been developed for HIV. This is largely due to the high mutation rate of the virus. At this time, antiretroviral medications are necessary to suppress HIV-1 replication and prevent disease progression. However, antiretroviral medications must be taken lifelong and on a daily basis to be effective. These drugs are costly, frequently induce unpleasant or toxic side effects, must be taken with strict adherence to be effective, and are often rendered ineffective due to the development of drug resistance by the virus. These drugs are also not a cure. When taken off drugs, virus levels rebound and disease progression resumes, sometimes at an increased rate. The estimated cost of treating an HIV infected individual over their lifetime is estimated to approach $500,000. Thus, there is an urgent need to develop better treatment options and a cure for HIV.

SUMMARY

(3) Compositions and methods for improving immunotherapy are described herein. For example, kits are provided that include vectors encoding a chimeric antigen receptor (CAR) construct and a CXCR5 polypeptide. The kits also can include any or all of the reagents needed to transduce cells. In addition, methods are provided that include transducing patient cells with a vector as described herein, followed by infusion back into the patient.

(4) In one aspect, a CAR construct is provided that further includes a nucleic acid molecule encoding a CXCR5 polypeptide. In one embodiment, the CXCR5 polypeptide has the sequence shown in SEQ ID NO: 1 or 3. In one embodiment, the nucleic acid molecule encoding the CXCR5 polypeptide has the nucleic acid sequence shown in SEQ ID NO: 2 or 4. In some embodiments, the CAR construct is an HIV-based CAR construct. In some embodiments, the HIV-based CAR construct has the sequence shown in SEQ ID NO: 6, 8 or 10. In some embodiments, the HIV-based CAR construct is encoded by the nucleic acid sequence shown in SEQ ID NO: 7, 9 or 11.

(5) In another aspect, a vector including a CAR construct and a nucleic acid molecule encoding a CXCR5 polypeptide is provided. In some embodiments, the vector is a lentiviral vector.

(6) In still another aspect, a kit including a vector is provided. Such a vector typically includes a CAR construct and a nucleic acid molecule encoding a CXCR5 polypeptide. In some embodiments, a kit can further include reagents for transducing cells (e.g., patient T cells).

(7) In yet another aspect, a method of inhibiting replication of HIV in cells is provided. Such a method typically includes providing cells from a subject having HIV; transducing the cells with a vector comprising a CAR construct and a nucleic acid molecule encoding a CXCR5 polypeptide; and culturing the cells. In some embodiments, the method can further include monitoring the cells for the presence of HIV In some embodiments, the method can further include monitoring the cells for the amount of HIV (particle number).

(8) In one aspect, a method of (functionally) curing HIV in a subject is provided. Such a method typically includes infusing cells into the subject, wherein the cells are autologous to the subject and comprise a vector, and wherein the vector comprises a CAR construct and a nucleic acid molecule encoding a CXCR5 polypeptide. In some embodiments, the method can further include monitoring the subject for the presence of HIV. In some embodiments, the method can further include

monitoring the subject for the amount of HIV (viral load).
(9) In some embodiments, any of the methods described herein can further include obtaining the cells from the subject. In some embodiments, any of the methods described herein can further include transducing the cells with the vector.
(10) Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods and compositions of matter belong. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the methods and compositions of matter, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

Description

DESCRIPTION OF DRAWINGS
(1) Part A—Preliminary Results
(2) FIG. **1**A and FIG. **1**B are schematics showing a representative genetic CAR construct composed of a targeting region consisting of an extracellular region of CD4 (domains 1+2) attached to the carbohydrate-recognition domain of mannose-binding lectin (MBL), followed by a transmembrane (TM) and intracellular signaling domains from CD28 and CD3 zeta chain. The CAR construct is followed by an internal ribosome entry site for translation of a second gene, the ZSGreen reporter (FIG. **1**A) or the IRES-driven CXCR5 (FIG. **1**).
(3) FIG. **2**A and FIG. **2**B show the successful co-expression of an anti-HIV CAR and human CXCR5 in human PBMC. FIG. **2**A are graphs showing the viability of the transduced cells (based on the mean fluorescent intensity of a live/dead cell stain that stains dead cells). FIG. **2**B shows the transduction efficiency, with transduced cells detected using antibodies against MBL (for the CAR construct) and CXCR5. The CAR construct also expresses the ZSGreen reporter (not shown).
(4) FIG. **3**A and FIG. **3**B show the successful co-expression of an anti-SIV CAR and rhesus CXCR5 in rhesus PBMC. FIG. **3**A are graphs showing the viability of the transduced cells (based on mean fluorescent intensity of a live/dead cell stain). FIG. **3**B shows the transduction efficiency, with transduced cells detected using antibodies against MBL (for the CAR construct) and CXCR5. The CAR construct also induced expression of ZSGreen (not shown).
(5) FIG. **4**A-FIG. **4**E shows that CXCR5 induced migration was detected in an ex vivo B cell follicle migration assay. Rhesus macaque PBMCs were transduced with CAR or CAR/CXCR5 vectors, labeled with CellTrace Violet (CTV), and layered onto freshly cut slabs of rhesus macaque lymph nodes. Panels A and B show tissues stained with anti-CD20 antibodies to delineate B cell follicles (“F”) and anti-CD3 antibodies to delineate the T cell zone and extra-follicular areas (“EF”). Panel C shows that similar total levels of CFSE-labeled cells were detected in tissues treated with CAR or CAR/CXCR5 transduced cells. Panel D shows that, while CAR-transduced cells showed similar levels inside and outside of follicles, CAR/CXCR5-transduced cells primarily located within B cell follicles. Panel E shows that CAR/CXCR5-transduced cells exhibited much higher F:EF ratios compared to CAR-transduced cells.
(6) Part B—Simian Immunodeficiency Virus (SIV)-Specific Chimeric Antigen Receptor-T Cells Engineered to Target B Cell Follicles and Suppress SIV Replication
(7) FIG. **5**A is a schematic of the construct design, and expression in rhesus macaque T cells. Schematics showing constructs encoding the chimeric antigen receptor (CAR) (rhCD4-MBL CAR), rhCXCR5, and the bi-cistronic CAR/CXCR5 encoding both proteins, with CXCR5 expression mediated by IRES or P2A. In all cases, the targeting domains are linked to domains from rhesus CD28 including a short extracellular hinge, transmembrane TM, and cytoplasmic

signaling, followed by the CD3 activation domain.
(8) FIG. **5**B-FIG. **5**F are graphs of flow cytometry of cells transduced with gamma-retroviral vectors encoding (Panel B) CAR; (Panel C) CXCR5; (Panel D) CAR/CXCR5 (IRES); (Panel E) CAR/CXCR5 (P2A); or (Panel F) mock-transduced. Cells were pre-gated sequentially on lymphocytes, singlets, live cells, and CD3+ cells (T cells) and evaluated for CAR and CXCR5 expression using antibodies against mannose binding lectin (MBL) and CXCR5, respectively.
(9) FIG. **5**G is a histogram depicting fluorescent intensities of CXCR5 expression from samples shown in Panels D-F.
(10) FIG. **5**H is a graph of the median percentage of T cells that expressed the CAR and CXCR5 in activated PBMCs transduced with CAR (n=3), CXCR5 (n=3), CAR/CXCR5 (IRES) (n=5), CAR/CXCR5 (P2A) (n=5) and mock-transduced (n=5).
(11) FIG. **5**I shows the percentage of T cells that co-expressed the CAR and CXCR5 in activated PBMCs transduced with either CAR/CXCR5 (IRES) or CAR/CXCR5 (P2A).
(12) FIG. **6**A-FIG. **6**C are graphs showing that co-expression of CXCR5 promotes selective migration of chimeric antigen receptor (CAR)-T cells toward CXCL13 in vitro. The percentage of CAR- or CAR/CXCR5-transduced PBMC that migrated toward (FIG. **6**A) CXCL12 (SDF-1) or (FIG. **6**B) CXCL13 was measured in transwell plates. FIG. **6**C shows the relationship between the percentage of cells expressing CXCR5 and the percentage of cells that migrated. In all panels, each data point symbol represents the mean value of duplicate samples obtained with cells from individual animals, with colors indicating transduction with CAR (blue) or CAR/CXCR5 (red).
(13) FIG. **7**A are images showing that CXCR5 co-expression enhances CD8+ CAR-T cell migration to B cell follicles ex vivo. FIG. **7**A shows chimeric antigen receptor (CAR; left) or CAR/CXCR5-transduced (right) rhesus macaque CD8+ T cells stained with cell trace violet dye (CTV) (pseudo-colored yellow), pipetted on to fresh rhesus macaque lymph node sections and incubated for 6 h at 37° C. Sections were then fixed and stained with anti-CD20 antibodies (green) to delineate B cell follicles (“F”) and anti-CD3 antibodies (blue) to delineate the T cell zone and extrafollicular areas (“EF”). Arrowheads indicate CTV+ cells. Confocal images were collected with a 20× objective. Scale bars equal 100 μm.
(14) FIG. **7**B-FIG. **7**F shows the experimental data demonstrating that CXCR5 co-expression enhances CD8+ CAR-T cell migration to B cell follicles ex vivo. Panel B shows similar total levels of CFSE-labeled CD8+ T cells were detected in tissues incubated with CAR- and CAR/CXCR5-transduced cells. Panel C demonstrates that CAR-transduced cells showed higher levels in the extrafollicular regions than in the follicles. Panel D demonstrates that, by contrast, CAR/CXCR5-transduced cells showed increased levels within B cell follicles. Panel E demonstrates that CAR/CXCR5-transduced cells showed higher F:EF ratios compared to CAR-transduced cells. Panel F shows the relationship between the percentage of transduced cells that expressed CXCR5 and F:EF ratios. Each symbol represents individual animals from which CD8+ T cells were derived.
(15) FIG. **8** are graphs showing that chimeric antigen receptor (CAR)/CXCR5-transduced T cells suppress simian immunodeficiency virus (SIV) in vitro. PBMC target cells were infected with the indicated SIVmac239 (top row) and SIVE660 (bottom row) isolates for 24 h. The cells were then washed and mixed with the effector cells transduced as indicated, at effector-to-target ratios (E:T) of 1:1 (left panels) or 0.2:1 (right panels). Culture supernatants were collected on the indicated days, and the presence of virus was determined by p27 ELISA. The effector cells were transduced either with the CD4-mannose-binding lectin (MBL)-CAR alone or CD4-MBL-CAR plus CXCR5. As negative controls, no effector cells, or cells transduced with the negative control 139 CAR were used.
(16) Part C—Nine Day Transduction and Expansion Protocol
(17) FIG. **9** shows flow cytometric analysis of Rhesus macaque PBMCs transduced and expanded in 9 days sorted on live, singlet, CD3 T cells co-labeled with MBL antibodies (binds CD4-MBL-

CAR; x-axis) and CXCR5 antibodies (y-axis).
(18) FIG. **10** shows that the 9-day protocol described herein yields sufficient cell numbers for cellular immunotherapy.
(19) FIG. **11** shows that co-expression was maintained after the 9-day protocol described herein.
(20) FIG. **12** shows that viability was maintained after the 9-day protocol described herein.
(21) FIG. **13** showing representative result central memory T cells (TCM) maintained after 9 day transduction and expansion. Phenotype definitions: CM defined as CD95+, CD28+, CCR7+; EM1 defined as CD95+, CD28+, CCR7−; EM2 defined as CD95+, CD28−, CCR7−.
(22) FIG. **14** shows CTV-labeled CD4-CD3-CAR/CXCR5-transduced PBMCs (pseudo-colored red and indicated with arrow heads) inside B cell follicles of spleen (left) and LN (middle) but not in lung (right). Sections were stained with either IgM antibodies or anti-CD20 antibodies to label B cells and identify follicles (F).

DETAILED DESCRIPTION

(23) The methods described herein improve existing immunotherapy, particularly immunotherapy directed to B cells or B cell follicles. The methods described herein can be applied to any type of disease that includes B cell follicles, i.e., any type of disease in which the immunotherapy needs to reach B cell follicles, such as B cell lymphomas and other viral infections that include B cell follicles such as human immunodeficiency virus (HIV) or herpes viruses (e.g., HHV8, which causes Kaposi's sarcoma, or EBV, which can result in lymphomas).
(24) The methods described herein are expected to result in a functional cure for HIV. As used herein, functional cure refers to suppressed viral replication in the absence of antiretroviral drugs. In some instances, functional cure refers to non-detectable levels of virus in the absence of antiretroviral drugs. Accordingly, the methods described herein can replace antiretroviral drug therapy to treat HIV The methods described herein are expected to be substantially cheaper and have fewer side effects than antiretroviral drug therapy, and are expected to replace the need for conventional drugs. As used herein, HIV refers to both HIV type 1 (HIV-1) and HIV type 2 (HIV-2).
(25) During HIV infection, virus-producing cells accumulate inside B cell follicles within lymphoid tissues; the same is true in the simian immunodeficiency virus (SIV)-infected rhesus macaque model of HIV B cell follicles have been shown to be, at least to some extent, immune-privileged sites, in that CD8 T cells are largely excluded from B cell follicles, which thereby permit ongoing viral replication (Connick et al., 2007, J. Immunol., 178(11):6975-83)). The immune-privileged status of B cell follicles can explain the inability of HIV- and SIV-specific CD8 T cells to fully suppress virus replication.
(26) Chimeric Antigen Receptor (CAR) T cell therapy is a type of immunotherapy that changes a subject's own T cells so they are better able to recognize and attack cancer cells or pathogens. It is demonstrated herein that engineering CAR-expressing CD8 T cells to also express CXCR5 (e.g., referred to herein as CAR/CXCR5 T cells) results in a substantial increase in the effectiveness of those immunotherapeutic T cells, to increase migration into B cell follicles. It has been shown that increasing levels of SIV-specific CD8 T cells in follicles is associated with a decrease in virus-producing cells in follicles, and also a decrease in plasma viral load. The same phenomena would be expected to occur in humans with HIV.
(27) Thus, methods are provided that include collecting (e.g., obtaining, providing) cells from a subject having HIV, transducing those cells with both a CAR nucleic acid and a nucleic acid encoding a CXCR5 polypeptide, and returning those cells to the patient.
(28) Cells collected from a subject can be any number of cells including, without limitation, a population of peripheral blood mononuclear cells (PMBCs; e.g., CD8 T cells, CD4 T cells and a few NK cells) or T cells (e.g., CD8 T cells). In addition or as an alternative to T cells, the methods described herein can be applied to NK cells (e.g., autologous or allogeneic NK cells) and/or hematopoietic stem cells (HSCs). This method also could be applied to engineered T cells that are

not specific for a particular subject (e.g., universal T cells).
(29) Cells can be collected from a subject using known methods. Typically, cells are collected in a biological sample. Biological samples can include, without limitation, blood, serum, cerebrospinal fluid, or tissues (e.g., tissue biopsy, e.g., muscle biopsy, skin biopsy). In some instances, apheresis is used to obtain the desired cells from the blood of a subject, which then can be returned back to the subject.
(30) Following collection, cells can be transduced with one or more nucleic acid vectors or constructs (e.g., a CAR/CXCR5 construct, or a CAR construct and a construct encoding CXCR5). Methods of transducing cells are known in the art. For example, and without limitation, cells can be transduced with one or more nucleic acid molecules using, for example, mechanical means (e.g., electroporation), viral mechanisms (e.g., retroviruses carrying the CAR/CXCR5 construct), or gene editing (e.g., CRISPR or TALEN) (see, for example, Eyquem et al., 2017, Nature, 543:113-7).
(31) CAR constructs are known in the art, and typically include an antigen-binding targeting portion, sometimes referred to as a ligand-binding targeting portion, and a signaling domain that results in T cell activation following binding by the antigen. The signaling domain can be a transmembrane domain, a cytoplasmic domain, or an intracellular domain.
(32) CAR constructs and methods of making CAR constructs are known in the art. See, for example, Brentjens et al., 2010, Mol. Ther., 18:4:666-8; Till et al., 2008, Blood, 12:2261-71; Park et al., 2011, Trends Biotechnol., 29:550-7; Grupp et al., 2013, N. Engl. J. Med., 368:1509-18; Han et al., 2013, J. Hematol. Oncol., 6:47; Tumaini et al., 2013, Cytotherapy, 15:1406-17; Haso et al., 2013, Blood, 121:1165-74; WO 2012/079000; WO 2013/059593; US 2012/0213783; and U.S. Pat. No. 9,629,877, all of which are incorporated herein by reference in their entirety.
(33) Specifically with respect to HIV, there are a number of CAR constructs that are known in the art. For example, and without limitation, see Liu et al., 2015, J. Virology, 89(13):6685-94; Hale et al., 2017, Mol. Ther., 25(3):570-9; Zhen et al., 2015, Mol. Ther., 23(8):1358-67; U.S. Pat. Nos. 7,115,262; 7,151,087; and 8,420,099; US 2013/0189264; and US 2017/0267739, all of which are incorporated herein by reference in their entirety. A number of representative HIV CAR constructs are shown in SEQ ID NOs: 6, 8 and 10, which are encoded by the HIV CAR nucleic acid constructs shown in SEQ ID NOs: 7, 9 and 11, respectively. Representative HIV CAR constructs are described in more detail in WO 2015/077789.
(34) A CAR construct (e.g., an existing CAR construct) can be genetically engineered to also include a nucleic acid molecule encoding a CXCR5 polypeptide, or a new vector can be generated that includes the necessary components of a CAR construct as well as a nucleic acid molecule encoding a CXCR5 polypeptide. Representative human CXCR5 polypeptides are shown in SEQ ID NO:1 (i.e., GenBank Accession No. NP_001707.1) and SEQ ID NO:3 (i.e., GenBank Accession No. NP_116743.1), which are encoded by the nucleic acid sequences shown in SEQ ID NO:2 (i.e., GenBank Accession No. NM_001716.4) and SEQ ID NO:4 (i.e., GenBank Accession No. NM_032966.2).
(35) In some instances, it may be desirable to codon optimize the CXCR5 nucleic acid sequence and/or the CAR construct. Codon optimization is known in the art and can be performed using, for example, computer software. A representative computer software for codon optimizing a sequence can be obtained from IDT (see, for example, idtdna.com/CodonOpt on the World Wide Web), but others are available. An example of a codon optimized human CXCR5 sequence is shown in SEQ ID NO: 5.
(36) In some embodiments, a nucleic acid encoding a further polypeptide can be delivered to the T cells (e.g., CD4 T cells) such that the T cells are made resistant to infection by HIV See, for example, Hale et al. (2017, Mol. Ther., 25:570); and Younan et al. (2013, Blood, 122:179-87). A representative further polypeptide that can impart resistance to HIV infection by T cells is the mC46 polypeptide. A further polypeptide, whether it be to impart resistance to infection by HIV or for another purpose, can be included in the CAR/CXCR5 construct (e.g., under one of the existing

promoters or under a different promoter) or, if CAR and CXCR5 are delivering on separate constructs, the further polypeptide can be included in one or the other or both of those constructs. Alternately, a further polypeptide can be included and delivered to the cells in its own construct.
(37) Such a construct (e.g., CAR/CXCR5) or constructs (e.g., a CAR and a CXCR5) can be contained within a host cell or a virion for propagation outside of T cells and/or for maintenance in the T cells.
(38) Methods of culturing and expanding the transduced cells are known in the art. In some instances, it may be desirable to include, without limitation, IL-15 or ALT-803, an IL-15 superagonist (NantKwest) in the culture. In some instances, it may be desirable to switch the cytokines that are present in the media during the culturing of the cells, which may lead to increased persistence of cells and fewer regulatory T cells (Treg cells). For example, various combinations of IL-15, IL-7, and/or IL-21, with or without IL-2, can be used in the culture media.
(39) Following transduction of the patients' cells and appropriate culturing and expansion of the transduced cells, those cells are returned back to the same patient. Returning cells to a patient is oftentimes performed via infusion. Infusion is known in the art, and typically includes suspending the cells in a pharmaceutically acceptable carrier and introduced the composition back into the subject intravenously. Pharmaceutically acceptable carriers are known in the art and include, without limitation, saline or other physiological buffers, nutrients, preservatives, and the like.
(40) In some instances, it may be desirable to lymphodeplete the subject prior to cellular immunotherapy to create space for the homeostatic proliferation of the infused cells. For example, lymphodepletion can be achieved using a chemotherapeutic such as cyclophosphamide (e.g., Cytoxan) or, alternatively, an anti-CD20 antibody (e.g., Rituximab), which can temporarily deplete B cells and B cell follicles (i.e., the virus-producing cells).
(41) As demonstrated herein, those T cells (e.g., CAR/CXCR5 T cells) migrate to B cell follicles and are able to significantly suppress the replication of HIV. As a part of the methods described herein, a subject can be monitored for the presence and/or amount (e.g., viral load) of HIV. In addition, because of the precise targeting provided by CXCR5, subjects receiving a CAR/CXCR5 construct as described herein would not require intravenous immunoglobulin (IVIG) treatments, as is needed for B cell depleting CARS like CD19-specific CARs, as they would not remain immunocompromised. For example, subjects (e.g., virally suppressed subjects on cART or infected subjects who have not received cART) can be infused with CAR/CXCR5 T cells (e.g., autologous CAR/CXCR5 T cells) or CAR/CXCR5 NK cells (e.g., autologous or allogeneic CAR/CXCR5 NK cells) as described herein. Upon receiving the CAR/CXCR5 T cells or CAR/CXCR5 NK cells, the subject typically will no longer receive antiviral therapy. The autologous CXCR5/CAR T cells, or autologous or allogeneic CAR/CXCR5 NK cells, home to lymphoid B cell follicles, where HIV-producing cells are most concentrated, and suppress viral replication. In many subjects, immunotherapy, which is significantly improved by the presence of the CXCR5 polypeptide described herein, can lead to a functional cure of HIV
(42) In accordance with the present invention, there may be employed conventional molecular biology, microbiology, biochemical, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. The invention will be further described in the following examples, which do not limit the scope of the methods and compositions of matter described in the claims.
EXAMPLES
(43) Part A—Preliminary Results
Example 1-CAR/CXCR5 Vectors and Transduced Cells
(44) An HIV-1-specific CAR has been developed (Liu et al., 2015, J. Virology, 89(13):6685-94). In the field, CAR constructs have been generated in both a lentiviral vector background as well as a mouse gamma retroviral vector background. These vectors have been constructed with an internal ribosome entry site (IRES) to drive expression of CXCR5 with the CAR or with a protease

cleavage site from which co-expressed proteins get cleaved by cellular proteases after expression. See, for example, Vagner et al., 2001, EMBO Reports, 2:893-8; Chinnasamy et al., 2006, Virology J., 3:14.

(45) Virus binding is primarily mediated by interaction of gp120, the external subunit of the HIV envelope glycoprotein (Env) with CD4 protein and various coreceptor molecules (one of several alternative chemokine receptors). These interactions then activate the gp41 transmembrane subunit of the envelope glycoprotein, to cause fusion between the virus and cell membranes.

(46) Human CXCR5 (SEQ ID NO:2) was sub-cloned into the plasmid vectors containing the HIV-specific CAR, and rhesus CXCR5 (encoding the polypeptide shown in GenBank Accession No. XP_001100017) was sub-cloned into the vectors containing the SIV-specific CAR to generate HIV- and SIV-based CXCR5/CAR vectors, respectively. FIG. **1** is a schematic of two of these vectors; FIG. **1**A shows a SIV-based CAR vector (Rh CD4 MBL2 CAR), while FIG. **1**B shows a SIV-based CXCR5/CAR vector (Rh CD4 MBL2 CAR+CXCR5). The plasmid vectors were verified by sequencing, used to generate pseudotyped retroviral vectors, and the virions were produced and titrated.

(47) Primary CD8 T cells from humans and rhesus macaques then were transduced with each vector (or a CAR-only vector). Transduction efficiency was determined by staining transduced (and mock-transduced) cells with antibodies directed against CXCR5 and CAR. The viability of transduced cells was determined using a live-dead cell stain. FIG. **2** shows the co-expression of human CAR and CXCR5 in human PBMC, and FIG. **3** shows the co-expression of corresponding rhesus proteins in rhesus PBMC.

Example 2—Efficacy of CAR/CXCR5 Vector Transduced Cells

(48) A comprehensive multicolor flow cytometric analysis of CXCR5-transduced cells is performed to determine the percentage and viability of transduced cells, as well as CD8 naïve, regulatory, memory, and memory stem cell subsets. Such flow cytometry strategies are well-established (Sallusto et al., 2004, Annu. Rev. Immunol., 22:745-63).

(49) Efficacy of the CAR/CXCR5 T cells can be demonstrated by showing that the CAR/CXCR5-transduced T cells 1) migrate towards the ligand for CXCR5, CXCL13; 2) migrate to B cell follicles in tissues processed in the lab; and/or 3) migrate to B cell follicles in SIV-infected rhesus macaques.

(50) The ability of transduced cells to migrate towards CXCL13 is assessed using a transwell chemotaxis assay as previously described (Meditz et al., 2008, AIDS Res. Hum. Retrovir., 24(7):977-85).

(51) Migration of CAR/CXCR5 transduced cells into B cell follicles was also demonstrated in an ex vivo migration assay. Here, rhesus macaque PBMCs were transduced with CAR or CAR/CXCR5 vectors, labeled with CTV, and dripped onto slabs of fresh rhesus macaque lymph nodes. Tissues were subsequently stained with anti-CD20 antibodies to delineate B cell follicles (“F”) and anti-CD3 antibodies to delineate the T cell zone and extra-follicular areas (“EF”) (FIGS. **4**A and **4**B). Similar total levels of CTV-labeled cells were detected in tissues treated with CAR and CAR/CXCR5 transduced cells (FIG. **4**C). CAR-transduced cells showed similar levels in follicles and outside of follicles, whereas CAR/CXCR5-transduced cells primarily located within B cell follicles (FIG. **4**D). CAR/CXCR5-transduced cells showed much higher F:EF ratios compared to CAR-transduced cells. Thus, these experiments demonstrated that CXCR5 induced migration in an ex vivo B cell follicle migration assay.

(52) Example 3—Treatment of SIV-Infected Rhesus Macaque Chronically SIVmac239- or SIVmac251 infected rhesus macaque are treated with CXCR5/CAR transduced cells. CXCR5/CAR T cells are labeled with a live cell stain, such as CFSE or CTV, prior to infusion so that the cells can be tracked. CXCR5/CAR T cells, CD4 and CD8 T cell counts, and viral loads in blood are evaluated before treatment and, following treatment, are monitored over time. In addition to blood PBMC, CXCR5/CAR cells are tracked and quantified in lymphoid tissues (lymph node, spleen,

GALT), and bronchoalveolar lavage (BAL) at multiple time points using flow cytometry (using CFSE+CD8+CXCR5+CD4+MBL+). On day 60 following the infusion, the animal is sacrificed and a necropsy performed in which multiple tissues are collected and examined. The tissues that are collected include lymph nodes (inguinal, axillary, iliac, mesenteric), spleen, gut associated lymphoid tissues, liver, brain (obex) and lung.

(53) In tissues, transduced cells are visualized in situ using CFSE and immunostaining with antibodies (e.g., the antibodies described above for use in flow cytometry) in combination with anti-CD20 to define B cell follicles morphologically as previously described (Connick et al., 2007, J. Immunol., 178)11):6975-83). In addition, anti-FITC antibodies may be used to amplify weak signal from CFSE-labeled cells that have gone through multiple cell divisions. As a secondary means to track transduced cells in tissues, in situ hybridization with a probe specific for the retroviral vector, combined with anti-CD20 staining, can be performed as described (Connick et al., 2007, J. Immunol., 178)11):6975-83). Similarly, SIV producing cells are quantified in tissues using in situ hybridization as described (Connick et al., 2007, J. Immunol., 178)11):6975-83).

(54) Part B—Simian Immunodeficiency Virus (SIV)-Specific Chimeric Antigen Receptor-T Cells Engineered to Target B Cell Follicles and Suppress SIV Replication

Example 4—Plasmid Constructs and Retroviral Vectors Encoding CARs

(55) All CAR targeting motifs were synthesized by GenScript, codon-optimized for expression in rhesus macaque cells, and sub-cloned into the plasmid pMSGV1 gamma-retrovirus vector backbone (Hughes et al., 2005, Hum. Gene Ther., 16:457-72). The active antiviral CAR employed in this study was a rhesus variant of the human bispecific CAR designated CD4-MBL (Ghanem et al., 2017, Cytother., doi:10.1016/j.jcyt.2017.11.001). As a non-reactive negative control, the previously described 139 CAR was used, which does not react with cells in this system. The targeting domains were linked to extracellular hinge, transmembrane and cytoplasmic co-stimulatory domain of rhesus CD8 followed by the activation domain of rhesus CD3 zeta, as previously described (Liu et al., 2015, J. Virol., 89:6685-94; Ghanem et al., 2017, Cytother., doi:10.1016/j.jcyt.2017.11.001).

(56) T cells were transduced to express either the rhCD4-MBL CAR, rhCXCR5, or the rhCD4-MBL CAR plus rhCXCR5. For co-expression, bi-cistronic plasmid constructs (produced by GenScript) were designed in which the rhCD4-MBL gene was linked to the downstream rhCXCR5 gene. CXCR5 expression was driven by either the ECMV internal ribosome entry site (IRES) or the self-cleaving P2A peptide from porcine teschovirus-1 with a GSG linker added at the N-terminus of the P2A peptide sequence (Kim et al., 2011, PLoS One, 6:e18556). The corresponding gamma-retroviruses were generated for expression of these genes in rhesus macaque T cells. In most experiments, these plasmids were co-transfected with the plasmid pBS-CMV-gagpol (Delang et al., 2016, Sci. Rep., 6:31819) (a gift from Dr. Patrick Salmon, Addgene plasmid #35614), a plasmid encoding RD114 envelope glycoprotein (Porter et al., 1996, Hum. Gene Ther., 7:913-9), and the plasmid pMD.G encoding VSV-G envelope (Gori et al., 2007, J. Pharmacol. Exp. Ther., 322:989-97) (a gift from Dr. Scott McIvor) at ratios of 3:1:1:0.4, respectively. Retroviral vector supernatants were collected 48 h after transfection, and were titrated by transducing HEK293T cells. Retrovirus was snap frozen and stored at −80° C. In the SIV suppression studies, gamma-retrovirus vector production was carried out as previously described (Liu et al., 2015, J. Virol., 89:6685-94).

Example 5—Transduction of Rhesus T Cells

(57) Primary rhesus macaque PBMC, or CD8+ T cells enriched by negative selection (Miltenyi), were activated for 2 to 3 days in six-well plates with plate-bound anti-CD3 (FN18) and soluble anti-CD28.2 (both from NUP Reagent Resource) in either RPMI supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 300 IU/ml IL-2, for early experiments, or in X-Vivo 15 completed with 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 IU/ml IL-2 for later experiments. RetroNectin

(TaKaRa)-mediated transduction was carried out on the activated T cells. Retroviral vector supernatants, diluted in serum-free media, were added (eventual MOI of 0.5) to RetroNectin-coated six-well plates and centrifuged for 2 h at 2,000×g to facilitate binding of the retrovirus. After removal of the unbound retrovirus, activated PBMC or CD8+ T cells (1.5×10E6 cells/well) were added to the wells and centrifuged at 1,000×g for 10 min. Mock-transduced cells were subjected to exactly the same procedures without the addition of retrovirus to the RetroNectin-coated wells. Cells were cultivated in the media listed above for 5-6 days prior to analysis by flow cytometry.

Example 6—Flow Cytometry

(58) Cells were analyzed using an LSR Fortessa flow cytometer (BD Bioscience). The following antibodies were used: CD4 (M-T477, reactive with endogenous rhCD4 and the rhCD4-MBL CAR), CD3 (SP34-2), CD8 (RPA-T8) (all from BD Bioscience), CXCR5 (MU5UBEE) (eBioscience), MBL2 (3E7) (Invitrogen). Viability was assessed with the Live/Dead Fixable Near IR Dead Cell Stain Kit (Invitrogen). A minimum of 70,000 events were acquired for each sample. Data analysis utilized FlowJo v10 (FlowJo, LLC).

Example 7—In Vitro Transwell Migration Assay

(59) Rhesus macaque PBMCs were transduced with the CAR or CAR/CXCR5 vectors, or mock-transduced. Samples were run in duplicate. For each sample, one million cells in 100 μl X-Vivo-15 media containing 0.1% BSA were placed in the upper chamber of a 24-well plate, with a 5.0 μm transwell membrane (Costar). To the lower chamber containing 600 μl X-Vivo 15 and 0.1% BSA, either CXCL12 at 1 μg/ml or CXCL13 at 2.5 μg/ml (both from ProSpec) were added. No chemokine was added to control wells. After incubation for 4 h at 37° C., cells were collected from the lower chamber, fixed with 1% paraformaldehyde, and counted on a Cytoflex flow cytometer (Beckman). All samples were normalized with the addition of AccuCheck Counting Beads (Invitrogen). Specific cell migration was determined by first subtracting the number of cells that migrated to media alone from the number of cells that migrated to the chemokine and then dividing by the number of cells added to the upper chamber.

Example 8—Ex Vivo B Cell Follicle Migration Assay

(60) Chimeric antigen receptor- and CAR/CXCR5-transduced rhesus CD8+ T cells were used in conjunction with fresh lymph node tissue sections from allogeneic rhesus macaques. A gelatin sponge (7 mm Gel foam by Pfizer) was cut to fit and placed into a six-well plate containing 3-4 ml of RPMI with 20% heat inactivated FBS. The sponge was hydrated for 1 h at 37° C. Fresh rhesus macaque lymph nodes, collected at the Wisconsin National Primate Research Center, were shipped in chilled RPMI containing 100 μg/ml heparin overnight on ice blocks. Lymph nodes were cut into 0.5 cm×0.5 cm pieces and embedded in 40° C. PBS-buffered 4% low-melt agarose and cut into 300-μm thick slices using a Compresstome, as previously described (Abdelaal et al., 2015, Biol. Proced. Online, 17:2). Tissue sections and associated agarose were laid flat on the hydrated sponge without being submerged. Transduced CD8+ T cells were stained with a 5-μM solution of Cell Trace Violet Dye (CTV) (Molecular Probes). The dye was added at a 1:1 ratio to 1×10E7 cells/ml suspended in PBS/10% FBS, and cells were incubated for 15 min at 37° C., followed by two washes with complete RPMI supplemented with 10% heat inactivated FBS 100 U/ml penicillin, and 100 μg/ml streptomycin. For each fresh tissue section, one million CTV-stained transduced CD8+ T cells were re-suspended in 20-30 μl complete RPMI and were slowly pipetted onto the surface of the tissue. Tissue sections were incubated at 37° C. for 6 h followed fixation with 4% PBS-buffered paraformaldehyde for 2 hours at room temp. After fixation, sections were washed with chilled PBS containing 100 μg/ml heparin (PBS-H). Antigen retrieval was carried out by boiling tissues 3× in 0.01 M urea for 30 s. Tissues were permeabilized and blocked with PBS-H containing 0.3% Triton x-100 and 2% normal goat serum for 1 h, then incubated overnight with mouse-anti-human CD20 (0.19 μg/ml, clone L26, Novocastra) to label B cells and rat-anti-human CD3 (2 μg/ml, CD3-12, Bio-Rad) to label T cells. After washing with PBS-H, secondary antibody staining was carried out by incubating tissues overnight with goat-anti-mouse-IgG/Alexa 488 (0.75

μg/ml Jackson ImmunoResearch Laboratories) and goat-anti-rat-IgG/Cy5 (0.3 μg/ml, Jackson ImmunoResearch Laboratories). All incubations were done at 4° C. on a rocking platform. Sections were imaged using a Leica confocal microscope. 512×512 pixel z-series were collected using a step size of 2 μm and with collection initiated at least 50 μm deep into each section. B cell follicles were identified morphologically as clusters of brightly stained closely aggregated CD20+ cells. Areas that showed loosely aggregated B cells that were ambiguous as to whether the area was a follicle were not included. Cell counts were done with individual z-scans. The total number of CTV-stained cells was counted inside follicles and the adjacent area outside of the follicles. For each sample, 2-3 tissue sections and a minimum of three follicles (range 3-8) were evaluated.

Example 9-SIV Suppression Assay

(61) To generate SIV-infected target cells, rhesus macaque PBMCs were re-suspended at 5×10E5/ml in complete medium, transferred to a T25 flask, and incubated at 37° C. in 5% CO2 for 2-3 days. The PBMCs were washed, adjusted to 3×10E6/ml in total of 4 ml volume in complete media containing 30 IU/ml IL-2, and incubated with 200-600 TCID50/ml of virus for 24 h at 37° C. in 5% CO2. Infected cells were washed three times using 20 ml of medium per wash and then re-suspended in complete medium at a density of 1.5×10E6 cells per ml in 96-well round bottom plates. To generate effector cells, T cells (derived from activated PBMCs) were transduced with the indicated gamma-retroviral vectors. In triplicate, 100 μl of SIV-infected targets were mixed with 100 μl of serially diluted effectors. Co-cultures were incubated at 37° C. in 5% CO2 for a total of 16 days. On the indicated days, supernatants were collected, and p27 content was determined by ELISA (ABL, Inc.).

Example 10—Statistical Analysis

(62) All statistical analyses assumed two-sided tests with $P<0.05$ considered significant. Paired t-tests with pooled variance were used to evaluate co-expression levels of the CAR and CXCR5 via IRES versus P2A constructs. An unpaired t-test with pooled variance was used to evaluate groups in the CXCL12 in vitro migration assays while an unpaired Welch's t-test of unequal variance was used to evaluate groups in the CXCL13 in vitro migration assays. Paired t-tests with pooled variance were used in all statistical analyses in the ex vivo migration assay. The F:EF ratios were log transformed before analysis. Statistical analyses were conducted using GraphPad Prism (Version 6.01; GraphPad Software, Inc., La Jolla, CA, USA).

Example 11—Results

(63) The goal of this work was to engineer rhesus macaque T cells to co-express a potent anti-SIV CAR along with CXCR5, in order to promote CAR-T cell trafficking to B cell follicles. To this end, constructs for expression of the CARs were designed, with or without co-expression of CXCR5. CAR-transduced T cells were analyzed using both an in vitro trans-well assay of chemokine-directed cell migration and a novel ex vivo B cell follicle migration assay. In addition, the ability of T cells expressing CAR and CXCR5 to suppress viral replication was tested in vitro.

Example 12-CAR and CXCR5 Expression in Transduced Primary Rhesus Macaque T Cells

(64) Gamma-retroviral vectors encoding the rhCD4-MBL CAR and rhCXCR5 constructs were developed, and vectors encoding bicistronic constructs to express both proteins were developed. Two variations of bicistronic vectors were developed, one with CXCR5 co-expression driven by an internal ribosome entry site (IRES) and the other via a P2A self-cleavage site (Kim et al., 2011, PLoS One, 6:e18556). For simplicity, the constructs encoding the rhCD4-MBL CAR alone or the bicistronic rhCD4-MBL CAR plus rhCXCR5 are, respectively, referred to as CAR or CAR/CXCR5; for the latter, the use of either the IRES or P2A modalities is indicated. The constructs are shown schematically in FIG. **5**A.

(65) In FIGS. **5**B-**5**F, T cells derived from activated rhesus PBMCs were transduced with gamma-retroviral vectors encoding the CAR or CXCR5 genes alone, or the bi-cistronic CAR/CXCR5 constructs (IRES or P2A). Cell viabilities post-transduction were 87-90% (data not shown). Antibodies directed against MBL or CXCR5 were used to detect surface expression of the CAR

and CXCR5, respectively. Transduction with the CAR (FIG. **5**B) or CXCR5 (FIG. **5**C) vectors gave the expected surface expression of the corresponding individual proteins. For vectors encoding the bi-cistronic CAR/CXCR5, the P2A-based construct yielded a clear population of cells expressing both CAR and CXCR5, with only a small fraction of cells expressing only one of the proteins; by contrast. The IRES-based construct appeared less effective at co-expressing CXCR5 relative to CAR, since the fraction of cells expressing only the CAR was comparable to that expressing both proteins, with a minimal fraction expressing only CXCR5 only (FIGS. **5**D, **5**E). These results are consistent with the efficient P2A system producing equivalent amounts of the two post-cleavage components of a bi-cistronic construct, as contrasted with the relatively inefficient expression of the downstream component in the IRES system (Mizuguchi et al., 2000, Mol. Ther., 1:37-82; Ibrahimi et al., 2009, Hum. Gene Ther., 20:845-60). Moreover, as indicated in FIG. **5**G, the P2A-based construct produced cells with nearly twofold higher surface expression levels of CXCR5 than obtained with the IRES-based construct (median 1.8-fold higher; range 1.4- to 2.2-fold).

(66) The percentages of T cells that expressed the CAR and CXCR5 with each construct are shown in FIG. **1**H. Transduction with the vectors encoding CAR-only or CXCR5-only yielded a median of 44.4% (range 40-47.6%) and 81.1% (range 51.8-84.2%) of cells expressing each protein, respectively. Cells transduced with the IRES-based bi-cistronic CAR/CXCR5 vector showed higher number of cells expressing the CAR compared to CXCR5, with a median cell expression of 37.2% (range 5.6-50.2%) for the CAR and 19.4% (range 4.7-19.7%) for CXCR5. In contrast, cells transduced with the P2A-based CAR/CXCR5 vector showed similar expression of the two proteins, with a median of 40.2% (range 37.3-52.2%) for the CAR and 38.9% (range 27.5-50.2%) for CXCR5. Similar transduction efficiencies were found with enriched rhesus CD8 T cells transduced with these vectors (data not shown). The percentage of cells that co-expressed CAR and CXCR5 is shown in FIG. **5**I. Cells transduced with the IRES-based construct showed a median co-expression efficiency of 18.1% (range 4.3-18.9%), whereas cells transduced with the P2A-based construct resulted in a significantly higher co-expression efficiency of 33.3% (range of 25.9-38.3). Thus, the data in FIG. **5** establish the suitability of the P2A-based bi-cistronic system for efficient co-expression of CAR and the B cell follicle-homing chemokine receptor CXCR5, and its superiority over the IRES-based system.

Example 13—CXCR5 Co-Expression Promotes CAR-T Cell Migration Selectively to CXCL13 In Vitro

(67) The ability of CXCR5 co-expression to promote migration of CAR-T cells toward CXCL13, the chemokine ligand for CXCR5, was next tested. To this end, an in vitro transwell migration assay was utilized. Using this assay, it was found that both CAR-transduced and CAR/CXCR5-transduced PBMCs similarly migrated toward a positive control chemokine CXCL12 (SDF-1 alpha) that is strongly chemotactic for lymphocytes (Bleul et al., 1996, 184:1101-9), demonstrating the ability of both CAR and CAR/CXCR5-transduced cells to migrate to a chemotactic stimulus (FIG. **6**A). In contrast, significantly more CAR/CXCR5-transduced than CAR-transduced PBMCs migrated toward CXCL13 (FIG. **6**B). Furthermore, increasing specific migration to CXCL13 was seen with an increase in the percentage of cells expressing CXCR5 (FIG. **6**C). For these studies, a median of 54% (range 12-64%) of CAR/CXCR5-transduced cells expressed CXCR5. By contrast, a median of only 2% (range 1-5%) of the CAR-transduced cells expressed CXCR5 and they showed minimal migration to the stimulus. These results demonstrate that co-expression of CXCR5 promotes selective migration of the CAR-T cells toward CXCL13 in vitro.

Example 14—CAR/CXCR5-Transduced CD8+ T Cells Selectively Migrate into B Cell Follicles Ex Vivo

(68) As an additional means to evaluate the ability of CXCR5 to promote selective migration of CAR-T cells, a novel ex vivo B cell follicle migration assay was developed. This method was adapted from previously described ex vivo live tissue migration assays that tracked T cells in

mouse thymus tissue using two-photon microscopy (Melichar et al., 2013, Sci. Signal, 6:ra92; Dzhagalov et al., 2013, PLoS Biol, 11:e1001566). For these studies, the migration of CTV-labeled CAR- and CAR/CXCR5-transduced primary rhesus macaque CD8+ T cells were evaluated in fresh lymph node tissue sections. FIG. **7**A shows representative images of sections incubated with CTV-labeled CAR and CAR/CXCR5-transduced cells. Similar levels of total CTV+ cells were detected in lymph node sections incubated with CAR versus CAR/CXCR5-transduced cells (FIG. **7**B). While total numbers of cells were similar, significant differences were observed in the levels of CAR- compared to CAR/CXCR5-transduced cells in follicular and extrafollicular compartments. Significantly lower levels of CTV+ cells were found in follicular compared to extrafollicular areas in sections incubated with CAR-transduced cells (FIG. **7**C). In contrast, significantly higher levels of CTV+ cells were found in follicular compared to extrafollicular areas in sections incubated with CAR/CXCR5-transduced cells (FIG. **7**D). As a result, significantly large increases in the follicular to extrafollicular ratios (F:EF) of CTV-labeled cells were detected in the tissue sections incubated with CAR/CXCR5- compared to CAR-transduced T cells. Sections incubated with CAR/CXCR5-transduced cells showed a median F:EF ratio of 2.8 (range of 1.5-6.9), whereas sections incubated with CAR-transduced T cells showed a median ratio of 0.4 (range 0.3-0.7) (FIG. **7**E). An increased follicular to extrafollicular ratio was seen with an increase in the percentage of cells expressing CXCR5 (FIG. **7**F). A median of 46% (range 23-71%) of CAR/CXCR5-transduced cells expressed CXCR5 and showed relatively high F:EF ratios. By contrast, a median of only 1.6% (range 0.2-4.1%) of the CAR-transduced cells expressed CXCR5 and showed correspondingly low F:EF ratios. Thus, in this novel ex vivo B cell follicle migration assay, CAR/CXCR5- but not CAR-transduced CD8+ T cells preferentially migrated to B cell follicles.

Example 15-CXCR5 Co-Expression Does Not Impair CAR-T Cell-Mediated Suppression of SIV Replication In Vitro

(69) The all-rhesus CD4-MBL CAR (rhCD4-MBL) displayed potent suppression of multiple SIV strains. For this study, it was examined whether co-expression of CXCR5 affected the potency of SIV suppression by T cells expressing the rhesus CD4-MBL CAR. PBMCs transduced with the CAR or CAR/CXCR5 vectors, were co-cultured with rhesus PBMC targets infected with two different pathogenic SIV isolates, SIVmac239 and SIVE660. The negative controls employed included adding no effector T cells, and adding effector T cells that were transduced with the 139 CAR that recognizes an irrelevant epitope (a glioma-specific variant of the epidermal growth factor receptor (Morgan et al., 2012, Hum. Gene Ther., 23:1043-53)). Robust spreading of viral infection by both SIV strains was evident in the presence of the negative control effector cells (no effector T cells and 139 CAR-transduced T cells). In contrast, CAR-transduced and CAR/CXCR5-transduced effectors suppressed infection by both strains with equivalent high potency over the 12-day infection, at E:T ratios of 1:1 or 0.2:1 (FIG. **8**). These data demonstrate that the antiviral activity of CAR-T cells is not altered by co-expression of the CXCR5 follicular trafficking chemokine receptor on the effector cell surface.

(70) Part C—Nine Day Transduction and Expansion Protocol

Example 16—Retroviral Vector Production

(71) Gamma-retroviruses were generated for expression of CAR and/or CXCR5 in rhesus macaque T cells. Lipofectamine 2000 (Invitrogen)-mediated transfection was carried out using CAR, CXCR5 or CAR P2A CXCR5 pMSGV plasmids as well as the plasmid pBS-CMV-gagpol (a gift from Dr. Patrick Salmon, Addgene plasmid #35614), a plasmid encoding RD114 envelope glycoprotein, and the plasmid pMD.G encoding VSV-G envelope (a gift from Dr. Scott McIvor) at ratios of 3:1:1:0.4, respectively. Retroviral vector supernatants were collected 48 hours after transfection and centrifuged to remove debris.

(72) Lentivirus preparations were generated using pCL20cSLFR MSCV transfer plasmid as well as pCAG-SIVgprre (gag/pol), pMDG (VSV-G envelope) and pCAG4-RTR-SIV (tat/rev) at a ratio of 1:0.6:0.2:0.2 using lipofectamine-mediated transfection. Media was exchanged after 18 hr. Viral

supernatants were collected 48 hr later and centrifuged to remove debris. All virus preparations were snap frozen and stored at −80° C. Functional titer was determined by transducing HEK293T cells with the virus.

Example 17—Activation and Transduction of Rhesus T Cells

(73) Primary rhesus macaque PBMC were activated for 2 days in 6 well plates with plate-bound anti-CD3 (FN18) and soluble anti-CD28.2 (both from NHP Reagent Resource) in X-Vivo 15 completed with 10% heat inactivated FBS, 100 U/ml penicillin, 100 g/ml streptomycin, 2 mM glutamine, and 50 IU/ml IL-2. For PBMC activated with CD3 and CD28 antibodies, the population becomes highly enriched for T cells. RetroNectin (TaKaRa)-mediated transduction was carried out on the activated T cells with the gamma-retrovirus at an MOI of 0.5. After coating 6 well plates with Retronectin, retroviral vector supernatants, diluted in serum-free media, were added and centrifuged for 2 hours at 2000×g to facilitate binding of the retrovirus. After removal of the unbound retrovirus, activated PBMC (1.5×10E6 cells/well) were added to the retrovirus-coated wells and centrifuged at 1000×g for 10 minutes. Mock-transduced cells were subjected to exactly the same procedures without the addition of retrovirus to the RetroNectin-coated wells. Cells were cultivated for 2 days prior to analysis by flow cytometry.

Example 18—Expansion of Transduced Rhesus T Cells

(74) Two days post transduction, cells were seeded in a G-Rex 6 well plate (Wilson Wolf) at a density of 1×10E6 cells/cm.sup.2 in 30 ml of X-Vivo 15 completed with 10% heat inactivated FBS, 100 U/ml penicillin, 100 g/ml streptomycin, 2 mM glutamine, and 50 M beta-mercaptoethanol, and 50 IU/ml IL-2. Cells were grown undisturbed for 4 days before collection and analysis by flow cytometry.

Example 19-CTV Staining of Transduced Cells

(75) Cells were stained with the Cell Trace Violet Cell Proliferation kit (Invitrogen). Briefly, cells were placed in aliquots of 60×10E6 cells suspended in PBS containing 10% FBS. Cell Trace Violet, diluted in PBS, was added to a final concentration of 5.5 M and cells were incubated for 15 min at 37° C. The reaction was stopped with a 5 min incubation in 5 times the reaction volume of complete media. Cells were centrifuged and re-suspended in complete medium and allowed to rest for 10 minutes before use.

Example 20 Flow Cytometry

(76) Cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter). For the 6 color standard panel, the following antibodies were used: CD4 (M-T477, reactive with endogenous rhCD4 and the rhCD4-MBL CAR), CD3 (SP34-2), CD8 (RPA-T8) (all from BD Bioscience), CXCR5 (MUSUBEE) (eBioscience), MBL2 (3E7) (Invitrogen). Viability was assessed with the Live/Dead Fixable Near IR Dead Cell Stain Kit (Invitrogen). For the 11 color panel, the following antibodies were used in addition to CTV and the Live/Dead stain: CD4 (M-T477), CD3 (SP34-2), CD8 (RPA-T8), CCR7 (150503), CD28 (CD28.2), CD95(DX2) (all from BD Bioscience), CXCR5 (MUSUBEE) (eBioscience), MBL2 (3E7) (Invitrogen), CD159a (Z199) (Beckman Coulter). A minimum of 70,000 events were acquired for each sample. Data analysis utilized FlowJo v10 (FlowJo, LLC).

Example 21—Results

(77) Methods have been developed that allow transduction (e.g., for CAR and CXCR5 co-expression) and expansion of cells in 9 days while maintaining viability and ˜30% central memory T cells (TCM) (less differentiated memory cells). Most other methods of transducing and expanding cells take weeks, so the methods described herein represent a significant improvement in time without any apparent loss of quality. Rhesus macaque CD4-MBL-CAR/CXCR5 T cells were used herein, but the methods could be applied to cells from other species using similar but species-specific vectors.

(78) Rhesus macaque PBMCs were transduced with CAR and CXCR5 and expanded in 9 days. Flow cytometric analysis of cells sorted on live, singlet, CD3 T cells co-labeled with MBL

antibodies, which bind CD4-MBL-CAR, and CXCR5 antibodies is shown in FIG. **9**.
(79) The 9-day procedure described herein yields sufficient cell numbers for cellular immunotherapy (FIG. **10**). This procedure was used to yield 10E8 cells/kg for treatment of an SIV-infected macaque. These experiments also demonstrated that co-expression (FIG. **11**) and viability (FIG. **12**) was maintained after expansion.
(80) Central memory T cells (TCM) were maintained after 9 day transduction and expansion (FIG. **13**). CD4-MBL-CAR/CXCR5 transduced cells were produced with the 9 day protocol. Briefly, rhesus PBMC were stimulated with anti CD3/anti CD28 beads or with plate bound anti CD3 and soluble anti CD28 for two days, subjected to one round of transduction with CAR/CXCR5 retrovirus and then expanded for 4 days in the GREX plate in 50 U/ml IL-2, 50 μM beta mercaptoethanol. Cells were cryopreserved on d9, thawed and used immediately in flow cytometry. Gating strategy: Lymphocytes, singlets, live cells, CD3+, CD8+ for total cells or lymphocytes, singlets, live cells, CD3+, MBL+, CD8+ for transduced cells. Phenotype definitions: CM defined as CD95+, CD28+, CCR7+; EM1 defined as CD95+, CD28+, CCR7−; EM2 defined as CD95+, CD28−, CCR7−.
(81) An SIV infected animal was treated with CD4-MBL-CAR/CXCR5 T cells labeled with a live cell stain (CTV) and the animal was sacrificed two days later and the localization of the labeled cells evaluated. CTV-labeled cells were detected in spleen, lymph nodes, liver, and very few in lung. In the spleen and lymph nodes, CTV-labeled cells were detected in B cell follicles. Clusters of CTV+ cells were detected in the T cell zones, likely proliferating (a site that T cells normally undergo expansion). These data also indicate that 9 day transduction and expansion protocol described herein does not lead to transduced cells accumulating in the lung, which is an ongoing problem.
(82) FIG. **14** shows that CTV-labeled CD4-CD3-CAR/CXCR5-transduced PBMCs (pseudo-colored red and indicated with arrow heads) were located inside B cell follicles of spleen (left) and LN (middle) but not in lung (right). Sections were stained with either IgM antibodies or anti-CD20 antibodies to label B cells and identify follicles (F).
(83) It is to be understood that, while the methods and compositions of matter have been described herein in conjunction with a number of different aspects, the foregoing description of the various aspects is intended to illustrate and not limit the scope of the methods and compositions of matter. Other aspects, advantages, and modifications are within the scope of the following claims.
(84) Disclosed are methods and compositions that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that combinations, subsets, interactions, groups, etc. of these methods and compositions are disclosed. That is, while specific reference to each various individual and collective combinations and permutations of these compositions and methods may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular composition of matter or a particular method is disclosed and discussed and a number of compositions or methods are discussed, each and every combination and permutation of the compositions and the methods are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed.

Claims

1. A method of preparing a cellular composition for suppressing replication of HIV in B cell follicles in a subject having HIV, comprising: providing peripheral blood mononuclear cells from the subject having HIV; transducing the cells with a vector comprising a HIV-based CAR construct and a nucleic acid molecule encoding a CXCR5 polypeptide as shown in SEQ ID NO: 5; culturing the transduced cells, and infusing the cultured transduced cells into the subject, wherein the

cultured transduced cells migrate to B cell follicles and suppress replication of HIV in B cell follicles to reduce levels of HIV in the subject in the absence of anti-viral HIV drugs relative to HIV levels in the subject prior to the infusing step.

2. The method of claim 1, further comprising monitoring the subject for the presence of HIV.

3. The method of claim 1, further comprising monitoring the subject for the amount of HIV.

4. The method of claim 1, further comprising obtaining the cells from the subject using apheresis.

5. The method of claim 1, wherein the HIV-based CAR construct comprises the sequence shown in SEQ ID NO: 6, 8, or 10.

6. The method of claim 1, wherein the HIV-based CAR construct is encoded by a nucleic acid having the sequence shown in SEQ ID NO: 7, 9, or 11.

7. The method of claim 1, wherein the vector is a lentiviral vector or a gamma retroviral vector.

8. The method of claim 1, wherein the transducing step comprises gene editing.

9. The method of claim 8, wherein the gene editing comprises CRISPR or TALEN.

10. A method of suppressing replication of HIV in B cell follicles in a subject having HIV, comprising: providing peripheral blood mononuclear cells from the subject having HIV; transducing the cells with a vector comprising a HIV-based CAR construct and a nucleic acid molecule encoding a CXCR5 polypeptide as shown in SEQ ID NO: 5, wherein the HIV-based CAR construct comprises an antigen-binding targeting portion that recognizes gp120 and/or gp41; culturing the transduced cells, and infusing the cultured transduced cells into the subject, wherein the cultured transduced cells migrate to B cell follicles, thereby suppressing replication of HIV in B cell follicles to reduce levels of HIV in the subject in the absence of anti-viral HIV drugs relative to HIV levels in the subject prior to the infusing step.

11. A method of suppressing replication of HIV in B cell follicles in a subject having HIV, comprising: providing peripheral blood mononuclear cells from the subject having HIV; transducing the cells with a vector comprising a HIV-based CAR construct and a codon-optimized nucleic acid molecule encoding a CXCR5 polypeptide as shown in SEQ ID NO: 5; wherein the HIV-based CAR construct comprises an antigen-binding targeting portion that recognizes gp120 and/or gp41; and culturing the transduced cells, infusing the cultured transduced cells into the subject, wherein the cultured transduced cells migrate to B cell follicles, thereby suppressing replication of HIV in B cell follicles to reduce levels of HIV in the subject in the absence of anti-viral HIV drugs relative to HIV levels in the subject prior to the infusing step.