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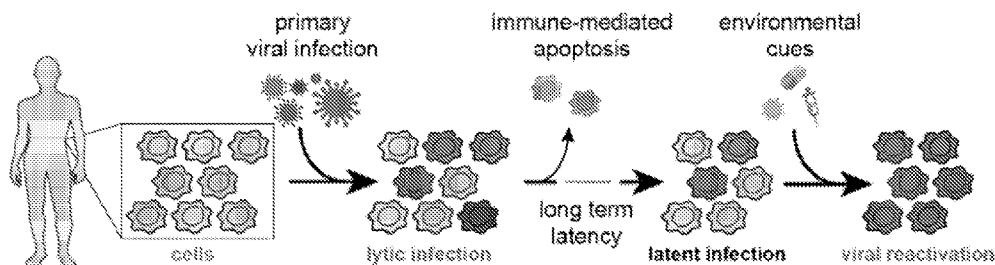


FIG. 1a

(57) Abstract: Provided are methods of monitoring a human subject receiving a T cell based therapy for HHV-6 reactivation in ther-
apeutic T cells present in the subject. In some embodiments, the methods comprise obtaining from the subject a biological sample
comprising the therapeutic T cells, and assessing the therapeutic T cells for HHV-6 reactivation. Also provided are methods comprising
assessing an *in vitro* culture comprising candidate therapeutic human T cells for HHV-6 reactivation, wherein the *in vitro* culture is
assessed for a level of one or more HHV-6 analytes by quantitative nucleic acid sequencing. The present disclosure further provides
non-transitory computer-readable media and computer devices that find use in practicing the methods of the present disclosure.

METHODS OF ASSESSING THERAPEUTIC T CELLS FOR LATENT AND REACTIVATED HUMAN HERPESVIRUS 6

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Patent Application No. 63/397,518, filed August 12, 2022, and U.S. Provisional Patent Application No. 63/397,515, filed August 12, 2022, which applications are incorporated herein by reference in their entireties.

INTRODUCTION

10 During the first years of life, humans become exposed to endemic pathogens that can often infect nearly 100% of the population. Though primary infection typically clears with sub-clinical symptoms, certain viruses that can infect humans, including viruses from the *Herpesviridae*, *Polyomaviridae*, *Adenoviridae*, and *Parvoviridae* families, can become 'quiescent,' resulting in a latent phase of the virus that is stably maintained in healthy individuals. However, under acute stress conditions, such as fever, hematopoietic stem cell transplant
15 (HSCT), or trauma, latent viruses can become reactivated, leading to a variety of complex clinical manifestations.

Cell therapies have yielded durable clinical benefits for patients with cancer but have been accompanied by unexpected side effects of treatment. There is a current lack of understanding of the mechanisms of toxicity observed in patients receiving cell therapies, including encephalitis
20 caused by human herpesvirus 6 (HHV-6).

SUMMARY

Provided are methods of monitoring a human subject receiving a T cell based therapy for HHV-6 reactivation in therapeutic T cells present in the subject. In some embodiments, the methods comprise obtaining from the subject a biological sample comprising the therapeutic T
25 cells, and assessing the therapeutic T cells for HHV-6 reactivation. Also provided are methods comprising assessing an *in vitro* culture comprising candidate therapeutic human T cells for HHV-6 reactivation, wherein the *in vitro* culture is assessed for a level of one or more HHV-6 analytes by quantitative nucleic acid sequencing. The present disclosure further provides non-transitory computer-readable media and computer devices that find use in practicing the methods of the
30 present disclosure.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A-1G: Petabase-scale analysis of viral nucleic acids reveals that HHV-6 is reactivated in human T cells. **1A:** Schematic of viral life cycles of endogenous human viruses. Viruses can enter a latent phase after primary infection and become reactivated based on

environmental cues. **1B**: Enumeration of BioSamples with human viruses capable of reactivation confidently expressed in human SRA samples. **1C**: Summary of BioSamples with viral expression in human T cells. Viruses shown in (b) but not (c) had no evidence of reactivation in T cells. **1D**: Reanalysis of Shytaj et al.¹⁸ RNA-seq data. CD4+ T cells from 3 separate donors were treated with either an HIV or mock infection and cultured for ~2 weeks. Shown is the % of RNA molecules aligning to the HHV-6B reference Serratus reference. **1E**: Reanalysis of the LaMere et al.¹⁷ data. Naive and memory CD4+ T cells were separated and cultured for two weeks. Shown is the % of RNA molecules aligning to the HHV-6B reference Serratus reference. **1F**: Quantification of HHV-6B episomal DNA from a reanalysis of ChIP-seq from LaMere et al.¹⁷ data. Shown are the % of DNA reads uniquely mapping to the HHV-6B transcriptome, noting the percent of total DNA reads mapping to the HHV-6B reference genome. **1G**: Coverage of the HHV-6B genome across two ChIP-seq libraries from different donors LaMere et al.¹⁷. Repetitive regions on the ends of the chromosome show no uniquely mapping reads.

FIG. 2A-2I: scRNA-seq identifies rare HHV-6 expressing cells in CAR T cell culture. **2A**: Longitudinal sampling of the HHV-6B U31 transcript CAR T cell culture from two donors. **2B**: Summary of HHV-6B qPCR expression at Day 19 for four donors normalized to the total cell count. Bars are sorted in increasing order of expression. **2C**: Schematic of single-cell sequencing workflow to detect HHV-6+ cells from the CAR T culture. Two models are presented that would explain HHV-6 reactivation: Model 1 (top) where cells all express HHV-6 transcripts or Model 2 (bottom) where only a subset of cells express HHV-6B. Both the host and HHV-6B viral RNA can be directly quantified using our 10x Genomics RNA-seq workflow. **2D**: Summary of HHV-6B expression from an individual donor (D98). The top 0.2% of cells contain 99% of the HHV-6B transcript UMIs from this experiment. **2E**: Tabulated summary of scRNA-seq profiling for four CAR T donors, including number of cells profiled, % expressing HHV-6, U31 transcript qPCR value, and number of shared TCR clones between the HHV-6B+ cells. **2F**: Extended longitudinal sampling of the HHV-6B U31 transcript CAR T cell culture for two donors. The fold increases from the first qPCR measurement (Day 21) and the last (Day 27) are noted for each donor. **2G**: Schematic and summary of HHV-6B expression in the D34 donor after 19 and 25 days, showing evidence of HHV-6B spreading in the culture as depicted in the schematic. **2H**: Correlation analyses of host factor gene expression with HHV-6B expression in individual cells. The per-gene correlation statistics are shown in black against a permutation of the HHV-6B expression in gray. Noteworthy genes are indicated. **2I**: Pathway enrichment analysis of GSEA/Msigdb Hallmark gene sets. A positive Normalized Enrichment Score (NES) corresponds to genes that are overexpressed in cells with high amounts of HHV-6B transcript.

FIG. 3A-3E: Clinically validated and FDA-approved CAR T cells harbor HHV-6 *in vivo*. **3A**: Schematic and summary of patient cohorts, noting different cell therapy products (axi-cel and tisa-cel) and two different timepoints for sampling (preinfusion product and 7 days post transfusion). The number of total cells analyzed and HHV-6+ cells detected are noted

underneath. **3B**: Summary heatmap of 7 HHV-6 positive cells from cohort 2 *ex vivo* follow up. Total HHV-6 expression is noted in blue boxes; non-zero host expression noted in green boxes (see **Methods**). **3C**: Heatmap of HHV-6B transcripts (columns) by same individual cells (rows, as in **3B**) grouped by viral gene class (immediate early; early; late expressing genes as previously described). **3D**: Summary of Axi-R-15 patient, noting 4 libraries of scRNA-seq (red dot) and the clinical period corresponding to altered mental status. Stars represent statistical significance of two-sided binomial tests (day 7 vs. day 0 $p = 0.00047$; day 7 vs. day 14 $p = 0.00032$). **3E**: Summary of SJCAR19-09 patients, including PCR of HHV-6 and T cell scRNA-seq abundance. Treatment regimen with Foscarnet is noted with initial dose administered at day +24.

FIG. 4A-4B: Characterization of OX40 expression in bulk sequencing experiments. **4A**: Expression of TNFRSF4 (OX40), the canonical receptor of HHV-6B in unstimulated and stimulated immune cell populations.⁴² OX40 is not expressed in unstimulated immune cells but highly expressed in CD4 and CD8 T cells after activation/stimulation of CD3/CD28 and IL-2. **4B**: Broad expression of OX40 across healthy tissues from the GTEx bulk atlas.

FIG. 5A-5B: Characterization of OX40 expression in resting and stimulated endothelial cells. **5A**: Refinement of HHV-6B expression using the single-cell GTEx atlas⁴⁰. **5B**: Pseudobulk expression of OX40 across the human fetal expression atlas⁴¹. Induction of OX40 expression on endothelial cell lines in the presence of TNF- α ; RNA-seq dataset from Richards *et al.*⁴³

FIG. 6A-6D: Supporting analyses for HHV-6 reactivation using Serratus. **6A**: Heatmap of HHV-6B transcripts across the four highest RNA-seq libraries from Serratus. Shown are the first 40 genes (based on genomic coordinate order) from the HHV-6B transcriptome and the number of reads that pseudoalign to each transcript. **6B**: Summary of naive CD4+ culture in the LaMere *et al.* dataset; compare to **FIG. 1E**. **6C**: Summary of HHV-6B expression in the Qu *et al* 2017 ATAC-seq atlas, showing sorted T cells from Patient 59, an individual with CTCL, had detectable levels of HHV-6B DNA within cells. **6D**: Smoothed coverage (rollmean of 500 base pairs) over the four libraries from Patient 59, indicating coverage across the HHV-6B reference genome.

FIG. 7A-7F: Supporting analyses for HHV-6 expression during in vitro CAR T cell culture. **7A**: Summary of observed (red) and permuted (gray) HHV-6B expression for four donors at day 19 in culture. Dotted line is 10 UMIs, the threshold for a super-expressor. **7B**: Heatmap of HHV-6B expression for selected cells across 3 donors with detectable super expressors. Columns are grouped based on HHV-6B gene programs (immediate early; early; late). **7C**: Uniform manifold approximation and projection for 3 samples, noting marker genes and HHV-6B UMI expression (log transformed). The WPRE feature indicates the presence of the CAR transgene. **7D**: Summary of HHV-6B +/- cells from differential testing for host factors. Arrows indicate lymphotoxin α (LT α /LTA) and downregulation of lymphotoxin β (LT β /LTB). **7E**: Summary of HHV-6B expression in re-cultured samples for donors D61 and D34. **7F**: Correlation statistics of HHV-6B transcript signatures with OX40 expression across 3 re-cultured samples, including p-values

from Pearson's product moment correlation coefficient. The consistently positive, significant correlation statistic represents an association uniquely between OX40 expression and the immediate early HHV-6B gene signature. From left to right in each of the four groups: D34 Day 25, D34 Day 27 and D61 Day 27.

5 **FIG. 8:** A flow diagram of a computer-implemented single cell RNA sequencing-based method for identifying individual human cells expressing HHV-6B RNA, according to embodiments of the present disclosure.

FIG. 9: A flow diagram of a second computer-implemented single cell RNA sequencing-based method for identifying individual human cells expressing HHV-6B RNA, according to
10 embodiments of the present disclosure.

FIG. 10: A flow diagram of a computer-implemented method for identifying a particular cell type susceptible to reactivation by a particular virus, according to embodiments of the present disclosure.

FIG. 11: Schematic of a re-culture experiment where CAR T cells were recovered from
15 the infusion product and then re-cultured with TransAct and IL7/15. Right: HHV-6B RNA counts per million (CPM) over five time points measured from scRNA-seq. HHV-6 was detected as soon as 3 days following re-culture and persisted for the full 14 days of additional culture.

FIG. 12A-12F: Mitigation of HHV-6 reactivation and spreading via foscarnet treatment *in vitro*. **12A:** Schematic of CAR T product re-culture experiment. Donor D97, which at day 19
20 showed a low but detectable level of HHV-6, was selected for re-culture for five days. **12B:** Summary of RT-qPCR at the control and two treatment levels of Foscarnet. Each dot represents a technical replicate. **12C:** Schematic of D34 re-culture +/- foscarnet at 1mM. **12D:** Difference between untreated and treated in the abundance of HHV-6+ cells. Comparing the two 10x Genomics scRNA-seq data channels, foscarnet-treated cells had a lower incidence of HHV-6
25 positive cells (OR=6.25; p=8.3e-122). **12E:** Reduced dimensionality analysis of treated and untreated D34 cells profiled with scRNA-seq. Host gene expression was used for the analysis, showing overlapping clustering of populations irrespective of treatment status. **12F:** Differential gene expression analysis comparing foscarnet treated and control CAR T cells. The three most significant differential genes are noted. 0 genes were differentially expressed with a minimum
30 log2 fold-change exceeding 1 (noted by the vertical bars).

DETAILED DESCRIPTION

Before the methods of the present disclosure are described in greater detail, it is to be understood that the methods are not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of
35 describing particular embodiments only, and is not intended to be limiting, since the scope of the methods will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the methods. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the methods, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the methods.

Certain ranges are presented herein with numerical values being preceded by the term “about.” The term “about” is used herein to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes. In determining whether a number is near to or approximately a specifically recited number, the near or approximating unrecited number may be a number which, in the context in which it is presented, provides the substantial equivalent of the specifically recited number.

Unless defined otherwise, 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 belong. Although any methods similar or equivalent to those described herein can also be used in the practice or testing of the methods, representative illustrative methods are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the materials and/or methods in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present methods are not entitled to antedate such publication, as the date of publication provided may be different from the actual publication date which may need to be independently confirmed.

It is noted that, as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation.

It is appreciated that certain features of the methods, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the methods, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments are specifically embraced by the present disclosure and are disclosed herein just as if each and every combination was individually and

explicitly disclosed, to the extent that such combinations embrace operable processes and/or compositions. In addition, all sub-combinations listed in the embodiments describing such variables are also specifically embraced by the present methods and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present methods. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

METHODS OF ASSESSING *IN VITRO* THERAPEUTIC T CELLS FOR LATENT AND REACTIVATED HHV-6

Aspects of the present disclosure include methods of assessing *in vitro* candidate therapeutic human T cells for latent and reactivated Human Herpesvirus 6 (HHV-6), e.g., HHV-6B. The methods are based in part on the surprising findings, demonstrated herein, that HHV-6 can become reactivated in human T cells in standard *in vitro* cultures, as well as the identification of a rare population of HHV-6 “super-expressor” cells that possess high viral transcriptional activity in chimeric antigen receptor (CAR) T cell culture that may spread rapidly to infect other cells *in vitro*. As will be appreciated with the benefit of the present disclosure, the methods find use in a variety of contexts, including the context of screening candidate therapeutic human T cells for latent and/or reactivated HHV-6 to determine whether such cells are suitable for administration to a subject in a cell based therapy. Details regarding the *in vitro* methods will now be described.

In certain aspects, provided are methods comprising assessing an *in vitro* culture comprising candidate therapeutic human T cells for one or more HHV-6 analytes, e.g., one or more HHV-6B analytes. As used herein, “candidate therapeutic human T cells” are human T cells to be used in a cell based therapy, subject to the result of the assessment for the one or more HHV-6 analytes and/or HHV-6 reactivation. Candidate therapeutic human T cells determined to be negative for the one or more HHV-6 analytes and/or HHV-6 reactivation may then be identified as therapeutic human T cells, which in turn may be administered to a subject in need thereof as a cell based therapy. A “cell based therapy” or “cell therapy” refers to the transfer of autologous or allogeneic cellular material into a subject for medical purposes. Non-limiting examples of cell-based therapies include CAR T cell therapy, engineered T cell therapy (e.g., T cells that express a recombinant T cell receptor (TCR)), a therapy comprising administering T cells which do not express a recombinant receptor, and the like.

According to some embodiments, the one or more HHV-6 analytes comprise an HHV-6B nucleic acid. The terms “nucleic acid” and “polynucleotide” are used interchangeably herein to

describe a polymer composed of nucleotides, e.g., deoxyribonucleotides or ribonucleotides. In certain embodiments, the one or more HHV-6 analytes comprise an HHV-6 deoxyribonucleic acid (DNA). According to some embodiments, the one or more HHV-6 analytes comprise an HHV-6B ribonucleic acid (RNA).

5 A variety of approaches are available for assessing the *in vitro* culture for one or more HHV-6B DNAs and/or RNAs. For example, the HHV-6B genome and transcriptome have been sequenced. The HHV-6B reference genome and transcriptome are available at Genbank (Genbank AF157706). Based on the available sequence information, the presence or absence of one or more selected HHV-6B DNAs and/or RNAs may be determined by hybridization (e.g.,
10 Southern analysis, Northern analysis, microarray analysis, or the like), nucleic acid sequencing, and/or the like.

Sequencing HHV-6B DNA and/or RNA may be performed using any of a variety of available high throughput nucleic acid sequencing machines and systems. Illustrative sequencing systems include the Illumina iSeq 100, Miniseq, MiSeq series, NextSeq series (e.g., NextSeq 500
15 series, NextSeq 1000, NextSeq 2000), and NovaSeq sequencing systems (Illumina, Inc., San Diego, Calif.), the Pacific Biosciences Sequel (e.g., Sequel II) sequencing system (Pacific Biosciences, Menlo Park, Calif.), the Oxford Nanopore Technologies MinION™, GridIONx5™, PromethION™, or SmidgION™ nanopore-based sequencing systems (Oxford Nanopore Technologies, Oxford, UK), and other systems having similar capabilities. In certain
20 embodiments, sequencing is achieved using a set of sequencing platform-specific oligonucleotides that hybridize to a defined region within amplified HHV-6B DNA and/or RNA DNA molecules.

In some embodiments, the raw sequence data is preprocessed to remove errors in the primary sequence of each read and to compress the data. A nearest neighbor algorithm can be
25 used to collapse the data into unique sequences by merging closely related sequences, to remove both PCR and sequencing errors. See, e.g., US2012/0058902; US2010/033057; WO2011/106738; US2015/0299785; or WO2012/027503, which is each incorporated by reference in its entirety.

According to some embodiments, the one or more HHV-6 analytes comprise an HHV-6B
30 protein. The terms “protein”, “polypeptide”, or “peptide” are used interchangeably herein to designate a linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues.

A variety of approaches are available for assessing the *in vitro* culture for one or more HHV-6B proteins. For example, based on the available amino acid sequence information for
35 proteins encoded by the HHV-6B transcriptome, the presence or absence of one or more selected HHV-6B proteins may be assessed, e.g., by a variety of immunoassays using available antibodies specific for one or more HHV-6B proteins. As used herein, an “immunoassay” (IA) is

a biochemical test that measures the presence or concentration of a macromolecule or a small molecule in a solution through the use of an antibody. In certain embodiments, the immunoassay is an enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay, a fluorescent immunoassay, a chemiluminescent immunoassay, a lateral flow immunoassay, or an immunoblot assay.

As such, the presence or amount of one or more HHV-6B proteins can be determined using antibodies and detecting specific binding to the one or more HHV-6B proteins. Any immunoassay may be utilized. The immunoassay may be an enzyme-linked immunoassay (ELISA), a competitive inhibition assay, such as forward or reverse competitive inhibition assays, or a competitive binding assay, for example. In some embodiments, one tag is attached to the capture antibody and the detection antibody. Alternately, a microparticle or nanoparticle employed for capture, also can function for detection, e.g., where it is attached or associated by some means to a cleavable linker.

A heterogeneous format may be used. For example, after a test sample from the *in vitro* culture comprising the candidate human therapeutic T cells is obtained, a first mixture is prepared. The mixture contains the test sample being assessed for a HHV-6B protein and a first specific binding partner, wherein the first specific binding partner and HHV-6B protein form a first specific binding partner-HHV-6B protein complex. The first specific binding partner may be an anti-HHV-6B protein antibody or a fragment thereof. The order in which the test sample and the first specific binding partner are added to form the mixture is not critical. The first specific binding partner may be immobilized on a solid phase. The solid phase used in the immunoassay (for the first specific binding partner and, optionally, the second specific binding partner) can be any solid phase known in the art, such as, but not limited to, a magnetic particle, a bead, a nanobead, a microbead, a nanoparticle, a microparticle, a membrane, a scaffolding molecule, a film, a filter paper, a disc, or a chip (e.g., a microfluidic chip).

After the mixture containing the first specific binding partner-HHV-6B protein complex is formed, any unbound analyte may be removed from the complex using any technique known in the art. For example, the unbound analyte can be removed by washing. Desirably, however, the first specific binding partner is present in excess of any HHV-6B protein present in the test sample, such that all HHV-6B protein that is present in the test sample is bound by the first specific binding partner.

After any unbound analyte is removed, a second specific binding partner is added to the mixture to form a first specific binding partner-HHV-6B protein-second specific binding partner complex. The second specific binding partner is preferably an anti-HHV-6B protein antibody that binds to an epitope on the HHV-6B protein that differs from the epitope on the HHV-6B protein bound by the first specific binding partner. Moreover, the second specific binding partner may be labeled with or contains a detectable label, e.g., tag attached by a cleavable linker.

The use of immobilized antibodies or fragments thereof may be incorporated into the immunoassay. The antibodies may be immobilized onto a variety of supports, such as magnetic or chromatographic matrix particles, latex particles or modified surface latex particles, polymer or polymer film, plastic or plastic film, planar substrate, a microfluidic surface, pieces of a solid substrate material, and the like.

As summarized above, the *in vitro* culture comprises candidate therapeutic human T cells. The type of human T cells may vary. Examples of T cells include naïve T cells (T_N), cytotoxic T cells (T_{CTL}), memory T cells (T_{MEM}), T memory stem cells (T_{SCM}), central memory T cells (T_{CM}), effector memory T cells (T_{EM}), tissue resident memory T cells (T_{RM}), effector T cells (T_{EFF}), regulatory T cells (T_{REGs}), helper T cells (T_H , T_{H1} , T_{H2} , T_{H17}), CD4+ T cells, CD8+ T cells, virus-specific T cells, alpha beta T cells ($T_{\alpha\beta}$), and gamma delta T cells ($T_{\gamma\delta}$). In some embodiments, the candidate therapeutic human T cells comprise candidate therapeutic CD4+ human T cells.

In certain embodiments, the candidate therapeutic human T cells are genetically modified. For example, according to some embodiments, the genetic modification comprises engineering the candidate therapeutic human T cells to express a receptor (e.g., a recombinant receptor) on the surface thereof. A variety of suitable approaches for genetically modifying cells are available. A “vector” is capable of transferring nucleic acid sequences to target cells (e.g., viral vectors, non-viral vectors, particulate carriers, and liposomes). Typically, “vector construct,” “expression vector,” and “gene transfer vector,” mean any nucleic acid construct capable of directing the expression of a nucleic acid of interest and which can transfer nucleic acid sequences to target cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

In order to express a desired polypeptide, a nucleotide sequence encoding the polypeptide can be inserted into appropriate vector, e.g., using recombinant DNA techniques known in the art. Illustrative examples of viruses useful as vectors include, without limitation, retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (e.g., herpes simplex virus), poxvirus, baculovirus, papillomavirus, and papovavirus (e.g., SV40). Illustrative examples of expression vectors include, but are not limited to pCIneo vectors (Promega) for expression in mammalian cells; pLenti4/V 5-DEST™, pLenti6/V 5- DEST™, murine stem cell virus (MSCV), MSGV, moloney murine leukemia virus (MMLV), and pLenti6.2/V5-GW/lacZ (Invitrogen) for lentivirus-mediated gene transfer and expression in mammalian cells. In particular embodiments, a nucleic acid sequence encoding a polypeptide to be expressed in the cells may be ligated into such expression vectors for the expression of the polypeptides in mammalian cells.

Expression control sequences, control elements, or regulatory sequences present in an expression vector are those non-translated regions of the vector – origin of replication, selection cassettes, promoters, enhancers, translation initiation signals (Shine Dalgarno sequence or Kozak sequence), introns, a polyadenylation sequence, 5' and 3' untranslated regions, and/or the like – which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host

utilized, any number of suitable transcription and translation elements, including ubiquitous promoters and inducible promoters may be used.

Components of the expression vector are operably linked such that they are in a relationship permitting them to function in their intended manner. In some embodiments, the term
5 refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, and/or enhancer) and a second polynucleotide sequence, e.g., a nucleic acid encoding the polypeptide, where the expression control sequence directs transcription of the nucleic acid encoding the polypeptide.

In certain embodiments, the expression vector is an episomal vector or a vector that is
10 maintained extrachromosomally. As used herein, the term "episomal" refers to a vector that is able to replicate without integration into the host cell's chromosomal DNA and without gradual loss from a dividing host cell also meaning that said vector replicates extrachromosomally or episomally. Such a vector may be engineered to harbor the sequence coding for the origin of DNA replication or "ori" from an alpha, beta, or gamma herpesvirus, an adenovirus, SV40, a
15 bovine papilloma virus, a yeast, or the like. The host cell may include a viral replication transactivator protein that activates the replication. Alpha herpes viruses have a relatively short reproductive cycle, variable host range, efficiently destroy infected cells and establish latent infections primarily in sensory ganglia. Illustrative examples of alpha herpes viruses include HSV 1, HSV 2, and VZV. Beta herpesviruses have long reproductive cycles and a restricted host
20 range. Infected cells often enlarge. Non-limiting examples of beta herpes viruses include CMV, HHV-6 and HHV-7. Gamma-herpesviruses are specific for either T or B lymphocytes, and latency is often demonstrated in lymphoid tissue. Illustrative examples of gamma herpes viruses include EBV and HHV-8.

According to some embodiments, the candidate therapeutic human T cells are
25 engineered to express a chimeric antigen receptor (CAR), a T cell receptor (TCR) such as a recombinant TCR, a chimeric cytokine receptor (CCR), a chimeric chemokine receptor, a synthetic notch receptor (synNotch), a Modular Extracellular Sensor Architecture (MESA) receptor, a Tango receptor, a ChaCha receptor, a generalized extracellular molecule sensor (GEMS) receptor, a growth factor receptor, a cytokine receptor, a chemokine receptor, a switch
30 receptor, an adhesion molecule, an integrin, an inhibitory receptor, a stimulatory receptor, an immunoreceptor tyrosine-based activation motif (ITAM)-containing receptor, an immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptor, a hormone receptor, a receptor tyrosine kinase, an immune receptor such as CD28, CD80, ICOS, CTLA4, PD1, PD-L1, BTLA, HVEM, CD27, 4-1BB, 4-1BBL, OX40, OX40L, DR3, GITR, CD30, SLAM, CD2, 2B4, TIM1, TIM2,
35 TIM3, TIGIT, CD226, CD160, LAG3, LAIR1, B7-1, B7-H1, and B7-H3, a type I cytokine receptor such as Interleukin-1 receptor, Interleukin-2 receptor, Interleukin-3 receptor, Interleukin-4 receptor, Interleukin-5 receptor, Interleukin-6 receptor, Interleukin-7 receptor, Interleukin-9 receptor, Interleukin-11 receptor, Interleukin-12 receptor, Interleukin-13 receptor, Interleukin-15

receptor, Interleukin-18 receptor, Interleukin-21 receptor, Interleukin-23 receptor, Interleukin-27 receptor, Erythropoietin receptor, GM-CSF receptor, G-CSF receptor, Growth hormone receptor, Prolactin receptor, Leptin receptor, Oncostatin M receptor, Leukemia inhibitory factor, a type II cytokine receptor such as interferon-alpha/beta receptor, interferon-gamma receptor, Interferon type III receptor, Interleukin-10 receptor, Interleukin-20 receptor, Interleukin-22 receptor, Interleukin-28 receptor, a receptor in the tumor necrosis factor receptor superfamily such as Tumor necrosis factor receptor 2 (1B), Tumor necrosis factor receptor 1, Lymphotoxin beta receptor, OX40, CD40, Fas receptor, Decoy receptor 3, CD27, CD30, 4-1BB, Decoy receptor 2, Decoy receptor 1, Death receptor 5, Death receptor 4, RANK, Osteoprotegerin, TWEAK receptor, TACI, BAFF receptor, Herpesvirus entry mediator, Nerve growth factor receptor, B-cell maturation antigen, Glucocorticoid-induced TNFR-related, TROY, Death receptor 6, Death receptor 3, Ectodysplasin A2 receptor, a chemokine receptor such as CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CX3CR1, XCR1, ACKR1, ACKR2, ACKR3, ACKR4, CCRL2, a receptor in the epidermal growth factor receptor (EGFR) family, a receptor in the fibroblast growth factor receptor (FGFR) family, a receptor in the vascular endothelial growth factor receptor (VEGFR) family, a receptor in the rearranged during transfection (RET) receptor family, a receptor in the Eph receptor family, a receptor that can induce cell differentiation (e.g., a Notch receptor), a cell adhesion molecule (CAM), an adhesion receptor such as integrin receptor, cadherin, selectin, and a receptor in the discoidin domain receptor (DDR) family, transforming growth factor beta receptor 1, and transforming growth factor beta receptor 2. In some embodiments, such a receptor is an immune cell receptor selected from a T cell receptor, a B cell receptor, a natural killer (NK) cell receptor, a macrophage receptor, a monocyte receptor, a neutrophil receptor, a dendritic cell receptor, a mast cell receptor, a basophil receptor, and an eosinophil receptor.

In certain embodiments, the candidate therapeutic human T cells are engineered to express a chimeric antigen receptor (CAR). According to some embodiments, the candidate therapeutic human T cells are engineered to express a recombinant TCR.

As described above, according to some embodiments, the candidate therapeutic human T cells may be engineered to express a CAR. The extracellular binding domain of the CAR may comprise a single chain antibody. The single-chain antibody may be a monoclonal single-chain antibody, a chimeric single-chain antibody, a humanized single-chain antibody, a fully human single-chain antibody, and/or the like. In one non-limiting example, the single chain antibody is a single chain variable fragment (scFv). In some embodiments, the extracellular binding domain of the CAR is a single-chain version (e.g., an scFv version) of an antibody approved by the United States Food and Drug Administration and/or the European Medicines Agency (EMA) for use as a therapeutic antibody. Non-limiting examples of single-chain antibodies which may be employed when the protein of interest is a CAR include single-chain versions (e.g., scFv versions) of Adecatumumab, Ascrinvacumab, Cixutumumab, Conatumumab, Daratumumab, Drozitumab,

Duligotumab, Durvalumab, Dusigitumab, Enfortumab, Enoticumab, Figitumumab, Ganitumab, Glembatumumab, Intetumumab, Ipilimumab, Iratumumab, Icrucumab, Lexatumumab, Lucatumumab, Mapatumumab, Narnatumab, Necitumumab, Nesvacumab, Ofatumumab, Olaratumab, Panitumumab, Patritumab, Pritumumab, Radretumab, Ramucirumab, Rilotumumab, Robatumumab, Seribantumab, Tarextumab, Teprotumumab, Tovetumab, Vantictumab, Vesencumab, Votumumab, Zalutumumab, Flanvotumab, Altumomab, Anatumomab, Arcitumomab, Bectumomab, Blinatumomab, Detumomab, Ibritumomab, Minretumomab, Mitumomab, Moxetumomab, Naptumomab, Nofetumomab, Pemtumomab, Pintumomab, Racotumomab, Satumomab, Solitomab, Taplitumomab, Tenatumomab, Tositumomab, Tremelimumab, Abagovomab, Igovomab, Oregovomab, Capromab, Edrecolomab, Nacolomab, Amatuximab, Bavituximab, Brentuximab, Cetuximab, Derlotuximab, Dinutuximab, Ensituximab, Futuximab, Girentuximab, Indatuximab, Isatuximab, Margetuximab, Rituximab, Siltuximab, Ublituximab, Echromeximab, Abituzumab, Alemtuzumab, Bevacizumab, Bivatuzumab, Brontictuzumab, Cantuzumab, Cantuzumab, Citatuzumab, Clivatuzumab, Dacetuzumab, Demcizumab, Dalotuzumab, Denintuzumab, Elotuzumab, Emactuzumab, Emibetuzumab, Enoblituzumab, Etaracizumab, Farletuzumab, Ficlatuzumab, Gemtuzumab, Imgatuzumab, Inotuzumab, Labetuzumab, Lifestuzumab, Lintuzumab, Lorvotuzumab, Lumretuzumab, Matuzumab, Milatuzumab, Nimotuzumab, Obinutuzumab, Ocaratuzumab, Otlertuzumab, Onartuzumab, Oportuzumab, Parsatuzumab, Pertuzumab, Pinatuzumab, Polatuzumab, Sibrotuzumab, Simtuzumab, Tacatuzumab, Tigatuzumab, Trastuzumab, Tucotuzumab, Vandortuzumab, Vanucizumab, Veltuzumab, Vorsetuzumab, Sofituzumab, Catumaxomab, Ertumaxomab, Depatuxizumab, Ontuxizumab, Blontuvmab, Tamtuvmab, or an antigen-binding variant thereof.

When the candidate therapeutic human T cells are engineered to express a recombinant receptor on the surface thereof, the receptor may include one or more linker sequences between the various domains. A “variable region linking sequence” is an amino acid sequence that connects a heavy chain variable region to a light chain variable region and provides a spacer function compatible with interaction of the two sub-binding domains so that the resulting polypeptide retains a specific binding affinity to the same target molecule as an antibody that includes the same light and heavy chain variable regions. A non-limiting example of a variable region linking sequence is a glycine-serine linker, such as a (G₄S)₃ linker. In certain embodiments, a linker separates one or more heavy or light chain variable domains, hinge domains, transmembrane domains, co-stimulatory domains, and/or primary signaling domains. In particular embodiments, the receptor (e.g., CAR) includes one, two, three, four, or five or more linkers. In particular embodiments, the length of a linker is about 1 to about 25 amino acids, about 5 to about 20 amino acids, or about 10 to about 20 amino acids, or any intervening length of amino acids. In some embodiments, the linker is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more amino acids in length.

In some embodiments, when the candidate therapeutic human T cells are engineered to express a recombinant receptor on the surface thereof, the antigen binding domain of the receptor (e.g., CAR) is followed by one or more spacer domains that moves the antigen binding domain away from the cell surface expressing the receptor to enable proper cell/cell contact, antigen binding and/or activation. The spacer domain (and any other spacer domains, linkers, and/or the like described herein) may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. In certain embodiments, a spacer domain is a portion of an immunoglobulin, including, but not limited to, one or more heavy chain constant regions, e.g., CH2 and CH3. The spacer domain may include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region. In some embodiments, the spacer domain includes the CH2 and/or CH3 of IgG1, IgG4, or IgD. Illustrative spacer domains suitable for use in the receptors (e.g., CARs) described herein include the hinge region derived from the extracellular regions of type 1 membrane proteins such as CD8 α and CD4, which may be wild-type hinge regions from these molecules or variants thereof. In certain embodiments, the hinge domain includes a CD8 α hinge region. According to some embodiments, the hinge is a PD-1 hinge or CD152 hinge. In certain embodiments, the hinge is an IgG4 hinge.

The "transmembrane domain" (Tm domain) is the portion of the receptor (e.g., CAR) that fuses the extracellular binding portion and intracellular signaling domain and anchors the receptor to the plasma membrane of the cell (e.g., T-cell, such as a Treg). The Tm domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. In some embodiments, the Tm domain is derived from (e.g., includes at least the transmembrane region(s) or a functional portion thereof) of the alpha or beta chain of the T-cell receptor, CD35, CD3 ζ , CD3 γ , CD3 δ , CD4, CD5, CD8 α , CD9, CD16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD134, CD137, CD152, CD154, or PD-1.

In one embodiment, a receptor (e.g., CAR) includes a Tm domain derived from CD28. In certain embodiments, a receptor includes a Tm domain derived from CD28 and a short oligo- or polypeptide linker, e.g., between 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids in length, that links the Tm domain and the intracellular signaling domain of the receptor. A glycine-serine linker may be employed as such a linker, for example.

The "intracellular signaling" domain of a receptor (e.g., a CAR) refers to the part of the receptor that participates in transducing the signal from binding to a target molecule/antigen into the interior of the cell to elicit cell function. Accordingly, the term "intracellular signaling domain" refers to the portion of a protein which transduces the signal and that directs the cell to perform a specialized function. To the extent that a truncated portion of an intracellular signaling domain is used, such truncated portion may be used in place of a full-length intracellular signaling domain as long as it transduces the signal. The term intracellular signaling domain is meant to include any truncated portion of an intracellular signaling domain sufficient for transducing signal.

Signals generated through the T cell receptor (TCR) alone are insufficient for full activation of the T cell, and a secondary or costimulatory signal is also required. Thus, T cell activation is mediated by two distinct classes of intracellular signaling domains: primary signaling domains that initiate antigen-dependent primary activation through the TCR (e.g., a TCR/CD3 complex) and costimulatory signaling domains that act in an antigen-independent manner to provide a secondary or costimulatory signal. As such, a receptor (e.g., CAR) expressed by a genetically modified cell may include an intracellular signaling domain that includes one or more (e.g., 1, 2, or more) “costimulatory signaling domains” and a “primary signaling domain.”

Primary signaling domains regulate primary activation of the TCR complex either in a stimulatory manner, or in an inhibitory manner. Primary signaling domains that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs (or “ITAMs”). Non-limiting examples of ITAM-containing primary signaling domains suitable for use in a receptor of the present disclosure include those derived from FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD3ζ, CD22, CD79α, CD79β, and CD66δ. In certain embodiments, a receptor includes a CD3ζ primary signaling domain and one or more costimulatory signaling domains. The intracellular primary signaling and costimulatory signaling domains are operably linked to the carboxyl terminus of the transmembrane domain.

In some embodiments, when the methods of the present disclosure are performed on candidate therapeutic human T cells engineered to express a recombinant receptor on the surface thereof, the receptor (e.g., CAR) includes one or more costimulatory signaling domains to enhance the efficacy and expansion of immune effector cells (e.g., T cells) expressing the receptor. As used herein, the term “costimulatory signaling domain” or “costimulatory domain” refers to an intracellular signaling domain of a costimulatory molecule or an active fragment thereof. Example costimulatory molecules suitable for use in receptors contemplated in particular embodiments include TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DAP10, LAT, KD2C, SLP76, TRIM, and ZAP70. In some embodiments, the receptor (e.g., CAR) includes one or more costimulatory signaling domains selected from the group consisting of 4-1BB (CD137), CD28, and CD134, and a CD3ζ primary signaling domain.

A receptor (e.g., CAR) expressed by a cell genetically modified according to the methods of the present disclosure may include any variety of suitable domains including but not limited to a leader sequence; hinge, spacer and/or linker domain(s); transmembrane domain(s); costimulatory domain(s); signaling domain(s) (e.g., CD3ζ domain(s)); ribosomal skip element(s); restriction enzyme sequence(s); reporter protein domains; and/or the like.

According to some embodiments, when the candidate therapeutic human T cells are genetically modified to express a receptor (e.g., a CAR) on their surface, the extracellular binding domain of the receptor specifically binds a tumor antigen expressed on the surface of a cancer

cell. Non-limiting examples of tumor antigens to which the extracellular binding domain of the receptor may specifically bind include 5T4, AXL receptor tyrosine kinase (AXL), B-cell maturation antigen (BCMA), c-MET, C4.4a, carbonic anhydrase 6 (CA6), carbonic anhydrase 9 (CA9), Cadherin-6, CD19, CD20, CD22, CD25, CD27L, CD30, CD33, CD37, CD44, CD44v6, CD56, CD70, CD74, CD79b, CD123, CD138, carcinoembryonic antigen (CEA), cKit, Cripto protein, CS1, delta-like canonical Notch ligand 3 (DLL3), endothelin receptor type B (EDNRB), ephrin A4 (EFNA4), epidermal growth factor receptor (EGFR), EGFRvIII, ectonucleotide pyrophosphatase/phosphodiesterase 3 (ENPP3), EPH receptor A2 (EPHA2), fibroblast growth factor receptor 2 (FGFR2), fibroblast growth factor receptor 3 (FGFR3), FMS-like tyrosine kinase 3 (FLT3), folate receptor 1 (FOLR1), GD2 ganglioside ("GD2"), glycoprotein non-metastatic B (GPNMB), guanylate cyclase 2 C (GUCY2C), human epidermal growth factor receptor 2 (HER2), human epidermal growth factor receptor 3 (HER3), Integrin alpha, lysosomal-associated membrane protein 1 (LAMP-1), Lewis Y, LIV-1, leucine rich repeat containing 15 (LRRC15), mesothelin (MSLN), mucin 1 (MUC1), mucin 16 (MUC16), sodium-dependent phosphate transport protein 2B (NaPi2b), Nectin-4, NMB, NOTCH3, p-cadherin (p-CAD), programmed cell death receptor ligand 1 (PD-L1), programmed cell death receptor ligand 2 (PD-L2), prostate-specific membrane antigen (PSMA), protein tyrosine kinase 7 (PTK7), solute carrier family 44 member 4 (SLC44A4), SLIT like family member 6 (SLITRK6), STEAP family member 1 (STEAP1), tissue factor (TF), T cell immunoglobulin and mucin protein-1 (TIM-1), Tn antigen, trophoblast cell-surface antigen (TROP-2), Wilms' tumor 1 (WT1), and VEGF-A.

In certain embodiments, the candidate therapeutic human T cells are genetically modified to express an antibody. The term "antibody" (also used interchangeably with "immunoglobulin") encompasses antibodies of any isotype (e.g., IgG (e.g., IgG1, IgG2, IgG3, or IgG4), IgE, IgD, IgA, IgM, etc.), whole antibodies (e.g., antibodies composed of a tetramer which in turn is composed of two dimers of a heavy and light chain polypeptide); single chain antibodies (e.g., scFv); fragments of antibodies (e.g., fragments of whole or single chain antibodies) which retain specific binding to the antigen, including, but not limited to single chain Fv (scFv), Fab, (Fab')₂, (scFv')₂, and diabodies; chimeric antibodies; monoclonal antibodies, humanized antibodies, human antibodies; and fusion proteins comprising an antigen-binding portion of an antibody and a non-antibody protein.

Immunoglobulin polypeptides include the kappa and lambda light chains and the alpha, gamma (IgG₁, IgG₂, IgG₃, IgG₄), delta, epsilon and mu heavy chains or equivalents in other species. Full-length immunoglobulin "light chains" (usually of about 25 kDa or about 214 amino acids) comprise a variable region of about 110 amino acids at the NH₂-terminus and a kappa or lambda constant region at the COOH-terminus. Full-length immunoglobulin "heavy chains" (of about 150 kDa or about 446 amino acids), similarly comprise a variable region (of about 116 amino acids) and one of the aforementioned heavy chain constant regions, e.g., gamma (of about 330 amino acids).

An immunoglobulin light or heavy chain variable region (V_L and V_H , respectively) is composed of a "framework" region (FR) interrupted by three hypervariable regions, also called "complementarity determining regions" or "CDRs". The extent of the framework region and CDRs have been defined (see, E. Kabat et al., *Sequences of proteins of immunological interest*, 4th ed. U.S. Dept. Health and Human Services, Public Health Services, Bethesda, MD (1987); and Lefranc et al. IMGT, the international ImMunoGeneTics information system®. Nucl. Acids Res., 2005, 33, D593-D597)). The sequences of the framework regions of different light or heavy chains are relatively conserved within a species. The framework region of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs. The CDRs are primarily responsible for binding to an epitope of an antigen. All CDRs and framework provided by the present disclosure are defined according to Kabat, *supra*, unless otherwise indicated.

An "antibody" thus encompasses a protein having one or more polypeptides that can be genetically encodable, e.g., by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. In some embodiments, an antibody of the present disclosure is an IgG antibody, e.g., an IgG1 antibody, such as a human IgG1 antibody. In some embodiments, the cell expresses an antibody that comprises a human Fc domain.

A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

According to some embodiments, the candidate therapeutic human T cells are not genetically modified to express an engineered receptor.

As will be appreciated with the benefit of the present disclosure, the candidate therapeutic human T cells may be expanding during a therapeutic T cell manufacturing process. By "expanding" or "expanded" is meant the cells are cultured under conditions in which the cells proliferate. Suitable conditions may vary depending upon, e.g., the type of T cells being expanded. Such conditions may include culturing the T cells in a suitable container (e.g., a cell culture plate or well thereof, a cassette, tube, bottle or bag suitable for use in an automated therapeutic cell manufacturing system, e.g., a closed automated therapeutic cell manufacturing system such as the CliniMACS Prodigy® system by Miltenyi Biotec, the Xuri® cell expansion system by Cytiva, the G-Rex® cell expansion system by Wilson Wolf, the Quantum® cell

expansion system from Terumo, the Cocoon® system by Lonza, or the like), in suitable medium (e.g., cell culture medium, such as RPMI, DMEM, IMDM, MEM, DMEM/F-12, or the like) at a suitable temperature (e.g., 32°C - 42°C, such as 37°C) and pH (e.g., pH 7.0 - 7.7, such as pH 7.4) in an environment having a suitable percentage of CO₂, e.g., 3% to 10%, such as 5%.

5 Methods for activating and expanding cells for therapy (e.g., therapeutic T cells and the like) are known in the art and are described, e.g., in U.S. Patent Nos. 6,905,874; 6,867,041; and 6,797,514; and PCT Publication No. WO 2012/079000, the contents of which are hereby incorporated by reference in their entirety. In the example of T cells, such methods may include
10 contacting PBMC or isolated T cells with a stimulatory agent and costimulatory agent, such as anti-CD3 and anti-CD28 antibodies, generally attached to a bead or other surface, in a culture medium with appropriate cytokines, such as IL-2. Anti-CD3 and anti-CD28 antibodies attached to the same bead serve as a “surrogate” antigen presenting cell (APC). One example is the Dynabeads® system, a CD3/CD28 activator/stimulator system for physiological activation of human T cells. In other embodiments, the T cells are activated and stimulated to proliferate with
15 feeder cells and appropriate antibodies and cytokines using methods such as those described in U.S. Patent Nos. 6,040,177 and 5,827,642 and PCT Publication No. WO 2012/129514, the contents of which are hereby incorporated by reference in their entirety.

 In certain embodiments, the candidate therapeutic human T cells are expanded using an automated system designed for the manufacture of therapeutic cells. Non-limiting examples of
20 such systems include the CliniMACS Prodigy® system by Miltenyi Biotec, the Xuri® cell expansion system by Cytiva, the G-Rex® cell expansion system by Wilson Wolf, the Quantum® cell expansion system from Terumo, the Cocoon® system by Lonza, etc. Detailed guidance and protocols for manufacturing therapeutic cells on such systems are available from the providers of such systems.

25 When the candidate therapeutic human T cells are being expanded during a therapeutic T cell manufacturing process, in certain embodiments, the methods comprise assessing the *in vitro* culture for the one or more HHV-6B analytes two or more times during expansion of the candidate therapeutic human T cells. For example, the *in vitro* culture may be assessed 2 or more, 3 or more, 4 or more, or 5 or more times during expansion of the candidate therapeutic
30 human T cells.

 When a manufacturing process comprises activating the candidate therapeutic human T cells, according to some embodiments, the methods comprise assessing the *in vitro* culture for the one or more HHV-6B analytes one or more times at from 0 to 15 days post-activation, e.g., at from 0 to 20 days post-activation.

35 Once the cells have been expanded to the desired extent, and if the assessment determines the absence of the one or more HHV-6 analytes and/or absence of HHV-6 reactivation, the methods may comprise harvesting the cells, i.e., removing the cells from the

container(s)/bioreactor(s) in which the cells were expanding at the time of harvest. Prior to or subsequent to harvesting the cells, the cells may be concentrated if desired, e.g., by centrifugation, a suitable cell separation technique (e.g., magnetic beads), and/or the like.

In some embodiments, the harvested cells are cryopreserved. As used herein, “cryopreserved” refers to cells that have been preserved or maintained by cooling to low sub-zero temperatures, such as 77 K or -196 deg. C. (the boiling point of liquid nitrogen). At these low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. Useful methods of cryopreservation and thawing cryopreserved cells, as well as processes and reagents related thereto, include but are not limited to e.g., those described in U.S. Patent Nos. 10370638; 10159244; 9078430; 7604929; 6136525; and 5795711, the disclosures of which are incorporated herein by reference in their entirety. In contrast, the term “fresh”, as used herein with reference to cells, may refer to cells that have not been cryopreserved and, e.g., may have been directly obtained and/or used (e.g., transplanted, cultured, etc.) following collection from a subject or organ thereof.

In some embodiments, for cryopreservation, a cell suspension is aliquoted into one or more vessels and pelleted by centrifugation. Cell pellets may then be resuspended in cryopreservation media under cold conditions to reach a desired final concentration, such as e.g., 10 million live cells per mL, and the resuspended cells kept at 4-8 deg. C. Cells prepared for cryopreservation may then be aliquoted into freezing containers and frozen using a controlled rate freezer. After controlled rate freezing is complete, cryopreserved may then be transferred to vapor phase liquid nitrogen for storage.

As described above, the methods of the present disclosure find use in screening the candidate therapeutic human T cells for the one or more HHV-6 analytes and/or HHV-6 reactivation to determine whether the candidate therapeutic human T cells are suitable for use in a cell based therapy. For example, when the assessing determines an absence of the one or more HHV-6B analytes in the *in vitro* culture, the methods may further comprise identifying the candidate therapeutic human T cells as therapeutic T cells. Such methods may further comprise administering to a subject in need thereof the therapeutic human T cells or progeny thereof. Also by way of example, when the assessing determines the presence of one or more HHV-6B analytes in the *in vitro* therapeutic human T cell culture, the methods may further comprise administering to a subject in need thereof therapeutic human T cells other than the candidate therapeutic T cells, wherein the administered therapeutic human T cells are known to be HHV-6B negative at the time of administration. By “HHV-6B negative” is meant it has been determined that the administered therapeutic human T cells do not comprise detectable latent or reactivated HHV-6B.

Aspects of the present disclosure further include methods comprising assessing an *in vitro* culture comprising candidate therapeutic human T cells for HHV-6B reactivation. By “HHV-6B reactivation” is meant a process by which latent HHV-6B switches to a lytic phase of

replication. In certain embodiments, assessing for HHV-6B reactivation comprises assessing the *in vitro* culture for a level of one or more HHV-6B analytes. The one or more HHV-6B analytes may comprise any of the HHV-6B analytes described elsewhere herein.

In certain embodiments, when the one or more HHV-6B analytes comprise an HHV-6B nucleic acid (e.g., an HHV-6B DNA and/or an HHV-6B RNA), the assessing comprises quantitative nucleic acid sequencing. In certain embodiments, the quantitative nucleic acid sequencing comprises single cell nucleic acid sequencing. When the one or more HHV-6B analytes comprise HHV-6B RNA, single cell RNA sequencing (scRNA-Seq) may be employed. scRNA-Seq is a genomic approach for the detection and quantitative analysis of messenger RNA molecules in a biological sample. As a proxy for studying the proteome, some research has turned to protein-encoding, mRNA molecules (collectively termed the “transcriptome”), whose expression correlates well with cellular traits and changes in cellular state. scRNA-seq permits comparison of the transcriptomes of individual cells. A variety of suitable approaches for scRNA-seq are available, non-limiting examples of which include C1 (SMARTer) (e.g., see Pollen et al. (2014) *Nat Biotechnol.* 32:1053-8), Smart-seq2 (e.g., see Picelli et al. (2013) *Nat Methods* 10:1096-8), MATQ-seq (e.g., see Sheng et al. (2017) *Nat Methods* 14:267-70), MARS-seq (e.g., see Jaitin et al. (2014) *Science* 343:776-9), CEL-seq (e.g., see Hashimshony et al. (2012) *Cell Rep.* 2:666-73), Drop-seq (e.g., see Macosko et al. (2015) *Cell* 161:1202-14), InDrop (e.g., see Klein et al. (2015) *Cell* 161:1187-201), Chromium (e.g., see Zheng et al. (2017) *Nat Commun.* 8:14049), SEQ-well (e.g., see Gierahn et al. (2017) *Nat Methods* 14:395-8), SPLIT-seq (e.g., see Rosenberg et al. (2017) *BioRxiv* doi.org/10.1101/105163), and others. Further details regarding single-cell RNA-sequencing can be found, e.g., in Haque et al. (2017) *Genome Med* 9, 75.

In certain embodiments, performing scRNA-seq comprising labeling the cells according to the Biolegend TotalSeq™-A protocol (www.biolegend.com/en-us/protocols/totalseq-a-antibodies-and-cell-hashing-with-10x-single-cell-3-reagent-kit-v3-3-1-protocol), performing the 10x 3' Chromium Single-Cell RNA-Sequencing Protocol (support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v31-chemistry), and sequencing at about 300-400 M reads per 10X library and about 25 M reads per Biolegend TotalSeq™ Library. Data from the single cell RNA sequencing may be deconvoluted into single cell transcriptomes, e.g., using barcode sequence information.

According to some embodiments, when the assessing comprises performing scRNA-Seq, the assessing comprises aligning RNA sequence reads of single cells of the candidate therapeutic human T cells to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences, and counting the number of RNA sequence reads of single cells mapping to the HHV-6B RNA sequences of the reference. According to such methods, reactivation may be determined to be present in a single cell when a threshold number of RNA sequence reads from the single cell map to the HHV-6B RNA sequences of the reference. In certain embodiments, the threshold number of RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11,

or about 10 RNA sequence reads. Such methods may be computer-implemented as described in further detail below.

According to some embodiments, when the assessing comprises performing scRNA-Seq, the assessing comprises pseudoaligning RNA sequence reads of single cells of the candidate therapeutic human T cells to an HHV-6B reference index, removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome, and counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index. HHV-6B reactivation is determined to be present in a cell of the candidate therapeutic human T cells when a threshold number of remaining RNA sequence reads are pseudoaligned to the HHV-6B RNA reference index. In some instances, the threshold number is the threshold number of RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

Nucleic acids may be obtained from a given sample (e.g., a candidate therapeutic T cell culture) using extraction methods known to those skilled in the art. Such methods may initially include lysis, inactivation of nucleases, and separation of nucleic acids from cell debris. Methods for isolating nucleic acids from extracts employ combinations of extraction/precipitation, chromatography, centrifugation, electrophoresis and affinity separation. Additional methods for isolating nucleic acids will be recognized by those skilled in the art. In some cases, separation of the total nucleic acid from other components of the sample may not be performed.

In some cases, extraction/precipitation methods may include solvent extraction performed to eliminate contaminants from nucleic acids (e.g., phenol-chloroform extraction), selective precipitation of nucleic acids using high concentrations of salt or changes in pH to precipitate proteins, and nucleic acid precipitation using isopropanol or ethanol.

In other cases, nucleic acids can be isolated using methods that combine affinity immobilization with magnetic separation. For example, poly(A) mRNA may be bound to streptavidin-coated magnetic particles by biotin-labeled oligo(dT) and the particle complex removed from unbound contaminants using a magnet. Such methods can replace several centrifugation, organic extraction and phase separation steps with a rapid magnetic separation step.

In some cases, chromatography methods to isolate nucleic acids may utilize gel filtration, ion exchange, selective adsorption or affinity binding. For example, nucleic acids may be isolated from extracts by adsorption chromatography which relies on the nucleic acid-binding properties of silica or glass particles in the presence of chaotropic agents (see, e.g., U.S. Patent Nos. 5,234,809 and 7,517,969, herein incorporated by reference). In certain cases, chromatography and affinity separation is used in combination to isolate nucleic acids from any given sample. For example, silica or glass coated magnetic particles may be added to a sample containing nucleic acids. Upon addition of a chaotropic agent, nucleic acids in the sample will bind to the silica or

glass coating. Nucleic acids are then separated from unbound contaminants using a magnet. Suitable chaotropic agents are substances that disrupt the structure of, and denatures, macromolecules such as proteins and nucleic acids, and includes, e.g., butanol, ethanol, guanidinium chloride, guanidinium isothiocyanate, lithium perchlorate, lithium acetate, magnesium chloride, phenol, propanol, sodium dodecyl sulfate, thiourea, urea, and the like.

In certain embodiments, total nucleic acid may be isolated from a given sample by first lysing the sample so that nucleic acids are released into solution. Silica or glass coated magnetic particles are added to the lysed sample together with an effective amount of a chaotropic agent (e.g., 8 M guanidinium chloride), to allow nucleic acids to adsorb onto the surfaces of the magnetic particles. Several wash steps are performed and the nucleic acid-bound magnetic particles are optionally dried (e.g., using a heater). A release agent is added to release the nucleic acids from the magnetic particles. Nucleic acids are then eluted into a buffer of choice before proceeding to downstream processing (e.g., nucleic acid amplification and detection).

In some embodiments, total nucleic acid may be isolated from a given sample by using a method described in Jangam, et al., known as filtration isolation of nucleic acids (FINA) (Jangam, et al., J. Clin. Microbiol., 47(8), 2363-2368 (2009)). Generally, a method for isolation of HIV proviral DNA from leukocyte DNA from whole blood includes the use of a cell separation membrane disk placed in direct contact with an absorbent pad, which drives fluid flow by capillary pressure. Upon transfer of a sample of whole blood onto the disk, leukocytes and erythrocytes are trapped in the cell separation membrane, while plasma flows through into the absorbent pad. Membrane-entrapped cells are lysed, and cell debris etc., are wicked into the absorbent pad. The released nucleic acids are trapped within the membrane for further elution and processing. In other cases, total nucleic acid may be isolated from a given sample by using commercially available nucleic acid isolation kits that result in isolated nucleic acids ready for downstream processing (e.g., sequencing).

In certain cases, extraction and purification of nucleic acids may be performed as described in Sur et al. J. Mol. Diagn., 2010, 12 (5): 620-628. A single pass of paramagnetic particles (PMPs), on which nucleic acids are adsorbed, through an immiscible hydrophobic liquid yields pure nucleic acid. Only two aqueous solutions are required: a lysis buffer, in which nucleic acids are captured on PMPs, and an elution buffer, in which they are released for amplification. The PMPs containing the nucleic acids are magnetically transported through a channel containing liquid wax that connects the lysis chamber to the elution chamber in a cartridge. Transporting PMPs through the immiscible phase yields DNA and RNA with equivalent purity as methods that utilize extensive wash steps.

In certain embodiments, extraction of nucleic acids from cells may be performed as described in U.S. Patent No. 8,017,340, which is herein incorporated by reference in its entirety. Briefly, nucleic acids may be isolated by exposing a sample comprising cells containing nucleic acids to an aqueous mixture comprising a lytic reagent and one or more beads capable of binding

the nucleic acid released from the cells to form a nucleic acid-bead complex; and passing the nucleic acid-bead complex through an immiscible liquid layer to separate the nucleic acid from the aqueous mixture, where the one or more beads are magnetic, and the nucleic acid-bead complex is passed through and separated from the immiscible liquid layer with an applied magnetic field. The immiscible liquid layer may be an organic liquid or a wax layer.

METHODS OF ASSESSING *IN VIVO* THERAPEUTIC T CELLS FOR HHV-6B REACTIVATION

Aspects of the present disclosure include methods of assessing *in vivo* therapeutic T cells for HHV-6B reactivation. In certain embodiments, provided are methods of monitoring a human subject receiving a T cell based therapy for HHV-6B reactivation in therapeutic T cells present in the subject. Such methods comprise obtaining from the subject a biological sample comprising the therapeutic T cells, and assessing the therapeutic T cells for HHV-6B reactivation. The manner of the assessment will vary depending upon the type of HHV-6B analyte(s) used to assess reactivation. For example, an immunoassay may be performed on the sample when an HHV-6B protein is used to assess for reactivation. Any of the immunoassays described elsewhere herein may be employed. Also by way of example, quantitative nucleic acid sequencing (e.g., quantitative single cell nucleic acid sequencing) may be performed on the sample when HHV-6B DNA and/or RNA is used to assess for reactivation.

In certain embodiments, assessing the therapeutic T cells for HHV-6B reactivation comprises performing scRNA-Seq on the therapeutic T cells. When scRNA-Seq is performed, according to some embodiments, the assessing comprises pseudoaligning RNA sequence reads of single cells of the therapeutic T cells to an HHV-6B reference index, and removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome. Such methods further comprise counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index. According to such methods, HHV-6B reactivation is determined to be present in a cell of the therapeutic T cells when a threshold number of remaining RNA sequence reads are pseudoaligned to the HHV-6B RNA reference index. In certain embodiments, the highly homologous regions of the HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A. According to some embodiments, the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

When scRNA-Seq is performed, according to some embodiments, the assessing comprises aligning RNA sequence reads of single cells of the therapeutic T cells to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences, and counting the number of RNA sequence reads of single cells mapping to the HHV-6B RNA sequences of the reference, where HHV-6B reactivation is determined to be present when a threshold number of RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the reference.

In some instances, when the assessment determines the presence of HHV-6B reactivation, the methods further comprise ceasing the T cell based therapy. Such methods may further comprise administering to the subject a new T cell based therapy with cells determined to be HHV-6B negative.

5 According to some embodiments, when the assessment determines the presence of HHV-6B reactivation, the method further comprises administering an antiviral therapy to the subject. Suitable antiviral therapies include, but are not limited to, administering to the subject an agent approved for treating HHV-6B infection in humans. In certain embodiments, the antiviral therapy comprises administering ganciclovir, cidofovir, foscarnet, or any combination thereof to
10 the subject. In some instances, the antiviral therapy comprises administering foscarnet to the subject.

In certain embodiments, when the assessment determines the absence of HHV-6B reactivation, the methods comprise continuing the T cell based therapy, e.g., by further administering therapeutic T cells from the same source as one or more previous administrations.

15 Biological samples of interest include those that comprise T cells, including but not limited to, whole blood samples (e.g., a peripheral blood sample), a fraction of whole blood comprising peripheral blood mononuclear cells (e.g., blood plasma), serum, a peripheral blood mononuclear cell (PBMC) sample, a gut tissue sample, urine, buffy coat, synovial fluid, bone marrow, cerebrospinal fluid, saliva, lymph fluid, seminal fluid, vaginal secretions, urethral secretions,
20 exudate, transdermal exudates, pharyngeal exudates, nasal secretions, sputum, sweat, bronchoalveolar lavage, tracheal aspirations, fluid from joints, or vitreous fluid. T cells can also be obtained from biological samples which may be derived from, for example, solid tissue samples. T cells may be helper T cells (effector T cells or Th cells), cytotoxic T cells (CTLs), memory T cells, and regulatory T cells. In some embodiments, peripheral blood mononuclear
25 cells (PBMC) are isolated by techniques known to those of skill in the art, e.g., by Ficoll-Hypaque® density gradient separation.

Nucleic acid, such as, genomic DNA or RNA may be extracted from lymphoid cells by methods known to those of skill in the art. Examples include using the QIAamp® DNA blood Mini Kit or a Qiagen DNeasy Blood extraction kit (both commercially available from Qiagen,
30 Gaithersburg, Md., USA) to extract genomic DNA. Alternatively, total nucleic acid can be isolated from cells, including both genomic DNA and mRNA. In other embodiments, cDNA is transcribed from mRNA and then used as templates for amplification. The RNA molecules can be transcribed to cDNA using known reverse-transcription kits, such as the SMARTer™ Ultra Low RNA kit for Illumina sequencing (Clontech, Mountain View, Calif.) essentially according to the supplier's
35 instructions.

Aspects of the present disclosure further include methods of monitoring a therapeutic T cell product for HHV-6B reactivation. In certain embodiments, the methods comprise

administering a first population of therapeutic T cells from the therapeutic T cell product to a subject in need thereof, and culturing a second population of therapeutic T cells from the therapeutic T cell product, wherein the culturing comprises culturing the therapeutic T cells during a period subsequent to administration of the first population of therapeutic T cells to the subject.

5 Such methods further comprise, during the period subsequent to administration of the first population of therapeutic T cells to the subject, monitoring the T cell culture for HHV-6B reactivation. In some instances, the culturing commences prior to administration of the first population of therapeutic T cells to the subject (e.g., 7 or fewer days prior to administration), at the time of administration of the first population of therapeutic T cells to the subject, or subsequent
10 to administration of the first population of therapeutic T cells to the subject, e.g., commencing within one, two, three, four or five days of administration of the first population of therapeutic T cells to the subject. The duration of the culturing, in some instances, is from three days to two weeks, e.g., from three to 14 days, from three to 13 days, from three to 12 days, from three to 11 days, from three to 10 days, from three to 9 days, from three to 8 days, from three to 7 days, from
15 three to 6 days, or from three to 5 days. In some embodiments, the culturing comprises continuation of the culture of the therapeutic T cells once a phase of manufacturing the therapeutic T cell product has concluded. For example, an aliquot (first population) of the expanded therapeutic T cells may be administered to the subject, and the remaining population (second population) may be cultured in parallel with administration of the first population to the
20 subject. Suitable culture conditions may include culturing the second population of therapeutic T cells from the therapeutic T cell product in a suitable container (e.g., a cell culture plate or well thereof, a cassette, tube, bottle, or the like) in suitable medium (e.g., cell culture medium, such as RPMI, DMEM, IMDM, MEM, DMEM/F-12, or the like) at a suitable temperature (e.g., 32°C - 42°C, such as 37°C) and pH (e.g., pH 7.0 - 7.7, such as pH 7.4) in an environment having a
25 suitable percentage of CO₂, e.g., 3% to 10%, such as 5%. Monitoring the T cell culture for HHV-6B reactivation may include one or more (e.g., two or more, or three or more) assessments for HHV-6B reactivation. Any convenient manner of assessment may be employed. In some embodiments, the one or more assessments are performed using any of the methods of the present disclosure described herein for assessing HHV-6B reactivation, e.g., any of the
30 quantitative nucleic acid sequencing approaches described herein, which are not reiterated here for purposes of brevity.

Aspects of the present disclosure further include methods of preventing or mitigating viral reactivation in therapeutic human cells during a therapeutic human cell manufacturing process. In certain embodiments, such methods comprise expanding the therapeutic human cells in the
35 presence of an anti-viral agent at a concentration effective to prevent or mitigate viral reactivation in the expanding therapeutic human cells. In some instances, the therapeutic human cells are therapeutic human T cells. When the therapeutic human cells are therapeutic human T cells, in some embodiments, HHV-6A, HHV-6B, and/or HHV-7 viral reactivation is prevented or mitigated.

In certain embodiments, the therapeutic human cells are therapeutic human induced pluripotent stem cells (iPSCs). Non-limiting examples of anti-viral agents that may be employed include ganciclovir, cidofovir, foscarnet, or any combination thereof. The effective concentration may vary depending upon the particular anti-viral agent employed and can be readily ascertained by one of skill in the art with the benefit of the present disclosure. In some embodiments, the therapeutic human cells are genetically modified to express an engineered receptor. Such cells are described elsewhere herein and not reiterated for purposes of brevity. The therapeutic human cell manufacturing process may comprise expanding the human therapeutic cells. Suitable manufacturing processes may be carried out, e.g., in an automated therapeutic cell manufacturing system, e.g., a closed automated therapeutic cell manufacturing system such as the CliniMACS Prodigy® system by Miltenyi Biotec, the Xuri® cell expansion system by Cytiva, the G-Rex® cell expansion system by Wilson Wolf, the Quantum® cell expansion system from Terumo, the Cocoon® system by Lonza, or the like), in suitable medium (e.g., cell culture medium, such as RPMI, DMEM, IMDM, MEM, DMEM/F-12, or the like) at a suitable temperature (e.g., 32°C - 42°C, such as 37°C) and pH (e.g., pH 7.0 - 7.7, such as pH 7.4) in an environment having a suitable percentage of CO₂, e.g., 3% to 10%, such as 5%.

COMPUTER-IMPLEMENTED METHODS, COMPUTER-READABLE MEDIA AND COMPUTER DEVICES

Also provided by the present disclosure are computer-implemented methods, computer-readable media and computer devices. The computer-readable media and computer devices find use, e.g., in practicing the computer-implemented methods of the present disclosure.

By “computer-implemented” is meant at least one step of the method is implemented using one or more processors and one or more non-transitory computer-readable media. The computer-implemented methods of the present disclosure may further comprise one or more steps that are not computer-implemented, e.g., obtaining a sample (e.g., a blood sample, or the like) from a subject, manufacturing T cells, preparing a sample for assessment (e.g., nucleic acid sequencing, immunoassay, and/or the like), administering a therapy to a subject based on the assessment, and/or the like.

According to some embodiments, provided are computer-implemented methods for assessing a population of candidate human T cells for HHV-6B reactivation. Such methods comprise aligning RNA sequence reads of single cells of the candidate human T cells to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences, and counting the number of RNA sequence reads of the single cells mapping to the HHV-6B RNA sequences of the reference. According to such methods, HHV-6B reactivation is determined to be present in a single cell when a threshold number of RNA sequence reads from the single cell map to the HHV-6B RNA sequences of the reference. Shown in FIG. 8 is a flow diagram illustrating a non-limiting embodiment of such methods. According to this embodiment, the steps of the method may be performed as follows:

Step 1: Create a joint HHV-6B and host reference genome/ transcriptome

To identify reads from the RNA-seq library that are from HHV-6, a software with the capacity to identify them is provided. Thus, a combined reference of both human and HHV-6 nucleic acids is created. This requires both a combined genome (.fa file) and transcriptome (.gtf).

5

Make joint reference genome

```
cat human_genome.fa > combined_genome.fa
```

```
cat hhv6_genome.fa >> combined_genome.fa
```

10

Create a joint reference transcriptome

```
cat human_genes.gtf > combined_genes.gtf
```

```
cat hhv6_genes.gtf >> combined_genes.gtf
```

Create a reference for single-cell analysis

15

We show two examples for this—cellranger (for 10x Genomics single-cell sequencing)

And STAR, which is more flexible for any single-cell sequencing chemistry).

```
cellranger mkref --genome=combined_genome --fasta=combined_genome.fa \
  --genes=combined_genes.gtf
```

20

```
STAR --runMode genomeGenerate \
```

```
  --genomeDir combined_genome \
```

```
  --genomeFastaFiles combined_genome.fa
```

```
  --sjdbGTFfile combined_genes.gtf
```

25

```
  --sjdbGTFtagExonParentTranscript Parent \
```

```
  --sjdbOverhang 99
```

Step 2: Align single-cell datasets to combined reference

Once this modified reference file is established, the single-cell sequencing library (e.g. sample_with_hhv6) is taken and read mapping is performed to look for molecules that align to the HHV-6B genome/transcriptome.

30

With CellRanger

```
cellranger count --id=sample_with_hhv6 \
```

35

```
  --fastqs=/path/to/fastqs \
```

```
  --sample=sample_with_hhv6 \
```

```
  --transcriptome=/path/to/step1_combined_reference/combined_genome
```

With STAR alone

40

```
STAR --genomeDir /path/to/combined_genome/ \
```

```
  sample_with_hhv6_R1.fastq.gz sample_with_hhv6_R2.fastq.gz \
```

```
  --soloType Droplet
```

Step 3: Count per single-cell reads mapping to the HHV-6B transcriptome

45

This can be achieved through a variety of means using different software environments, either from the processed scRNA-seq counts for from the resulting .bam file of the alignment. In either case, identifying potential super expressors or other HHV-6B+ cells requires summarizing the number of HHV-6B-associated reads by identifying single-cell barcodes with at least 10 HHV-6B reads.

50

Specific commands provided herein are executable in any major unix-based operating system assuming the executable files are installed. All software used are available as part of an open-source license.

Computer-readable media and computer devices related to the above-described methods are also provided. For example, provided are one or more non-transitory computer readable media having stored thereon instructions that cause a computer device to: align RNA sequence reads of single cells of a population of candidate human T cells to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences; and count the number of RNA sequence reads of single cells mapping to the HHV-6B RNA sequences of the reference. Similarly, provided is a computer device comprising one or more processors and one or more non-transitory computer readable media. The one or more non-transitory computer readable media have stored thereon instructions that cause the computer device to align RNA sequence reads of single cells of a population of human T cells to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences, and count the number of RNA sequence reads of single cells mapping to the HHV-6B RNA sequences of the reference.

According to some embodiments, provided are computer-implemented methods of assessing for HHV-6B reactivation in therapeutic T cells present in a subject receiving a T cell based therapy. Such methods comprise pseudoaligning RNA sequence reads of single cells of the therapeutic T cells to an HHV-6B reference index, removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome, and counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index. According to such methods, HHV-6B reactivation may be determined to be present in a cell of the therapeutic T cells when a threshold number of remaining RNA sequence reads are pseudoaligned to the HHV-6B RNA reference index. In certain embodiments, the highly homologous regions of the HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A. According to some embodiments, the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads. Shown in FIG. 9 is a flow diagram illustrating a non-limiting embodiment of such methods. According to this embodiment, the steps of the method may be performed as follows:

Step 1: Create HHV-6B reference index for pseudoalignment

The specific example here utilizes the kallisto | bustools environment for performing pseudoalignment, which is a k-mer based dictionary mapping utility. Other tools such as salmon and older tools like grep can also be used. For use with kallisto, an index must be built with a defined k-mer size (size 31 is used here, which is default):

```
kallisto index -k 31 -i HHV6b_transcriptome.idx AF157706.fa
```

Step 2: Pseudoalign scRNA-seq datasets to only the HHV-6B reference index

Next, every molecule from the scRNA-seq library is considered to infer whether a k-mer of length 31 is contained in the read using a fast pseudoalignment procedure. Additional commands using bustools are deployed to make the data more convenient for downstream interpretation. Here, these results are “raw” with no correction, meaning they will contain false-

positive off-target reads that are human derived but annotated to be derived from the HHV-6B virus.

Workflow with kallisto

```

5 i="sample_with_hhv6"
  r1="sample_with_hhv6_R1.fastq.gz"
  r2="sample_with_hhv6_R2.fastq.gz"
  (kallisto bus -i HHV6b_transcriptome.idx -o "${i}_kb" -t 8 -x 10xv2 $r1
    $r2) 2>"${i}.hhv6b.so.txt"
10 bustools sort -t 8 -o "${i}_kb2/output_sorted.bus" "${i}_kb/output.bus"
  bustools text "${i}_kb2/output_sorted.bus" -p > "${i}_HHV6b_nocorrection.txt"

```

Step 2b: Optional Mapping of scRNA-seq data to host transcriptome

While not required to identify HHV-6+ cells, this is a necessary step to derive host gene correlations with viral expression and/or reactivation. This can be performed using a standard command such as cellranger count:

```

15 cellranger count --id=sample_with_hhv6 \
  --fastqs=/path/to/fastqs \
  --sample=sample_with_hhv6 \
20 --transcriptome=/path/to/human_only/reference

```

Step 3: Remove reads mapping to highly homologous regions of HHV-6B and the human transcriptome

Returning to the output from step 2, excluded now are reads that map to the k-mer equivalence class that is redundant between the HHV-6B transcriptome and human reference genome. This occurs for the HHV-6B gene DR1 and the human gene KDM2A. In the upstream execution of the kallisto index (current version of AF157706 and k-mers of length 31), the equivalence classes 6 and 120 will be assigned all false positive reads. Thus, these molecules may be excluded from the quantifications via a simple exclusion criterion (i.e. column 3 of the step 2 output cannot be equal to 6 or 120). This exact exclusion can be performed by many software environments, but awk is used here for convenience within the unix environment.

```

cat "${i}_HHV6b_nocorrection.txt" | awk '$3 != 6 && $3 != 120 {print $0}'

```

For scRNA-seq libraries that have no HHV-6B expression, this will return nothing, which is the case for several entries in Table 1. However, for library with HHV-6 expression, this will return non-zero entries, which can be interpreted as real HHV-6B expression.

Step 4: Count number of HHV-6B aligned reads per single-cell barcode

This step similarly groups the HHV-6 expression by single-cell barcode to identify super expressor cells of HHV-6B transcripts. This works within the unix environment but can be similarly executed in various software environments.

```

45 cat "${i}_HHV6b_nocorrection.txt" | awk '$3 != 6 && $3 != 120 {print $0}' | \
  cut -f 1 | sort -gk 1 | uniq -c

```

Advantages of this method

- 0% false discovery rate of HHV-6+ cells when benchmarked on millions of cells and billions of sequencing reads from single-cell sequencing.
- Computationally more efficient, requiring only minutes of compute rather than hours/days in workflow 1. This enables massive scale screening of samples for HHV-6+ cells (noting again Table 1).
- Modularity of execution. Since our approach requires only a viral transcriptome to perform the first two steps, we can rapidly append other viruses or other pathogens into this workflow.

Computer-readable media and computer devices related to the above-described methods are also provided. For example, provided are one or more non-transitory computer readable media having stored thereon instructions that cause a computer device to: pseudoalign RNA sequence reads of single cells of therapeutic T cells to an HHV-6B reference index; remove RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and count the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index. Similarly, provided is a computer device comprising one or more processors and one or more non-transitory computer readable media. The one or more non-transitory computer readable media have stored thereon instructions that cause the computer device to pseudoalign RNA sequence reads of single cells of candidate therapeutic T cells to an HHV-6B reference index; remove RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and count the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index.

According to some embodiments, provided are computer-implemented methods of identifying a human therapeutic cell type susceptible to reactivation by a virus. Such methods comprise (i) obtaining from a sequencing database of nucleic acid sequencing samples having at least one annotated read from a virus known to infect humans; (ii) performing step (i) for one or more viruses known to infect humans; (iii) obtaining metadata for the annotated biosample that was linked to specific sequencing reads, wherein the metadata comprises sample name, descriptor, biosample ID, derived organism, or any combination thereof; and (iv) identifying relevant cell types for a specific virus and identifying virus-specific reactivation, or identifying viral reactivation for a specific cell type and identifying cell type-specific reactivation. Shown in FIG. 10 is a flow diagram illustrating a non-limiting embodiment of such methods. According to this embodiment, the steps of the method may be performed as follows:

Step 1: Perform a Serratus API call for a specific virus using a valid nuccore ID

Shown here is an example for NC_001401.1, the human adeno-associated virus. This command plugs into the Serratus API to stream all RNA-seq samples that have at least one annotated read being derived from the virus with the specific nuccore id.

```
wget -O NC_001401.1.txt
"https://api.serratus.io/matches/nucleotide?scoreMin=0&scoreMax=100&identityMin=50&identityMax=100&sequence=NC_001401.1"
```

The overall process of Step 1 requires executing the API call for multiple viruses, such as all of those that have been known to infect humans (see attached table).

Step 2: Annotate relevant meta data onto the Serratus hits

From this API call in Step 1, there will likely be hundreds to thousands or more 'hits' of RNA-seq libraries where one or more reads may be viral derived. Many of these will not be relevant for human samples. Explicitly, there are nearly 5.7 million RNA-seq libraries in Serratus, and it

is not feasible to investigate each of them without specific methodologies. Performed here is a secondary API call to the NCBI Gene Expression Omnibus to include meta data (i.e. sample names, descriptors, biosample IDs, derived organism, etc.). Second, it was determined that more stringent filtering of the Serratus values per RNA-seq sample most reliably yields high-confidence viral reactivation pairs. Explicitly required is a minimum of 100 reads in the overall library to be viral derived and with a minimum Serratus identity score of 50. These thresholds were found to be both sufficiently inclusive so as not to miss potential reactivation but that reduce the search space from 5.7M samples to ~6,000 for viruses from the Herpesviridae, Polyomaviridae, Adenoviridae, and Parvoviridae families.

```

10 library(data.table)
   library(dplyr)
   library(stringr)

15 # Import SRA meta data
   sra <- fread("../data/hu_SraRunInfo.csv.gz")
   nc_virus <- gsub(".txt", "", list.files("../data/nc_pulls/"))

20 pull_viral_info <- function(which_virus){
   virus <- fread(paste0("../data/nc_pulls/", which_virus, ".txt"))
   human_reactivation <- merge(virus, sra, by.x = "run_id", by.y = "Run")

   human_reactivation_filt <- human_reactivation %>%
     filter(n_reads >= 100 & score > 50) %>%
25   arrange(desc(n_reads)) %>%
     mutate(which_virus = which_virus)
   human_reactivation_filt
}

30 lapply(nc_virus, pull_viral_info) %>%
   rbindlist() %>% data.frame() -> all_viral_df
   table(all_viral_df$which_virus)
   all_viral_df$which_virus_short <- sapply(strsplit(all_viral_df$which_virus, "[.]"), function(x) x[1])

35 # Import virus annotation
   tbl <- fread("../data/Table_human_viruses.txt", header = FALSE)

   # Optional!
   # Grep for those that can be latent / reactivated
40   tbl <- tbl[grepl(c("Herpesviridae|Polyomaviridae|Parvoviridae|Adenoviridae"),tbl$V2),]
   tbl <- tbl[tbl$V6 != "Not available",]

   #-----
   # Chunk: annotate viral name
45   lapply(1:dim(tbl)[1], function(x){
     ncs <- str_trim(strsplit(tbl[["V6"]][x], ",")[1])
     lapply(ncs, function(one_nc){
       tbl$what <- one_nc
       tbl[x,]
50   }) %>% rbindlist()
   }) %>% rbindlist() -> all_annotations

   virus_vec <- all_annotations[["V1"]]; names(virus_vec) <- all_annotations[["what"]]
   all_viral_df$virus_name <- virus_vec[as.character(all_viral_df$which_virus_short)]

55   all_viral_df[is.na(all_viral_df$virus_name),c("virus_name", "BioSample")] %>%
     distinct() %>%

```

```

group_by(virus_name) %>% summarize(count = n()) -> new_count_df

# Count the hits per virus
cc <- as.character(new_count_df$virus_name)
5 new_count_df$virus_name <- factor(cc, levels = rev(cc))

write.table(new_count_df, file = "../output/source_data_bar_graph.tsv",
            sep = "\t", quote = FALSE, row.names = FALSE, col.names = TRUE)

10 # Filter for GEO samples
all_viral_df_geo <- all_viral_df[grepl("GSM", all_viral_df$SampleName),]

library(GEOquery)

15 # Deeper dive with GEO meta data
length(unique(all_viral_df_geo$SampleName))
table(all_viral_df_geo$virus_name)

all_viral_df_geo_get <- all_viral_df_geo[!(all_viral_df_geo$SampleName%in% gsub(".soft", "",
20 list.files("../metadata/"))),]
dim(all_viral_df_geo_get)

# Remove GSM IDs that cause problems with the filtering
bl <- c("GSM4467089", "GSM4467088", "GSM4467090", "GSM4467091")
25 all_viral_df_geo <- all_viral_df_geo[!c(all_viral_df_geo$SampleName %in% bl),]
titles_df <- lapply(all_viral_df_geo$SampleName, function(gsm){
  print(gsm)
  gds <- GEOquery::getGEO(gsm, destdir = "../metadata/")
  data.frame(gsm,
30 title = gds@header$title,
  name1 = gds@header$source_name_ch1
)
}) %>% rbindlist() %>% data.frame()

35 # merge Serratus data with GEO meta data to find enriched patterns
mdf <- merge(all_viral_df_geo, titles_df, by.x = "SampleName", by.y =
"gsm")[c("virus_name", "run_id", "SampleName", "score", "n_reads", "title", "name1")] %>%
  arrange(desc(n_reads))
data.frame(sort(table(mdf$name1))) %>% arrange(desc(Freq)) %>% head()
40

mdf$known_infection <- grepl("dpi|nfect|hpi|p.i.", mdf$title) | grepl("dpi|nfect|hpi|p.i.",
mdf$name1)

# Make T cell count
45 mdf[complete.cases(mdf) & (grepl("T-cell|T cell|Tcell|CD4T|CD8T|^T cell", mdf$name1) |
grepl("T-cell|T cell|Tcell|CD4T|CD8T|^T cell", mdf$title)),] %>% distinct()
mdf[grepl("T-cell|T cell|Tcell|CD4T|CD8T", mdf$name1) | grepl("T-cell|T
cell|Tcell|CD4T|CD8T", mdf$title),] %>% distinct()

50 # See all HHV6 hits
mdf %>% filter(virus_name == "Human herpesvirus 6")

# Look at the top hits per virus
mdf[complete.cases(mdf),] %>%
55 distinct() %>%
  group_by(virus_name) %>%
  filter(!known_infection) %>% top_n(5, wt = n_reads) %>%

```

```

arrange((virus_name)) %>%
data.frame() %>%
write.table(file = "../output/top_hits_per_virus.tsv", sep = "\t", quote = FALSE, row.names =
FALSE, col.names = TRUE)
5
# Export T cells
mdf[complete.cases(mdf) & (grepl("T-cell|\ T\ cell|Tcell|CD4T|CD8T|^T cell", mdf$name1) |
grepl("T-cell|\ T\ cell|Tcell|CD4T|CD8T|^T cell", mdf$title)),] %>% distinct() %>%
group_by(virus_name) %>%
10 write.table(file = "../output/Tcell_hits_per_virus.tsv", sep = "\t", quote = FALSE, row.names =
FALSE, col.names = TRUE)

# export iPSC cells
mdf[(grepl("iPSC|luripotent", mdf$name1) | grepl("iPSC|luripotent", mdf$title)) &
complete.cases(mdf),] %>% distinct() %>%
15 write.table(file = "../output/iPSC_hits_per_virus.tsv", sep = "\t", quote = FALSE, row.names =
FALSE, col.names = TRUE)

```

Step 3: Specific filtering of API output

20 To finally identify high-confidence annotations of viral reactivation in specific BioSamples, we must filter on one of two axes, outlined in Steps a and b. The attached code provides specific examples for both of these modes of filtering to nominate the highest likelihood viruses and cell types subject to reactivation.

25 Step 3a: Identification of virus-specific reactivation

To determine the cell types that a specific virus can become reactivated in we filtered for a specific virus (e.g. Human cytomegalovirus). Rather than examining only the virus by itself as an output for step 1, there are various RNA-seq samples that have reads mapping to dozens of viruses in low complexity repeat regions that we can exclude only when considering multiple viruses within the same analytical environment. Thus, there is an advantage to aggregation and filtering into the total data frame.

```
df %>% filter(which_virus %in% c( "Human cytomegalovirus"))
```

35 Step 3b: Identification of cell type-specific reactivation

Here, we try to identify any virus that may reactivate in a specific celltype (e.g. T cells, iPSCs, etc.). Using the concatenation of all viral+ RNA-seq samples, we perform regular expression filtering for a specific cell type. Examples of this for T cells are shown below:

```
40 df[grepl("T-cell|\ T\ cell|Tcell|CD4T|CD8T", mdf$name1) |
grepl("T-cell \ T\ cell|Tcell|CD4T|CD8T", mdf$title) ,] %>% distinct()
```

Viruses that may be screened according to the above-described methods include, but are not limited, to those in the table below:

Virus	Family
Adeno-associated virus	Dependovirus, Parvoviridae
Aichi virus	Kobuvirus, Picornaviridae
Australian bat lyssavirus	Lyssavirus, Rhabdoviridae
BK polyomavirus	Polyomavirus, Polyomaviridae

Banna virus	Seadornavirus, Reoviridae
Barmah forest virus	Alphavirus, Togaviridae
Bunyamwera virus	Orthobunyavirus, Bunyaviridae
Bunyavirus La Crosse	Orthobunyavirus, Bunyaviridae
Bunyavirus snowshoe hare	Orthobunyavirus, Bunyaviridae
Cercopithecine herpesvirus	Lymphocryptovirus, Herpesviridae
Chandipura virus	Vesiculovirus, Rhabdoviridae
Chikungunya virus	Alphavirus, Togaviridae
Cosavirus A	Cosavirus, Picornaviridae
Cowpox virus	Orthopoxvirus, Poxviridae
Coxsackievirus	Enterovirus, Picornaviridae
Crimean-Congo hemorrhagic fever virus	Nairovirus, Bunyaviridae
Dengue virus	Flavivirus, Flaviviridae
Dhori virus	Thogotovirus, Orthomyxoviridae
Dugbe virus	Nairovirus, Bunyaviridae
Duvenhage virus	Lyssavirus, Rhabdoviridae
Eastern equine encephalitis virus	Alphavirus, Togaviridae
Ebolavirus	Ebolavirus, Filoviridae
Echovirus	Enterovirus, Picornaviridae
Encephalomyocarditis virus	Cardiovirus, Picornaviridae
Epstein-Barr virus	Lymphocryptovirus, Herpesviridae
European bat lyssavirus	Lyssavirus, Rhabdovirus
GB virus C/Hepatitis G virus	Pegivirus, Flaviviridae
Hantaan virus	Hantavirus, Bunyaviridae
Hendra virus	Henipavirus, paramyxoviridae
Hepatitis A virus	Hepatovirus, picornaviridae
Hepatitis B virus	Orthohepadnavirus, Hepadnaviridae
Hepatitis C virus	Hepacivirus, Flaviviridae
Hepatitis E virus	Hepevirus, Unassigned
Hepatitis delta virus	Deltavirus, Unassigned
Horsepox virus	Orthopoxvirus, Poxviridae
Human adenovirus	Mastadenovirus, Adenoviridae

Human astrovirus	Mamastrovirus, Astroviridae
Human coronavirus	Alphacoronavirus, Coronaviridae
Human cytomegalovirus	Cytomegalovirus, Herpesviridae
Human enterovirus 68, 70	Enterovirus, Picornaviridae
Human herpesvirus 1	Simplexvirus, Herpesviridae
Human herpesvirus 2	Simplexvirus, Herpesviridae
Human herpesvirus 6	Roseolovirus, Herpesviridae
Human herpesvirus 7	Roseolovirus, Herpesviridae
Human herpesvirus 8	Rhadinovirus, Herpesviridae
Human immunodeficiency virus	Lentivirus, Retroviridae
Human papillomavirus 1	Mupapillomavirus, Papillomaviridae
Human papillomavirus 2	Alphapapillomavirus, Papillomaviridae
Human papillomavirus 16, 18	Alphapapillomavirus, Papillomaviridae
Human parainfluenza	Respirovirus, Paramyxoviridae
Human parvovirus B19	Erythrovirus, Parvoviridae
Human respiratory syncytial virus	Pneumovirus, Paramyxoviridae
Human rhinovirus	Enterovirus, Picornaviridae
Human SARS coronavirus	Betacoronavirus, Coronaviridae
Human spumaretrovirus	Spumavirus, Retroviridae
Human T-lymphotropic virus	Deltaretrovirus, Retroviridae
Human torovirus	Torovirus, Coronaviridae
Influenza A virus	Influenzavirus A, Orthomyxoviridae
Influenza B virus	Influenzavirus B, Orthomyxoviridae
Influenza C virus	Influenzavirus C, Orthomyxoviridae
Isfahan virus	Vesiculovirus, Rhabdoviridae
JC polyomavirus	Polyomavirus, Polyomaviridae
Japanese encephalitis virus	Flavivirus, Flaviviridae
Junin arenavirus	Arenavirus, Arenaviridae
KI Polyomavirus	Polyomavirus, Polyomaviridae
Kunjin virus	Flavivirus, Flaviviridae
Lagos bat virus	Lyssavirus, Rhabdoviridae
Lake Victoria marburgvirus	Marburgvirus, Filoviridae

Langat virus	Flavivirus, Flaviviridae
Lassa virus	Arenavirus, Arenaviridae
Lordsdale virus	Norovirus, Caliciviridae
Louping ill virus	Flavivirus, Flaviviridae
Lymphocytic choriomeningitis virus	Arenavirus, Arenaviridae
Machupo virus	Arenavirus, Arenaviridae
Mayaro virus	Alphavirus, Togaviridae
MERS coronavirus	Betacoronavirus, Coronaviridae
Measles virus	Morbilivirus, Paramyxoviridae
Mengo encephalomyocarditis virus	Cardiovirus, Picornaviridae
Merkel cell polyomavirus	Polyomavirus, Polyomaviridae
Mokola virus	Lyssavirus, Rhabdoviridae
Molluscum contagiosum virus	Molluscipoxvirus, Poxviridae
Monkeypox virus	Orthopoxvirus, Poxviridae
Mumps virus	Rubulavirus, Paramyxoviridae
Murray valley encephalitis virus	Flavivirus, Flaviviridae
New York virus	Hantavirus, Bunyavirus
Nipah virus	Henipavirus, Paramyxoviridae
Norwalk virus	Norovirus, Caliciviridae
O'Nyong-nyong virus	Alphavirus, Togaviridae
Orf virus	Parapoxvirus, Poxviridae
Oropouche virus	Orthobunyavirus, Bunyaviridae
Pichinde virus	Arenavirus, Arenaviridae
Poliovirus	Enterovirus, Picornaviridae
Punta toro phlebovirus	Phlebovirus, Bunyaviridae
Puumala virus	Hantavirus, Bunyavirus
Rabies virus	Lyssavirus, Rhabdoviridae
Rift valley fever virus	Phlebovirus, Bunyaviridae
Rosavirus A	Rosavirus, Picornaviridae
Ross river virus	Alphavirus, Togaviridae
Rotavirus A	Rotavirus, Reoviridae
Rotavirus B	Rotavirus, Reoviridae

Rotavirus C	Rotavirus, Reoviridae
Rubella virus	Rubivirus, Togaviridae
Sagiyama virus	Alphavirus, Togaviridae
Salivirus A	Salivirus, Picornaviridae
Sandfly fever sicilian virus	Phlebovirus, Bunyaviridae
Sapporo virus	Sapovirus, Caliciviridae
Semliki forest virus	Alphavirus, Togaviridae
Seoul virus	Hantavirus, Bunyavirus
Simian foamy virus	Spumavirus, Retroviridae
Simian virus 5	Rubulavirus, Paramyxoviridae
Sindbis virus	Alphavirus, Togaviridae
Southampton virus	Norovirus, Caliciviridae
St. louis encephalitis virus	Flavivirus, Flaviviridae
Tick-borne powassan virus	Flavivirus, Flaviviridae
Torque teno virus	Alphatorquevirus, Anelloviridae
Toscana virus	Phlebovirus, Bunyaviridae
Uukuniemi virus	Phlebovirus, Bunyaviridae
Vaccinia virus	Orthopoxvirus, Poxviridae
Varicella-zoster virus	Varicellovirus, Herpesviridae
Variola virus	Orthopoxvirus, Poxviridae
Venezuelan equine encephalitis virus	Alphavirus, Togaviridae
Vesicular stomatitis virus	Vesiculovirus, Rhabdoviridae
Western equine encephalitis virus	Alphavirus, Togaviridae
WU polyomavirus	Polyomavirus, Polyomaviridae
West Nile virus	Flavivirus, Flaviviridae
Yaba monkey tumor virus	Orthopoxvirus, Poxviridae
Yaba-like disease virus	Orthopoxvirus, Poxviridae
Yellow fever virus	Flavivirus, Flaviviridae
Zika virus	Flavivirus, Flaviviridae

A variety of processor-based systems may be employed to implement the embodiments of the present disclosure. Such systems may include system architecture wherein the components of the system are in electrical communication with each other using a bus. System

architecture can include a processing unit (CPU or processor), as well as a cache, that are variously coupled to the system bus. The bus couples various system components including system memory, (e.g., read only memory (ROM) and random access memory (RAM), to the processor.

5 System architecture can include a cache of high-speed memory connected directly with, in close proximity to, or integrated as part of the processor. System architecture can copy data from the memory and/or the storage device to the cache for quick access by the processor. In this way, the cache can provide a performance boost that avoids processor delays while waiting for data. These and other modules can control or be configured to control the processor to
10 perform various actions. Other system memory may be available for use as well. Memory can include multiple different types of memory with different performance characteristics. Processor can include any general purpose processor and a hardware module or software module, such as first, second and third modules stored in the storage device, configured to control the processor as well as a special-purpose processor where software instructions are incorporated into the
15 actual processor design. The processor may essentially be a completely self-contained computing system, containing multiple cores or processors, a bus, memory controller, cache, etc. A multi-core processor may be symmetric or asymmetric.

 To enable user interaction with the computing system architecture, an input device can represent any number of input mechanisms, such as a microphone for speech, a touch-sensitive
20 screen for gesture or graphical input, keyboard, mouse, motion input, speech and so forth. An output device can also be one or more of a number of output mechanisms. In some instances, multimodal systems can enable a user to provide multiple types of input to communicate with the computing system architecture. A communications interface can generally govern and manage the user input and system output. There is no restriction on operating on any particular hardware
25 arrangement and therefore the basic features here may easily be substituted for improved hardware or firmware arrangements as they are developed.

 The storage device is typically a non-volatile memory and can be a hard disk or other types of computer-readable media which can store data that are accessible by a computer, such as magnetic cassettes, flash memory cards, solid state memory devices, digital versatile disks,
30 cartridges, random access memories (RAMs), read only memory (ROM), and hybrids thereof.

 The storage device can include software modules for controlling the processor. Other hardware or software modules are contemplated. The storage device can be connected to the system bus. In one aspect, a hardware module that performs a particular function can include the software component stored in a computer-readable medium in connection with the necessary
35 hardware components, such as the processor, bus, output device, and so forth, to carry out various functions of the disclosed technology.

Embodiments within the scope of the present disclosure may also include tangible and/or non-transitory computer-readable storage media or devices for carrying or having computer-executable instructions or data structures stored thereon. Such tangible computer-readable storage devices can be any available device that can be accessed by a general purpose or
5 special purpose computer, including the functional design of any special purpose processor as described above. By way of example, and not limitation, such tangible computer-readable devices can include RAM, ROM, EEPROM, CD-ROM or other optical disk storage, magnetic disk storage or other magnetic storage devices, or any other device which can be used to carry or store desired program code in the form of computer-executable instructions, data structures, or
10 processor chip design. When information or instructions are provided via a network or another communications connection (either hardwired, wireless, or combination thereof) to a computer, the computer properly views the connection as a computer-readable medium. Thus, any such connection is properly termed a computer-readable medium. Combinations of the above should also be included within the scope of the computer-readable storage devices.

15 Computer-executable instructions include, for example, instructions and data which cause a general purpose computer, special purpose computer, or special purpose processing device to perform a certain function or group of functions. Computer-executable instructions also include program modules that are executed by computers in stand-alone or network environments. Generally, program modules include routines, programs, components, data structures, objects,
20 and the functions inherent in the design of special-purpose processors, etc. that perform tasks or implement abstract data types. Computer-executable instructions, associated data structures, and program modules represent examples of the program code means for executing steps of the methods disclosed herein. The particular sequence of such executable instructions or associated data structures represents examples of corresponding acts for implementing the functions
25 described in such steps.

Other embodiments of the disclosure may be practiced in network computing environments with many types of computer system configurations, including personal computers, hand-held devices, multi-processor systems, microprocessor-based or programmable consumer electronics, network PCs, minicomputers, mainframe computers, and the like. Embodiments may
30 also be practiced in distributed computing environments where tasks are performed by local and remote processing devices that are linked (either by hardwired links, wireless links, or by a combination thereof) through a communications network. In a distributed computing environment, program modules may be located in both local and remote memory storage devices.

The present disclosure also provides the following embodiments.

1. A method of monitoring a human subject receiving a T cell based therapy for HHV-6B reactivation in therapeutic T cells present in the subject, the method comprising:
obtaining from the subject a biological sample comprising the therapeutic T cells; and
assessing the therapeutic T cells for HHV-6B reactivation.
- 5 2. The method according to embodiment 1, wherein the biological sample is a peripheral blood sample obtained from the subject.
3. The method according to embodiment 1 or 2, wherein the obtaining and assessing steps are performed two or more times while subject is receiving the T cell based therapy.
4. The method according to any one of embodiments 1 to 3, wherein the assessing is by
10 quantitative nucleic acid sequencing.
5. The method according to embodiment 4, wherein the assessing is by single cell nucleic acid sequencing.
6. The method according to embodiment 5, wherein the single cell nucleic acid sequencing is single cell RNA sequencing (scRNA-Seq).
- 15 7. The method according to embodiment 6, wherein the assessing comprises:
aligning RNA sequence reads of single cells of the therapeutic T cells to a reference
comprising human genomic DNA sequences and HHV-6B RNA sequences; and
counting the number of RNA sequence reads of single cells mapping to the HHV-6B
RNA sequences of the reference,
20 wherein HHV-6B reactivation is determined to be present when a threshold number of
RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the
reference.
8. A computer-implemented method of assessing for HHV-6B reactivation in therapeutic T
cells present in a subject receiving a T cell based therapy, the method comprising:
25 aligning RNA sequence reads of single cells of the therapeutic T cells to a reference
comprising human genomic DNA sequences and HHV-6B RNA sequences; and
counting the number of RNA sequence reads of single cells mapping to the HHV-6B
RNA sequences of the reference,
wherein HHV-6B reactivation is determined to be present when a threshold number of
30 RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the
reference.
9. The method according to embodiment 7 or 8, wherein the threshold number of RNA
sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence
reads.
- 35 10. The method according to embodiment 6, wherein the assessing comprises:
pseudoaligning RNA sequence reads of single cells of the therapeutic T cells to an
HHV-6B reference index;

removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index,

5 wherein HHV-6B reactivation is determined to be present in a cell of the therapeutic T cells when a threshold number of remaining RNA sequence reads are pseudoaligned to the HHV-6B RNA reference index.

11. A computer-implemented method of assessing for HHV-6B reactivation in therapeutic T cells present in a subject receiving a T cell based therapy, the method comprising:

10 pseudoaligning RNA sequence reads of single cells of the therapeutic T cells to an HHV-6B reference index;
removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and
counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B
15 reference index,
wherein HHV-6B reactivation is determined to be present when a threshold number of remaining RNA sequence reads of a cell of the therapeutic T cells are pseudoaligned to the HHV-6B RNA reference index.

12. The method according to embodiment 10 or 11, wherein the highly homologous regions
20 of the HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A.

13. The method according to any one of embodiments 10 to 12, wherein the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

25 14. The method according to any one of embodiments 1 to 13, wherein when the assessment determines the presence of HHV-6B reactivation, the method further comprises ceasing the T cell based therapy.

15. The method according to embodiment 14, further comprising administering to the subject a new T cell based therapy with cells known to be HHV-6B negative.

16. The method according to any one of embodiments 1 to 15, wherein when the assessment determines the presence of HHV-6B reactivation, the method further comprises administering an antiviral therapy to the subject.

17. The method according to embodiment 16, wherein the antiviral therapy comprises
5 administering to the subject an agent approved for treating HHV-6B infection in humans.

18. The method according to embodiment 16 or embodiment 17, wherein the antiviral therapy comprises administering ganciclovir, cidofovir, foscarnet, or any combination thereof to the subject.

19. The method according to any one of embodiments 1 to 13, wherein when the
10 assessment determines the absence of HHV-6B reactivation, the method comprises continuing the T cell based therapy.

20. A method comprising assessing an *in vitro* culture comprising candidate therapeutic human T cells for HHV-6B reactivation, wherein the *in vitro* culture is assessed for a level of one or more HHV-6B analytes by quantitative nucleic acid sequencing.

15 21. The method according to embodiment 20, wherein the assessing is by single cell nucleic acid sequencing.

22. The method according to embodiment 21, wherein the single cell nucleic acid sequencing is single cell RNA sequencing (scRNA-Seq).

23. The method according to embodiment 22, wherein the assessing comprises:
20 aligning RNA sequence reads of single cells of the candidate therapeutic human T cells to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences; and
counting the number of RNA sequence reads of single cells mapping to the HHV-6B RNA sequences of the reference,
25 wherein HHV-6B reactivation is determined to be present when a threshold number of RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the reference.

24. A computer-implemented method of assessing an *in vitro* culture comprising candidate therapeutic human T cells for HHV-6B reactivation, the method comprising:
30 aligning RNA sequence reads of single cells of the candidate therapeutic human T cells to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences; and
counting the number of RNA sequence reads of single cells mapping to the HHV-6B RNA sequences of the reference,
35 wherein HHV-6B reactivation is determined to be present when a threshold number of RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the reference.

25. The method according to embodiment 23 or 24, wherein the threshold number of RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

26. The method according to embodiment 22, wherein the assessing comprises:

5 pseudoaligning RNA sequence reads of single cells of the candidate therapeutic human T cells to an HHV-6B reference index;

removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and

10 counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index,

wherein HHV-6B reactivation is determined to be present in a cell of the candidate therapeutic human T cells when a threshold number of remaining RNA sequence reads are pseudoaligned to the HHV-6B RNA reference index.

27. A computer-implemented method of assessing an *in vitro* culture comprising candidate therapeutic human T cells for HHV-6B reactivation, the method comprising:

15 pseudoaligning RNA sequence reads of single cells of the candidate therapeutic human T cells to an HHV-6B reference index;

removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and

20 counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index,

wherein HHV-6B reactivation is determined to be present when a threshold number of remaining RNA sequence reads of a cell of the candidate therapeutic human T cells are pseudoaligned to the HHV-6B RNA reference index.

25 28. The method according to embodiment 26 or 27, wherein the highly homologous regions of the HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A.

29. The method according to any one of embodiments 26 to 28, wherein the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or
30 about 10 RNA sequence reads.

30. The method according to any one of embodiments 20 to 29, wherein the candidate therapeutic human T cells comprise CD4+ human T cells.

31. The method according to any one of embodiments 20 to 30, wherein the candidate therapeutic human T cells are genetically modified.

35 32. The method according to embodiment 31, wherein the candidate therapeutic human T cells are genetically modified to express an engineered receptor.

33. The method according to embodiment 32, wherein the engineered receptor is a chimeric antigen receptor (CAR), an engineered T cell receptor (TCR), a chimeric cytokine receptor

(CCR), a chimeric chemokine receptor, a synthetic notch receptor (synNotch), a Modular Extracellular Sensor Architecture (MESA) receptor, a Tango receptor, a ChaCha receptor, or a generalized extracellular molecule sensor (GEMS) receptor.

34. The method according to embodiment 32, wherein the engineered receptor is a CAR.

5 35. The method according to any one of embodiments 32 to 34, wherein the engineered receptor comprises an extracellular binding domain that binds to a tumor antigen.

36. The method according to any one of embodiments 20 to 31, wherein the candidate therapeutic human T cells are not genetically modified to express an engineered receptor.

10 37. The method according to any one of embodiments 20 to 36, wherein the candidate therapeutic human T cells are being expanded during a therapeutic T cell manufacturing process.

38. The method according to embodiment 37, comprising assessing the *in vitro* culture for the one or more HHV-6B analytes two or more times during expansion of the candidate therapeutic human T cells.

15 39. The method according to embodiment 37 or 38, wherein the manufacturing process comprises activating the candidate therapeutic human T cells, and wherein the method comprises assessing the *in vitro* culture for the one or more HHV-6B analytes one or more times at from 0 to 20 days post-activation.

20 40. The method according to any one of embodiments 20 to 39, further comprising, when the assessing determines an absence of HHV-6B reactivation, identifying the candidate therapeutic human T cells as therapeutic T cells.

41. The method according to embodiment 40, further comprising administering to a subject in need thereof the therapeutic human T cells or progeny thereof.

25 42. The method according to any one of embodiments 20 to 39, further comprising, when the assessing determines the presence of HHV-6B reactivation, administering to a subject in need thereof therapeutic human T cells other than the candidate therapeutic human T cells of the *in vitro* culture, wherein HHV-6B reactivation is known to be absent in the administered therapeutic human T cells.

30 43. A computer-implemented method of identifying a human therapeutic cell type susceptible to reactivation by a virus, the method comprising:

(i) obtaining from a sequencing database of nucleic acid sequencing samples having at least one annotated read from a virus known to infect humans;

(ii) performing step (i) for one or more viruses known to infect humans;

35 (iii) obtaining metadata for the annotated biosample that was linked to specific sequencing reads, wherein the metadata comprises sample name, descriptor, biosample ID, derived organism, or any combination thereof; and

(iv) identify relevant cell types for a specific virus and identifying virus-specific reactivation, or identify viral reactivation for a specific cell type and identifying cell type-specific reactivation.

44. A method of monitoring a therapeutic T cell product for HHV-6B reactivation, the method comprising:

administering a first population of therapeutic T cells from the therapeutic T cell product to a subject in need thereof;

culturing a second population of therapeutic T cells from the therapeutic T cell product, wherein the culturing comprises culturing the therapeutic T cells during a period subsequent to administration of the first population of therapeutic T cells to the subject; and

during the period subsequent to administration of the first population of therapeutic T cells to the subject, monitoring the T cell culture for HHV-6B reactivation.

45. The method according to embodiment 44, wherein the monitoring is by quantitative nucleic acid sequencing.

46. The method according to embodiment 45, wherein the monitoring is by single cell nucleic acid sequencing.

47. The method according to embodiment 46, wherein the single cell nucleic acid sequencing is single cell RNA sequencing (scRNA-Seq).

48. The method according to embodiment 47, wherein the monitoring comprises: aligning RNA sequence reads of single cells of the therapeutic T cell product to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences; and

counting the number of RNA sequence reads of single cells mapping to the HHV-6B RNA sequences of the reference,

wherein HHV-6B reactivation is determined to be present when a threshold number of RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the reference.

49. A computer-implemented method of monitoring a therapeutic T cell product for HHV-6B reactivation, the method comprising:

aligning RNA sequence reads of single cells of the therapeutic T cell product to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences; and

counting the number of RNA sequence reads of single cells mapping to the HHV-6B RNA sequences of the reference,

wherein HHV-6B reactivation is determined to be present when a threshold number of RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the reference.

50. The method according to embodiment 48 or 49, wherein the threshold number of RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

51. The method according to embodiment 47, wherein the monitoring comprises:

5 pseudoaligning RNA sequence reads of single cells of the therapeutic T cell product to an HHV-6B reference index;
removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and
counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B
10 reference index,
wherein HHV-6B reactivation is determined to be present in a cell of the therapeutic T cell product when a threshold number of remaining RNA sequence reads are pseudoaligned to the HHV-6B RNA reference index.

52. A computer-implemented method of monitoring a therapeutic T cell product for HHV-6B
15 reactivation, the method comprising:

pseudoaligning RNA sequence reads of single cells of the therapeutic T cell product to an HHV-6B reference index;
removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and
20 counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index,
wherein HHV-6B reactivation is determined to be present when a threshold number of remaining RNA sequence reads of a cell of the therapeutic T cell product are pseudoaligned to the HHV-6B RNA reference index.

25 53. The method according to embodiment 51 or 52, wherein the highly homologous regions of the HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A.

54. The method according to any one of embodiments 51 to 53, wherein the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or
30 about 10 RNA sequence reads.

55. A method of preventing or mitigating viral reactivation in therapeutic human cells during a therapeutic human cell manufacturing process, the method comprising expanding the

therapeutic human cells in the presence of an anti-viral agent at a concentration effective to prevent or mitigate viral reactivation in the expanding therapeutic human cells.

56. The method according to embodiment 55, wherein the therapeutic human cells are therapeutic human T cells.

5 57. The method according to embodiment 56, wherein HHV-6A, HHV-6B, and/or HHV-7 viral reactivation is prevented or mitigated.

58. The method according to embodiment 56 or embodiment 57, wherein the anti-viral agent is selected from ganciclovir, cidofovir, foscarnet, or any combination thereof.

59. The method according to embodiment 55, wherein the therapeutic human cells are
10 therapeutic human induced pluripotent stem cells (iPSCs).

60. The method according to embodiment 59, wherein HSV-1 viral reactivation is prevented or mitigated.

61. The method according to any one of embodiments 55 to 60, wherein the therapeutic human cells are genetically modified.

15 62. The method according to embodiment 61, wherein the therapeutic human cells are genetically modified to express an engineered receptor.

63. The method according to embodiment 62, wherein the engineered receptor is a chimeric antigen receptor (CAR), an engineered T cell receptor (TCR), a chimeric cytokine receptor (CCR), a chimeric chemokine receptor, a synthetic notch receptor (synNotch), a Modular
20 Extracellular Sensor Architecture (MESA) receptor, a Tango receptor, a ChaCha receptor, or a generalized extracellular molecule sensor (GEMS) receptor.

64. The method according to embodiment 62, wherein the engineered receptor is a CAR.

65. The method according to any one of embodiments 62 to 64, wherein the engineered receptor comprises an extracellular binding domain that binds to a tumor antigen.

25 66. The method according to any one of embodiments 55 to 61, wherein the therapeutic human cells are not genetically modified to express an engineered receptor.

67. The method according to any one of embodiments 55 to 66, further comprising administering the expanded therapeutic human cells to a subject in need thereof.

30 68. One or more non-transitory computer readable media having stored thereon instructions that cause a computer device to:

align RNA sequence reads of single T cells to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences; and

count the number of RNA sequence reads of single T cells mapping to the HHV-6B RNA sequences of the reference,

35 wherein HHV-6B reactivation is determined to be present when a threshold number of RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the reference.

69. The one or more non-transitory computer readable media of embodiment 68, wherein the threshold number of RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

70. A computer device comprising
5 one or more processors; and
one or more non-transitory computer readable media having stored thereon instructions that cause the computer device to:

align RNA sequence reads of single T cells to a reference comprising human
genomic DNA sequences and HHV-6B RNA sequences; and

10 count the number of RNA sequence reads of single T cells mapping to the HHV-
6B RNA sequences of the reference,

wherein HHV-6B reactivation is determined to be present when a threshold
number of RNA sequence reads from a single cell map to the HHV-6B RNA
sequences of the reference.

71. The computer device of embodiment 70, wherein the threshold number of RNA
15 sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence
reads.

72. One or more non-transitory computer readable media having stored thereon instructions
that cause a computer device to:

20 pseudoalign RNA sequence reads of single cells of therapeutic T cells to an HHV-6B
reference index;

remove RNA sequence reads pseudoaligned to highly homologous regions of the HHV-
6B reference index and human T cell transcriptome; and

count the number of remaining RNA sequence reads pseudoaligned to the HHV-6B
25 reference index.

73. The one or more non-transitory computer readable media of embodiment 72, wherein
the highly homologous regions of the HHV-6B reference index and human T cell transcriptome
comprise a homologous region between HHV-6B DR1 and human KDM2A.

74. The one or more non-transitory computer readable media of embodiment 72 or
30 embodiment 73, wherein the instructions further cause the computer device to assess the
single cells for HHV-6B reactivation, wherein HHV-6B reactivation is determined to be present
when a threshold number of remaining RNA sequence reads for a cell of the therapeutic T cells
are pseudoaligned to the HHV-6B RNA reference index.

75. The one or more non-transitory computer readable media of embodiment 74, wherein
35 the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to
12, 9 to 11, or about 10 RNA sequence reads.

76. A computer device comprising
one or more processors; and

one or more non-transitory computer readable media having stored thereon instructions that cause the computer device to:

pseudoalign RNA sequence reads of single cells of candidate therapeutic T cells to an HHV-6B reference index;

5 remove RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and
count the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index.

77. The computer device of embodiment 76, wherein the highly homologous regions of the
10 HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A.

78. The computer device of embodiment 76 or embodiment 77, wherein the instructions further cause the computer device to assess the single cells for HHV-6B reactivation, wherein HHV-6B reactivation is determined to be present when a threshold number of remaining RNA
15 sequence reads of a cell of the candidate therapeutic T cells are pseudoaligned to the HHV-6B RNA reference index.

79. The computer device of embodiment 78, wherein the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

20 80. One or more non-transitory computer readable media having stored thereon instructions that cause a computer device to:

(i) obtain from a sequencing database of nucleic acid sequencing samples having at least one annotated read from a virus known to infect humans;

(ii) perform step (i) for one or more viruses known to infect humans;

25 (iii) obtain metadata for the annotated biosample that was linked to specific sequencing reads, wherein the metadata comprises sample name, descriptor, biosample ID, derived organism, or any combination thereof; and

(iv) identify relevant cell types for a specific virus and identifying virus-specific reactivation, or identify viral reactivation for a specific cell type and identifying cell
30 type-specific reactivation.

81. A computer device, comprising:
one or more processors; and
one or more non-transitory computer readable media having stored thereon instructions that cause the computer device to:

35 (i) obtain from a sequencing database of nucleic acid sequencing samples having at least one annotated read from a virus known to infect humans;

(ii) perform step (i) for one or more viruses known to infect humans;

- (iii) obtain metadata for the annotated biosample that was linked to specific sequencing reads, wherein the metadata comprises sample name, descriptor, biosample ID, derived organism, or any combination thereof; and
- (iv) identify relevant cell types for a specific virus and identifying virus-specific reactivation, or identify viral reactivation for a specific cell type and identifying cell type-specific reactivation.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Cell therapies have yielded durable clinical benefits for patients with cancer but have been accompanied by unexpected side effects of treatment, including neurotoxicity. While modes of antigen-dependent toxicity have been characterized¹⁻³, a comprehensive understanding of the antigen-independent mechanisms of toxicity regularly observed in clinical trials is lacking. For example, at least eight patients have been reported to experience encephalitis caused by human herpesvirus 6 (HHV-6)⁴. However, the source of this virus is unknown. Described herein is comprehensive viral RNA data mining to examine the landscape of human latent viral reactivation, which surprisingly revealed that HHV-6B can become reactivated in human CD4+ T cells in standard *in vitro* cultures. Using single-cell sequencing, a rare population of HHV-6 'super-expressors' (~1 in 360-10,000 cells) that possess high viral transcriptional activity were identified in chimeric antigen receptor (CAR) T cell culture before spreading rapidly to infect other cells *in vitro*. Further, through the reanalysis of single-cell sequencing data from FDA-approved cell therapy products⁵, the presence of CAR+, HHV-6 super-expressor T cells were identified in human patients *in vivo*. Together, the present examples implicate cell therapy products as the source of lytic HHV-6 repeatedly reported in clinical trials^{4,6-8} which has broad implications for the design, screening, and interpretation of unexpected toxicities in cell therapies.

Example 1 – HHV-6 is reactivated in human T cells

During the first years of life, humans become exposed to endemic pathogens that can often infect nearly 100% of the population.^{9,10} Though primary infection typically clears with sub-clinical symptoms, viruses from the *Herpesviridae*, *Polyomaviridae*, *Adenoviridae*, and *Parvoviridae* families all can become 'quiescent,' resulting in a latent phase of the virus that is stably maintained in healthy individuals¹⁰. However, under acute stress conditions, such as fever, hematopoietic stem cell transplant (HSCT)¹¹, or trauma⁹, latent viruses can become reactivated, leading to a variety of complex clinical manifestations¹² (FIG. 1A). Though anecdotes of latent viral reactivation have been reported, a complete understanding of the factors and mechanisms underlying latency and reactivation is lacking. Further, studies of viral latency and reactivation in emerging therapeutic modalities, such as cell therapy, have not been characterized.

In an effort to study human viral reactivation systematically, a petabase-scale resource, Serratus¹³, that quantified viral abundance from RNA sequencing data from the Sequence Read Archive (SRA) was mined. Here, the occurrence of high-confidence viral RNA from 129 curated human viruses¹⁴ was considered, focusing on the 19 viruses across the *Herpesviridae*,
 5 *Polyomaviridae*, *Adenoviridae*, and *Parvoviridae* families that are known to have lytic replication cycles and reactivate¹⁰ (Methods). For 15 of these 19 viruses, a high confidence annotation of viral RNA in a BioSample was observed, totaling 5,724 samples with plausible viral RNA expression, and in turn, lytic reactivation (FIG. 1b; Methods). After defining this landscape, examined were samples with the highest levels of viral RNA, which predominantly encompassed
 10 Epstein-Barr Virus (EBV) expression in B cells or intentional over-expression of a specific virus in a model cell line system. Surprisingly, it was observed that for human herpesvirus 6 (HHV-6), the top positive sample came from primary T cell culture where no record of the HHV-6 virus was noted in the original publication, suggesting that the virus may have been reactivated (FIG. 1c; Methods). Further, high levels of HHV-6 expression from various independent studies of T cells
 15 with and without *ex vivo* culture were observed, prompting a closer examination of a potential link between HHV-6 reactivation and primary T cells.

Whereas HHV-6A infections are largely confined to sub-Saharan Africa¹⁵, the HHV-6B virus accounts for 97-100% of HHV-6 infections reported in the United States, United Kingdom, and Japan. The canonical receptor for HHV-6B, TNFRSF4 (CD134 or OX40), is upregulated
 20 during both CD4 and CD8 T cell activation (among other healthy tissues; FIG. 4, 2), supporting the notion that this viral strain could spread during *in vitro* T cell culture and expansion.¹⁶ Sequencing data from the two studies with high levels of HHV-6^{17,18} that performed longitudinal sampling of cultured T cells in standard conditions (anti-CD3/anti-CD28 bead activation with IL-2) was examined to understand the kinetics of viral reactivation in T cell cultures. First, Shytaj *et al.*¹⁸ cultured total CD4+ T cells for 14 days from three separate donors where cells were initially
 25 treated with either an HIV or mock infection (transcriptional homology between HIV and HHV-6B could not account for the observed HHV-6B expression). Here, two donors (14 and 49) showed clear evidence of HHV-6 reactivation over the 2 weeks in culture, including a sample where nearly 3.5% of all RNA molecules were derived from HHV-6B and mapped to virtually all of the HHV-6B transcripts (FIG. 1d; FIG. 6a). Separately, analysis of the LaMere *et al.*¹⁷ study, in which naive and memory CD4+ T cells were separated and cultured for two weeks before profiling with RNA-seq (FIG. 1e; FIG. 6b) and chromatin immunoprecipitation with sequencing (ChIP-seq; FIG. 1f,g) indicated that one donor expressed HHV-6B transcripts at high levels (~4.27% of all RNAs) after
 30 2 weeks of memory CD4+ T cell culture *in vitro*. As this study utilized the same cell cultures to perform ChIP-seq against a variety of histone modifications, sequencing reads were remapped to estimate the abundance of HHV-6 derived DNA from these samples (Methods). It was found that all three donors increased their HHV-6B DNA copy number over the duration of culture, including donor 5131 that had the highest viral copy number for both DNA and RNA (FIG. 1e,f;

Methods). It was confirmed that these mapped DNA molecules were derived from the HHV-6B virus, noting the uniform coverage across the HHV-6B reference genome outside repeat regions (FIG. 1g; Methods) and across all transcripts. Finally, while the petabase-scale mining identified HHV-6 as a latent virus that may be reactivated in T cells *in vitro*, BioSamples with high HHV-6
5 derived from patients with Bronchiolitis¹⁹, graft versus host disease (GvHD)²⁰, and purified T cell populations from patients with cutaneous T-cell lymphoma (CTCL)²¹ were also observed (FIG. 6c,d). In summary, analysis of these public datasets implicates the reactivation and lytic activity of HHV-6B in T cells *in vitro* and *in vivo*.

Example 2 – Identification of rare HHV-6 expressing cells in CAR T cell culture

10 In light of eight cases of HHV-6 encephalitis from patients receiving CAR T cell therapy, including in three clinical trials,^{4,6-8} it was hypothesized that the lytic HHV-6 may be derived from cell therapy products. Using research grade CAR T cell culture conditions, cells were cultured over a 19 day period and screened by qPCR to detect the U31 transcript from the HHV-6B virus (Methods). Over the course of screening, four exemplar donors that by day 19 expressed a range
15 of HHV-6B expression from 0.0015-0.90 copies of U31 per cell were identified (FIG. 2a). For two of these donors, longitudinal qPCR values from the cultures were also collected, which demonstrated low, if any, HHV-6 expression before a rapid increase after approximately two weeks in culture (FIG. 2b). These data directly support the observations from the Serratus analysis that HHV-6 can become more transcriptionally active in T cell cultures over time.

20 Sought next was a better understanding of the dynamics and cell states of reactivated HHV-6B during these CAR T cell cultures. Assessed specifically was whether HHV-6 would be uniformly reactivated in positive samples (Model 1) or enriched in only a subset of cells that spread the virus throughout the culture (Model 2; FIG. 2c). Consistent with recent efforts that quantify both viral and host gene expression at a single-cell resolution²², an efficient
25 pseudoalignment pipeline after single-cell RNA-seq (scRNA-seq) was established to quantify HHV-6B transcripts with no detectable false positive quantification (Methods). Strikingly, this single-cell characterization revealed the presence of rare HHV-6+ cells sometimes referred to herein as 'super-expressors', directly supporting Model 2 where only a small percent of cells initially express HHV-6B (FIG. 2c; FIG. 7a). Expression was detected in 90 HHV-6B transcripts,
30 including super-expressors with predominant expression of immediate early (IE) genes, suggesting that these cells had recently been infected or had reactivated HHV-6B (FIG. 7b). In total, these rare super-expressors account for only 0.2% of total cells in culture for donor D98 despite containing a vast majority (99.2%) of HHV-6 viral RNA (FIG. 2d). Across three total donors, the existence of this rare cell population was confirmed as between 0.01%-0.3% (or 1 in
35 360-10,000) of cells that reactivate or express HHV-6 transcripts at high levels was identified. Utilizing the host (T cell) gene expression signatures, standard dimensionality reduction and annotation was performed and revealed that super-expressors were confined to the CD4+ T cell

compartment, consistent with OX40 expression in T cell subsets (FIG. 4a; FIG. 7c; Methods). A fourth donor (D38) had no detectable HHV-6B^{hi} cells, corresponding to the lowest U31 RNA qPCR value (donor D38) (FIG. 2d). Sequencing of TCR α/β chain transcripts revealed that none of these HHV-6B⁺ cells from these donors shared a clonotype receptor sequence (FIG. 2e). As both viral and host gene expression could be quantified in single-cells, host genes could be further linked with HHV-6B expression (Methods). Differential gene expression of HHV-6 super-expressors compared to other HHV-6B⁻ cells showed a consistent up-regulation of lymphotoxin α (LT α) and downregulation of lymphotoxin β (LT β ; FIG. 7d; Methods), but host factors, such as surface genes that would clearly segregate cells capable of HHV-6 reactivation from other cells, were unable to be nominated.

For two donors, D34 and D61, the time of T cell expansion was extended followed by collection of scRNA-seq libraries after a total of 25 and/or 27 total days from the initial conditions. Analysis of these later time points revealed an increase in abundance of HHV-6B transcripts by ~1200-2000-fold, compared to day 20 (FIG. 2f). At these later timepoints, it was determined that 49% and 62% of all cells were HHV-6 super-expressors, indicating that the lytic reactivation of the virus spread rapidly in this culture, suggesting that HHV-6 can rapidly spread and overtake certain T cell cultures (FIG. 2g; FIG. 7e). In these samples with greater numbers of HHV-6B⁺ cells, a correlation statistic was utilized to identify hundreds of genes associated with HHV-6B reinfection in culture (FIG. 2h). Of note, the results included proviral genes like HSPD1 and SLC3A2 that have been characterized as positive regulators of in other infections^{23,24} (FIG. 2h). Conversely, the results showed upregulation of genes such as LY6E²⁵, STAT1²⁶, and IL-32²⁷ in cells low for HHV-6B, each of which has previously been implicated in antiviral control for other diverse infection contexts (FIG. 2h). Further, after stratifying the HHV-6B transcripts into IE, early, and late using a prior annotation²⁸, the only gene signature correlated with OX40 expression in single-cells was the IE signature, aligning with the expectation that recently infected cells would express higher levels of the viral receptor (FIG. 7f; Methods). Integrated analyses of altered gene expression signatures associated with HHV-6B expression levels corroborated roles of various pro- and antiviral pathways, including type I interferon response²⁹ and oxidative phosphorylation³⁰ (FIG. 2i; Methods). Collectively, the host-viral association analyses indicate that these T cells in culture are transcriptionally inducing an anti-viral response, spanning metabolic transporters, transcription factors, cytokines, and surface molecules. Ultimately, despite these associations, an actionable molecular target capable of removing HHV-6-expressing cells from culture was not nominated.

Example 3 – Clinically validated and FDA-approved CAR T cells harbor HHV-6 *in vivo*

Having characterized this rare HHV-6⁺ population in T cell culture, it was hypothesized that these cells could be a potential source of HHV-6 previously reported in CAR T trials and treatments. ^{4,6-8} To test this, three cohorts of scRNA-seq datasets were collected from B cell

cancer patients receiving one of three CAR T cell products, including U.S. Food and Drug Administration (FDA) approved axicabtagene ciloleucel (axi-cel) and tisagenlecleucel (tisa-cel), as well as the clinically-validated SJCAR19 therapy. It should be noted that all CAR T cells were engineered to target the CD19 antigen.

5 Across these three cohorts, 924,694 cells from 72 patients were screened for HHV-6B expression, including T cells from the pre-infusion products and cells from peripheral blood draws weeks after the infusion (FIG. 3a; Table 1). While HHV-6+ cells were not in the pre-infusion products, a total of 13 cells from 4 donors expressed 1 or more HHV-6B transcripts in the follow-up blood draws, including eight 'super-expressor'-like cells (FIG. 3b; see Methods for discussion
10 of cells with 1 or 2 UMIs). Through a combination of CAR protein, CAR transcript, TCR α/β transcripts, and CD3 expression, the cellular identities of these HHV-6B+ cells were verified as engineered T cells (FIG. 3b; Methods). It was additionally confirmed that these cells expressed a diversity of HHV-6B-associated transcripts among these *in vivo* super expressors (FIG. 3c). Thus, having confirmed the presence of HHV-6B+, CAR+ T cells *in vivo*, further analyses were
15 performed on the two patients with detectable super-expressors from the post-infusion blood draws.

First, additional analysis was performed on samples from the subject, axi-R-15, that contained cells with the highest number of HHV-6B transcripts. The existing scRNA-seq profiles were complemented by generating another 31,953 cells for this sample spanning four timepoints,
20 including weekly blood draws out to day +21 (FIG. 3d; Methods). Along this time course, HHV-6+ cells were observed only at day 7 where the HHV6+ super-expressors were present with an overall frequency of ~1 in 1,000 T cells (a total of 10 super-expressors were detected among 9,757 T cell scRNA-seq profiles). Notably, this patient was clinically diagnosed with immune effector associated neurotoxicity syndrome (ICANS) following axi-cel treatment and subsequently
25 developed altered mental status with transient hypotension beginning on day +9, which peaked on day +12 (grade 3a by CTCAE criteria, grade 2 by ASTCT) before returning to baseline by day +14 (FIG. 3d). Similarly, a spike in HHV-6+ cells at day +7 was observed that was resolved by day +14.

Second, three HHV-6 super-expressors were detected in cohort 3 from SJCAR19-09.
30 Though two patients were tested for HHV-6 via qPCR from this cohort, only SJCAR19-09 tested positive (FIG. 3e), corroborating the detection of HHV6+ CAR T cells from this individual. SJCAR19-09 tested positive via qPCR at day +21 post- CAR T cell infusion and was started on Foscarnet at day +24 (FIG. 3e). After another positive qPCR test on day +27, the patient did not have detectable levels of HHV-6 at day +34 (FIG. 3e). Similarly, HHV6+ super-expressors at
35 days +14 were detected that persisted *in vivo* until day +21 before these cells were undetectable at day 27. As the products used in cohorts 2 and 3 were cultured for 7-10 days before infusion, one can infer that HHV-6 may be reactivated in some cells when the therapy is administered but the virus was not yet transcriptionally active or abundant enough to be detected via single-cell

sequencing. However, after 1-2 weeks of persistence and expansion *in vivo*, these rare HHV6+ super expressors were detectable in peripheral blood draws. Importantly, the SJCAR19-09 case vignette suggests that Foscarnet may be an efficacious agent to mitigate the effects of viral infection during the course of therapy if HHV-6 expressing cells are identified during the course of treatment with CAR T cells.

Table 1 – Summary of HHV-6B expression in large-scale single-cell datasets

Description	# Cells	# Reads	#HHV-6B UMIs	#HHV-6+ cells
24 patient CAR T cell pre-infusion product	137,326	8,972,716,245	0	0
32 patient (CAR) T cell, pre- and post- infusion	602,577	15,403,016,654	919	7
16 patient (CAR) T cell, pre- and post- infusion	184,791	81,875,146,341	374	6
Human cell landscape, 60 tissue types	599,926	15,680,467,351	0	0
~1,000 donor PBMCs	1,449,385	53,872,337,003	6	0

Though the population of HHV-6+ CAR T cells from these patients are rare, these results directly link the CAR T cell therapy to a potential cellular reservoir of lytic HHV-6 *in vivo*. Nevertheless, other possible cellular sources of viral expression were examined throughout human tissues and estimated HHV-6B transcript abundance from large consortiums profiled with scRNA-seq (Table 1). First, it was confirmed that among ~1,000 peripheral blood mononuclear cell (PBMC) samples, each stemming from a different donor, did not harbor any HHV-6+ cells (Table 1) or from the apheresis sequencing samples from other CAR T datasets. These analyses suggest that HHV-6 is not transcriptionally active in peripheral blood and unlikely to be the source of HHV-6 without the T cell therapy culture *in vitro*. The distinction should be noted that while nearly 100% of individuals in North America (where most of the T cells were sourced for these studies) are seropositive for prior HHV-6 infection and often have detectable viral DNA from PCR, the results indicate that transcriptionally active virus is a rare phenomenon in peripheral blood of adults. Second, the human cell landscape (Han et al. Nature 2020), consisting of >700,000 cells from 60 tissue types (Methods). A single HHV-6B transcript in this dataset was not detected. (Table 1). Though a variety of epithelial cells express the OX40 receptor (FIG. 5), this negative result further supports the possibility that lytic HHV-6 in patients with encephalitis comes from the cell therapy.

The present work implicates the cell therapy as the source of a lytic virus, HHV-6. From the inferences using the Serratus database, latent viral reactivation may be limited but is likely to

extend beyond HHV-6 and T cells to other viruses or forms of cell therapy. For example, reactivation of HSV-1 from reprogrammed induced pluripotent stem cells (iPSCs) in 18 RNA-seq libraries, as well as EBV expression in T cells (FIG. 1c), was observed. The EBV receptor, CD21, is expressed in gamma-delta T cells at levels exceeding B cell subsets in resting and stimulated conditions (FIG. 4b), raising the possibility that EBV may become reactivated in gamma-delta T cell therapies. Further, an increase of lytic HHV-6 was observed over time both *in vitro* (FIG. 2) and *in vivo* (FIG. 3), indicating that screening the cell therapy product for live virus at a single time point (e.g., pre-infusion product) may not be fully adequate to eliminate virus-positive cells from the final therapeutic. Indeed, it was determined that while HHV-6 may initially be transcriptionally active in a subset of cells, in a matter of 4-5 days, these rare cells can infect and overwhelm the remaining cells in culture, leading to a majority of cells to become HHV-6+ (FIG. 2g). This result suggests that longitudinal viral screening may be warranted both in patients receiving cell therapies as well as the therapeutic product itself. Taken together, the present work demonstrates the utility of comprehensive genomics sequencing and analyses to disentangle complex clinical phenotypes and identifies a previously unknown route of viral infection via cell therapy.

Example 4 – HHV-6 is reactivated in clinical autologous CAR T cell products

Having characterized HHV-6+ CAR T cells *in vitro*, it was hypothesized that these cells could be a potential source of HHV-6 encephalitis previously reported in CAR T trials and case studies. To assess this, three cohorts of scRNA-seq datasets collected from B cell lymphoma and leukemia patients receiving one of three autologous CAR T cell products (all targeting CD19 antigen), including U.S. Food and Drug Administration (FDA) approved axicabtagene ciloleucel (axi-cel) and tisagenlecleucel (tisa-cel), as well as from one early phase clinical study (SJCAR19; NCT03573700), were reanalyzed. Across these three cohorts, screened were single-cell expression profiles of 619,392 CAR T cells from pre-infusion products and 829,535 cells from peripheral blood draws weeks after the infusion. Of the 72 patient products, no HHV-6 viral transcripts were detected through reanalysis of the pre-infusion products. However, a total of 28 cells were detected that expressed one or more HHV-6B transcripts from 4 out of 35 donors with high-quality post-infusion scRNA-seq profiles, including thirteen 'super-expressor'-like cells. Using CAR protein (from flow-enriched cells), CAR transcript, TCR α/β transcripts, and CD3 expression, the cellular identity of 28 total HHV-6B+ cells were verified as engineered CAR T cells that expressed a diversity of HHV-6B transcripts. Having confirmed the presence of HHV-6B+, CAR+ T cells *in vivo*, the course of two patients with super-expressors in the post-infusion blood draws was more closely examined.

Examined first were additional samples from the subject, axi-R-15, whose cells showed the highest number of captured HHV-6B transcripts. The existing scRNA-seq profiles were complemented by sequencing another 31,953 cells for this subject spanning four time points,

including weekly blood draws out to day +21. Along this time course, HHV-6+ cells were observed only at day 7 where the HHV-6+ super-expressors were present with an overall frequency of ~1 in 1,000 T cells (a total of 10 super-expressors were detected among 9,757 T cell scRNA-seq profiles). Notably, this patient was clinically diagnosed with immune effector-associated neurotoxicity syndrome (ICANS) following axi-cel treatment and subsequently developed altered mental status with tremulousness and word-finding difficulties beginning on day +9, which peaked on day +12 (grade 3a by CTCAE criteria, grade 2 by ASTCT) before returning to baseline by day +14. Similarly, a spike in HHV-6+ cells was observed at day +7 that was resolved by day +14, and the dynamics of viral reactivation and clearance could be observed within 1 week of each other. Though the symptoms correlated with the presence of HHV-6+ CAR T cells in the blood, it could not be concluded that the detection of HHV-6 in peripheral blood is causal for the patient's neurotoxicity. As delirium and neurocognitive decline are common symptoms in patients with HHV-6 viremia receiving HSCT, whereas ICANS is not, HHV-6 is unlikely to be the pathogenic agent for ICANS in most patients receiving CAR T cell therapy.

In a second subject, three HHV-6 super-expressing cells were detected from patient SJCAR19-09 in cohort 3. Of the two patients tested for HHV-6 via qPCR from this cohort, only SJCAR19-09 tested positive, aligning with our single-cell-resolved detection of HHV-6+ CAR T cells in this individual. SJCAR19-09 continued to test positive via qPCR at day +21 post-CAR T cell infusion and was started on foscarnet at day +24. After another positive qPCR test on day +27, the patient no longer had detectable levels of HHV-6 at day +34. In line with these findings, HHV-6+ super-expressors were detected at day +14 that persisted *in vivo* until day +21 before these cells were undetectable at day +27. As the CAR T cells used in cohorts 2 and 3 were only cultured for 7-10 days before infusion, it was reasoned that HHV-6 may reactivate *in vivo* only after the therapy is administered. It was hypothesized that an extension of the culture, as is performed in many allogeneic manufacturing contexts to achieve higher cell numbers for multiple patients, could lead to viral reactivation *in vitro*. To test this, the culture of the SJCAR19-09 infusion was extended for an additional two weeks and examined at five time points during that period using scRNA-seq. Indeed, between five and 14 days of additional culture, expression of HHV-6B spanning 58 of the 103 distinct viral genes was consistently detected (FIG. 11). These data support a model where between two and three weeks after the initial manufacturing of a CAR T cell product, HHV-6 can be transcriptionally detected. In instances of SJCAR19-09 and axi-R-15, the CAR T cell infusion occurs before this window where detectable reactivation typically occurs. As a consequence, HHV-6+ CAR T cells could only be detected in peripheral blood draws after an additional 1-2 weeks of propagation *in vivo*.

Example 5 – Mitigation of HHV-6 spreading in CAR T cultures

The SJCAR19-09 case results suggested that foscarnet may be a productive agent to mitigate the effects of viral infection during therapy. It was hypothesized that the addition of

foscarnet to the allogeneic CAR T cell cultures could mitigate viral reactivation and spreading. Based on prior reports in other contexts of cell cultures being treated with 1, 2.5, and 5mM concentrations of foscarnet (Stenberg et al. (1985) *Antimicrob. Agents Chemother.* 28:802-806), additional day 19 vials from the allogeneic CAR T cell donors were thawed and the culture was extended for another 5 days to assess viral levels. The results confirmed that addition of foscarnet during culture led to a lower viral RNA abundance compared to the untreated control via qPCR (FIG. 12a-b). Using scRNA-seq to study another donor treated with 1mM of foscarnet, we replicated the reduction of HHV-6+ cells from 63.6% in the control to 21.8% in the foscarnet-treated cells was replicated and minimal changes in cell states were observed after the treatment (FIG. 12c-f). These results indicate a path forward to mitigate viral reactivation that occurs during culture and manufacturing.

Methods

Serratus viral screen analyses

To examine human viruses that may become transcriptionally activated in a systematic approach, the Serratus¹³ database for 129 human viruses curated from ViralZone¹⁴ was queried using the NC genome identification provided on the ViralZone webpage as input in the Serratus API. To focus on viral reactivation, assessed were 19 viruses across the *Herpesviridae*, *Polyomaviridae*, *Adenoviridae*, and *Parvoviridae* families contained in the query. A sample was categorized as a potential hit (FIG. 1b) given the Serratus output returned a minimum of 10 mapping reads with a homology score of at least 90% for the specific BioSample/virus pair.

As the ViralZone genome annotation for HHV-6 corresponded to HHV-6A (NC_001664) as the dominant strain in sub-Saharan Africa¹⁵, the Serratus database was queried for HHV-6B (NC_000898) for the T cell specific analyses shown in FIG. 1c-e. To annotate BioSamples as belonging to specific classes, regular expression mapping was performed for both the GEO sample title and the description. For T cells, the regular expression was "T-cell|\ T cell|Tcell|CD4T|CD8T|^T cell" whereas for iPSCs, "iPSC|luripotent" was queried. Notably, expression of EBV from annotated T cell populations was observed (FIG. 1c), including intestinal intraepithelial cytotoxic CD8 T cells.³⁵ As EBV infects cells via CD21, a canonical B cell marker, the expression of CD21 was examined across all resting and stimulated immune cell subsets, revealing that gamma-delta T cells can express CD21 at higher levels than any B cell subset (FIG. 4b), suggesting the possibility of EBV reactivation in gamma-delta T cells. More generally, the systematic approach described herein of pairing viral expression to specific BioSamples via Serratus is expected to enable the identification of cell types to become infected that have not been previously characterized.

The two primary studies that showed HHV-6 reactivation in T cell culture, Shytaj *et al.*¹⁸ and LaMere *et al.*¹⁷, took place on different continents, making it highly unlikely that other factors enabled the reactivation of HHV-6 in their cultures (e.g., same donor) aside from T cell culture

induced HHV-6 reactivation. For a deeper analyses of these datasets (FIG. 1d,e), the Serratus read abundance was paired with metadata from GEO, including the total number of reads per library.

For ATAC-seq and ChIP-seq libraries, DNA was not processed via the Serratus workflow, requiring realignment to the HHV-6B reference genome (e.g., FIG. 1 f,g). Thus, raw .fastq files were downloaded from GEO and reads were aligned to the HHV-6B reference genome (Genbank AF157706) using bwa and retaining only uniquely mapped reads (MAPQ >30) for downstream analysis and quantification. To screen for reads that were outside the repetitive regions (which have high homology with regions of the human genome), the read was required to map within the (8788, 153321) coordinates.

Primary human T cell cultures

Peripheral blood mononuclear cells (PBMCs) were isolated from leukopaks obtained from healthy donors and stored in liquid nitrogen prior to use. PBMCs were thawed, resuspended in X-vivo media (Lonza) supplemented with 5% human serum (Access Cell Culture) and IL-2 (Miltenyi), and stimulated with T Cell TransAct (Miltenyi). Lentivirus containing proprietary CAR sequences were administered early in culture. These T cell cultures were allowed to expand for up to 19 days and then frozen using a controlled rate freezer prior to storage in liquid nitrogen. For selected donors (FIG. 2f), the 19 day cryovial was thawed and re-cultured and expanded up to 7 additional days.

U31 qPCR protocol

Primary human T cells were centrifuged at 540 x *g* for 5 min before removal of culture supernatant, leaving the cell pellet for quantitative polymerase chain reaction (qPCR). Cells were processed for DNA using the manufacturer's protocol from Qiagen DNeasy Blood & Tissue Kit (Qiagen). Concentration and 260/280 ratio of the final product was measured using a NanoDrop Spectrophotometer (ThermoFisher).

For qPCR, the master mix used was TaqMan Fast Virus 1-Step Master Mix (ThermoFisher). Manufacturer's protocol was followed to add the appropriate volumes of master mix, water, DNA and primers. All samples were prepared in triplicate and run on a QuantStudio 6/7 Flex Real-Time PCR System (ThermoFisher). Primer and probe sequences for designated HHV-6 marker (U31) were employed. Measurements were taken in triplicates and the mean value is shown for qPCR plots.

Single-cell RNA-seq

scRNA-seq libraries were generated using the 10x Chromium Controller and the Chromium Single Cell 5' Library Construction Kit and human B cell and T cell V(D)J enrichment kit according to the manufacturer's instructions. Briefly, the suspended cells were loaded on a

Chromium controller Single-Cell Instrument to generate single-cell Gel Bead-In-Emulsions (GEMs) followed by reverse transcription and sample indexing using a C1000 Touch Thermal cycler with 96-Deep Well Reaction Module (BioRad). After breaking the GEMs, the barcoded cDNA was purified and amplified, followed by fragmenting, A-tailing and ligation with adaptors.

5 Finally, PCR amplification was performed to enable sample indexing and enrichment of scRNA-Seq libraries. For T cell receptor sequencing, target enrichment from cDNA was conducted according to the manufacturer's instructions. The final libraries were quantified using a Qubit dsDNA HS Assay kit (Invitrogen) and a High Sensitivity DNA chip run on a Bioanalyzer 2100 system (Agilent). 10x scRNA-seq libraries were sequenced as recommended by the

10 manufacturer (~20,000 reads / cell) via a Nova-seq 6000 using an S4 flow cell. Raw sequencing data was demultiplexed using CellRanger mkfastq and aligned to the host reference genome using CellRanger v6.0 and TCR sequences were processed using the CellRanger vdj pipeline with default settings.

Single-cell HHV-6B quantification workflow

15 To quantify HHV-6B reads, it was sought to develop a workflow that a) would utilize pseudoalignment rather than standard transcriptomic alignment, b) could be mapped to only the HHV-6B reference transcriptome (rather than a mixed transcriptome), and c) had no detectable off-target expression (i.e., 0 reported HHV-6B expression for scRNA-seq samples from human transcripts). The rationale for these constraints was to optimize computational efficiency in order

20 to reprocess consortium-level scRNA-seq libraries, noting >152 billion paired end reads were processed in the four studies noted in Table 1.

A kallisto|bustools^{36,37} workflow was developed to rapidly quantify reads from either single-cell or bulk transcriptomes. The GenBank AF157706 reference transcriptome was downloaded and a kallisto index using the default -k 31 (kmer) parameter was created. For single-

25 cell libraries, raw sequencing reads were processed using the kallisto `bus` command with appropriate hyperparameters for each version of the single-cell chemistry (either 14 or 16 bp sequence barcode and 10 or 12 bases of UMI sequence). After barcode and UMI correction, a plain text sparse matrix was emitted, corresponding to unique HHV-6B reads mapping to individual cells in the single-cell sequencing library. For bulk libraries, the same index could be

30 utilized with the standard kallisto `quant` execution.

To assess specificity (i.e., no human transcript expression being reported as an HHV-6B transcript), quantified were 4 healthy PBMC libraries that had no expected HHV-6B expression. Non-zero expression was recurrently observed at only the AF157706.1_cds_AAD49614.1_1 and AF157706.1_cds_AAD49682.1_98 coding features, both genic transcripts that encode for the

35 DR1 gene, which corresponded to AF157706 equivalence classes 6 and 120 in the kallisto index. By examining reads that mapped to both HHV-6B transcriptome and the human genome, it was determined that a region of the DR1 gene is highly homologous to the human gene KDM2A,

which is broadly expressed across tissues, including lymphocytes, leading to ~1-3 reads per million errantly aligning to the HHV-6B transcriptome. By excluding these two equivalence classes, identically 0 HHV-6B UMIs was consistently observed across libraries derived from peripheral blood mononuclear cells³⁴ and across 60 healthy tissue types from various donors (Han et al. Nature 2020). The number of UMIs per cell shown in FIG. 2 and FIG. 3 excludes these potential host-derived genes annotated as viral expression. Finally, verification of the *in vitro* expression of the CAR transgene was confirmed via single-cell quantification of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) in all sequencing libraries and in single-cells.

To assign whether a cell is a super expressor, a semi-arbitrary cutoff of 10 HHV-6B UMIs per cell based on empirical expression (e.g., FIG. 2d), and the ease of use/interpretation of the number 10, were used. One cell in the *in vivo* dataset expressed 8 UMIs (FIG. 3b), which likely is a super expressor but under sequenced based on the number of overall UMIs detected for this cell. For visualization across the Day 19 samples (FIG. 7a), shown are both the rank-ordered expression per cell based on total HHV-6B UMIs as well as a null model where HHV-6B reads are allocated proportional to the number of total UMIs detected for that particular cell barcode. For HHV-6B transcript heatmaps (FIG. 7b; FIG. 3c), the per-gene annotation from a prior report was utilized.²⁸ For simplicity in viewing expression signatures, the “intermediate early early” and “immediate early early” were collapsed into a single gene set for visualization in these two heatmaps.

Integrated viral / host gene expression analyses

To determine differentially expressed genes between HHV-6+ cells and HHV-6- cells from the Day 19 time points, D34 and D38, which had the highest number of HHV-6+ cells, were considered. This was achieved using the `FindMarkers` function from Seurat after segregating HHV-6+ cells as those that have a minimum of 2 UMIs (to increase power) and HHV-6- as those with identically 0 HHV-6 UMIs. From the FindMarkers output, a statistic was computed, $-\log_{10}(p.val) * \log_2 FC$, that preserves the direction of over/under expression while partially scaling the gene expression difference by the magnitude of the statistical association, resulting in the plot (FIG. 7d). From this association, the lymphotoxin genes LTA and LTB were distinguished as the consistent associations. As the homotrimer $LT\alpha_3$ is secreted but the heterotrimer ($LT\alpha_1\beta_2$) is membrane-bound³⁸, this suggests that changes in the LT expression upon HHV-6 reactivation lead to a secreted signal via $LT\alpha_3$, but due to the pleiotropic effects of this cytokine trimer³⁸, the impact of its possible increased secretion is not evident.

To more comprehensively examine host gene association with HHV-6B transcript abundance, also computed were Pearson correlation statistics per-gene and performed pathway analyses using the Pearson correlation between the log transformed total HHV-6B UMIs with the Seurat-normalized host expression as a means of rank-ordering genes linked to HHV-6B activity.

This was performed for all three re-cultured samples. After broadly verifying the top genes, the data for D34 at day 25 total of culture was presented as it was the earliest time point. Using this correlation-based approach, a significant enrichment of genes downstream of type I interferon activity were identified as being more expressed in cells low for HHV-6B, consistent with the antiviral properties of this pathway²⁹. In contrast, targets of the E2F transcription factor were upregulated as the HHV-6B infection spread throughout culture, consistent with prior reports of E2F1-induced HHV-6A expression in T cells³⁹. Furthermore, observed was an increase in genes associated with oxidative phosphorylation, a pathway previously reported to be co-opted during active viral infection³⁰. Overall, the results largely recapitulate known pro- and anti-viral responses, indicating that the host transcriptome at the single-cell level permits or resists HHV-6B infection from spreading within the T cell culture.

Reanalysis of public single-cell data

The data presented in Table 1 represents a summation of four large datasets reanalyzed for HHV-6B expression. In brief, the number of cells and raw sequencing reads analyzed are reported, alongside the number of high-confidence HHV-6B UMIs from the quantification pipeline. The number of HHV-6+ cells are barcodes annotated as cells before quality-control filtering in the original publication with at least one HHV-6 UMI. Many super-expressors contain high levels of mitochondrial and ribosomal transcripts, potentially due to these cells nearing apoptosis.

For reanalysis of the Healthy Cell Landscape (Han et al. Nature 2020), .fastq files were processed through the same workflow, using the HHV-6B reference index and kallisto | bustools pseudoalignment workflow. For the ~1,000 donor PBMC dataset³⁴, .fastq files were similarly downloaded from GEO and quantified with the resulting libraries for reads that pseudoaligned to the HHV-6B transcriptome. For either dataset, a single HHV-6B read that was annotated by a barcode that was a cell in the processed metadata was not detected for either study though 6 total HHV-6B reads from the PBMC cohort (all mapping to different gel emulsion bead barcodes) were observed.

For the public CAR T cell datasets, all .fastq files were downloaded through either the EGA portal⁹ or reanalyzed using a Terra workspace.³² Each dataset was processed with the same HHV-6B kallisto | bustools workflow. No cells annotated in the complete metadata in the pre-infusion only study⁵ were observed, resulting in an annotation of 0 HHV-6+ cells from this dataset. For the 7 days post infusion blood draw, a total of 26 unique 10x barcodes were observed with at least 1 HHV-6B UMI whereas only 7 of the 26 barcodes were annotated as cells in the original study.³² While the high UMI abundance for the Axi-R-15 donor has no ambiguous interpretation, it is noted that the other two donors (Tisa-R-32 and Axi-N-07) were pooled with the Axi-R-15 cells and then demultiplexed using genetic variants.³² As a consequence, the possibility that the residual 1-2 UMIs detected in these 3 cells are from barcode contamination from the Axi-R-15

donor cells cannot be excluded. Regardless, the characterization of the Axi-R-15 cells confirms the presence of HHV-6^{hi} cells in an FDA approved CAR T product.

To confirm that HHV-6+ cells form the *in vivo* fusion product were from CAR T cells, the presence of the CAR transgene was assessed via RNA (kallisto pseudoalignment to the axi-cell transcript) and host gene expression values were noted (FIG. 3). The CAR 'FACS' cell was from the 10x channel that enriched for CAR+ cells via flow cytometry but due to the mechanisms of 10x Genomics library preparation protocol, the specific protein abundance for this individual cell is not discernable. The 'cytotoxic' signature was the sum of the UMIs for GZMK, GNLY, KLRG1, ZEB2, and NKG7 genes. The TCR α/β transcript number was the total number of UMIs aligning per barcode in the TCR vdj libraries.

Expression of viral receptors across atlases

To evaluate other possible sources of HHV-6 reactivation and expression, the landscape of cell types that express TNFRSF4 (OX40), the canonical receptor of HHV-6B, and CR2 (CD21), the canonical receptor of EBV, was considered. Processed count data and/or summary plots were downloaded from the GTEx portal⁴⁰, fetal development⁴¹, sorted resting and stimulated immune cells⁴², and endothelial cell lines⁴³.

Analyses of these atlases revealed that OX40 is expressed specifically in activated CD4 and CD8 T cells in the immune system but had detectable levels across all tissues (FIG. 4). When examining single-cell and single-nucleus expression across the GTEx atlas, surprisingly observed was consistent expression in endothelial cells across various tissues, including in the pancreas and brain. 15 of the top 25 cell types / tissues were further corroborated as endothelial. While the overall expression of OX40 appears to be 1-2 orders of magnitude higher in activated T cells, the application of the present atlas-level analyses of the HHV-6B viral receptor suggests that endothelial cells may be targets of active HHV-6B infection, either from the primary infection or from cell therapy-mediated reactivation. Moreover, brain endothelial cell expression of OX40 might underly the encephalitis observed in severe infections/reactivation.

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Accordingly, the preceding merely illustrates the principles of the present disclosure. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same

function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein.

WHAT IS CLAIMED IS:

1. A method of monitoring a human subject receiving a T cell based therapy for HHV-6B reactivation in therapeutic T cells present in the subject, the method comprising:
obtaining from the subject a biological sample comprising the therapeutic T cells; and
5 assessing the therapeutic T cells for HHV-6B reactivation.

2. The method according to claim 1, wherein the biological sample is a peripheral blood sample obtained from the subject.

10 3. The method according to claim 1 or 2, wherein the obtaining and assessing steps are performed two or more times while subject is receiving the T cell based therapy.

4. The method according to any one of claims 1 to 3, wherein the assessing is by quantitative nucleic acid sequencing.

15 5. The method according to claim 4, wherein the assessing is by single cell nucleic acid sequencing.

20 6. The method according to claim 5, wherein the single cell nucleic acid sequencing is single cell RNA sequencing (scRNA-Seq).

7. The method according to claim 6, wherein the assessing comprises:
aligning RNA sequence reads of single cells of the therapeutic T cells to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences; and
25 counting the number of RNA sequence reads of single cells mapping to the HHV-6B RNA sequences of the reference,
wherein HHV-6B reactivation is determined to be present when a threshold number of RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the reference.

30 8. A computer-implemented method of assessing for HHV-6B reactivation in therapeutic T cells present in a subject receiving a T cell based therapy, the method comprising:
aligning RNA sequence reads of single cells of the therapeutic T cells to a reference comprising human genomic DNA sequences and HHV-6B RNA sequences; and
35 counting the number of RNA sequence reads of single cells mapping to the HHV-6B RNA sequences of the reference,

wherein HHV-6B reactivation is determined to be present when a threshold number of RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the reference.

5 9. The method according to claim 7 or 8, wherein the threshold number of RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

10. The method according to claim 6, wherein the assessing comprises:
10 pseudoaligning RNA sequence reads of single cells of the therapeutic T cells to an HHV-6B reference index;
removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and
counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index,
15 wherein HHV-6B reactivation is determined to be present in a cell of the therapeutic T cells when a threshold number of remaining RNA sequence reads are pseudoaligned to the HHV-6B RNA reference index.

11. A computer-implemented method of assessing for HHV-6B reactivation in therapeutic T
20 cells present in a subject receiving a T cell based therapy, the method comprising:
pseudoaligning RNA sequence reads of single cells of the therapeutic T cells to an HHV-6B reference index;
removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and
25 counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index,
wherein HHV-6B reactivation is determined to be present when a threshold number of remaining RNA sequence reads of a cell of the therapeutic T cells are pseudoaligned to the HHV-6B RNA reference index.

30

12. The method according to claim 10 or 11, wherein the highly homologous regions of the HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A.

35 13. The method according to any one of claims 10 to 12, wherein the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

14. The method according to any one of claims 1 to 13, wherein when the assessment determines the presence of HHV-6B reactivation, the method further comprises ceasing the T cell based therapy.

5 15. The method according to claim 14, further comprising administering to the subject a new T cell based therapy with cells known to be HHV-6B negative.

16. The method according to any one of claims 1 to 15, wherein when the assessment determines the presence of HHV-6B reactivation, the method further comprises administering
10 an antiviral therapy to the subject.

17. The method according to claim 16, wherein the antiviral therapy comprises administering to the subject an agent approved for treating HHV-6B infection in humans.

15 18. The method according to claim 16 or 17, wherein the antiviral therapy comprises administering ganciclovir, cidofovir, foscarnet, or any combination thereof to the subject.

19. The method according to any one of claims 1 to 13, wherein when the assessment determines the absence of HHV-6B reactivation, the method comprises continuing the T cell
20 based therapy.

20. A method comprising assessing an *in vitro* culture comprising candidate therapeutic human T cells for HHV-6B reactivation, wherein the *in vitro* culture is assessed for a level of one or more HHV-6B analytes by quantitative nucleic acid sequencing.

25

21. The method according to claim 20, wherein the assessing is by single cell nucleic acid sequencing.

22. The method according to claim 21, wherein the single cell nucleic acid sequencing is
30 single cell RNA sequencing (scRNA-Seq).

23. The method according to claim 22, wherein the assessing comprises:
aligning RNA sequence reads of single cells of the candidate therapeutic human T cells
to a reference comprising human genomic DNA sequences and HHV-6B RNA
35 sequences; and
counting the number of RNA sequence reads of single cells mapping to the HHV-6B
RNA sequences of the reference,

wherein HHV-6B reactivation is determined to be present when a threshold number of RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the reference.

- 5 24. A computer-implemented method of assessing an *in vitro* culture comprising candidate therapeutic human T cells for HHV-6B reactivation, the method comprising:
aligning RNA sequence reads of single cells of the candidate therapeutic human T cells
to a reference comprising human genomic DNA sequences and HHV-6B RNA
sequences; and
10 counting the number of RNA sequence reads of single cells mapping to the HHV-6B
RNA sequences of the reference,
wherein HHV-6B reactivation is determined to be present when a threshold number of
RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the
reference.

- 15 25. The method according to claim 23 or 24, wherein the threshold number of RNA
sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence
reads.

- 20 26. The method according to claim 22, wherein the assessing comprises:
pseudoaligning RNA sequence reads of single cells of the candidate therapeutic human
T cells to an HHV-6B reference index;
removing RNA sequence reads pseudoaligned to highly homologous regions of the
HHV-6B reference index and human T cell transcriptome; and
25 counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B
reference index,
wherein HHV-6B reactivation is determined to be present in a cell of the candidate
therapeutic human T cells when a threshold number of remaining RNA sequence
reads are pseudoaligned to the HHV-6B RNA reference index.

- 30 27. A computer-implemented method of assessing an *in vitro* culture comprising candidate
therapeutic human T cells for HHV-6B reactivation, the method comprising:
pseudoaligning RNA sequence reads of single cells of the candidate therapeutic human
T cells to an HHV-6B reference index;
35 removing RNA sequence reads pseudoaligned to highly homologous regions of the
HHV-6B reference index and human T cell transcriptome; and
counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B
reference index,

wherein HHV-6B reactivation is determined to be present when a threshold number of remaining RNA sequence reads of a cell of the candidate therapeutic human T cells are pseudoaligned to the HHV-6B RNA reference index.

5 28. The method according to claim 26 or 27, wherein the highly homologous regions of the HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A.

10 29. The method according to any one of claims 26 to 28, wherein the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

30. The method according to any one of claims 20 to 29, wherein the candidate therapeutic human T cells comprise CD4⁺ human T cells.

15

31. The method according to any one of claims 20 to 30, wherein the candidate therapeutic human T cells are genetically modified.

20 32. The method according to claim 31, wherein the candidate therapeutic human T cells are genetically modified to express an engineered receptor.

33. The method according to claim 32, wherein the engineered receptor is a chimeric antigen receptor (CAR), an engineered T cell receptor (TCR), a chimeric cytokine receptor (CCR), a chimeric chemokine receptor, a synthetic notch receptor (synNotch), a Modular
25 Extracellular Sensor Architecture (MESA) receptor, a Tango receptor, a ChaCha receptor, or a generalized extracellular molecule sensor (GEMS) receptor.

34. The method according to claim 32, wherein the engineered receptor is a CAR.

30 35. The method according to any one of claims 32 to 34, wherein the engineered receptor comprises an extracellular binding domain that binds to a tumor antigen.

36. The method according to any one of claims 20 to 31, wherein the candidate therapeutic human T cells are not genetically modified to express an engineered receptor.

37. The method according to any one of claims 20 to 36, wherein the candidate therapeutic human T cells are being expanded during a therapeutic T cell manufacturing process.

5 38. The method according to claim 37, comprising assessing the *in vitro* culture for the one or more HHV-6B analytes two or more times during expansion of the candidate therapeutic human T cells.

39. The method according to claim 37 or 38, wherein the manufacturing process comprises
10 activating the candidate therapeutic human T cells, and wherein the method comprises assessing the *in vitro* culture for the one or more HHV-6B analytes one or more times at from 0 to 20 days post-activation.

40. The method according to any one of claims 20 to 39, further comprising, when the
15 assessing determines an absence of HHV-6B reactivation, identifying the candidate therapeutic human T cells as therapeutic T cells.

41. The method according to claim 40, further comprising administering to a subject in need
20 thereof the therapeutic human T cells or progeny thereof.

42. The method according to any one of claims 20 to 39, further comprising, when the
assessing determines the presence of HHV-6B reactivation, administering to a subject in need
thereof therapeutic human T cells other than the candidate therapeutic human T cells of the *in*
vitro culture, wherein HHV-6B reactivation is known to be absent in the administered
25 therapeutic human T cells.

43. A computer-implemented method of identifying a human therapeutic cell type
susceptible to reactivation by a virus, the method comprising:

- 30 (i) obtaining from a sequencing database of nucleic acid sequencing samples having at least one annotated read from a virus known to infect humans;
- (ii) performing step (i) for one or more viruses known to infect humans;
- (iii) obtaining metadata for the annotated biosample that was linked to specific sequencing reads, wherein the metadata comprises sample name, descriptor, biosample ID, derived organism, or any combination thereof; and
- 35 (iv) identify relevant cell types for a specific virus and identifying virus-specific reactivation, or identify viral reactivation for a specific cell type and identifying cell type-specific reactivation.

44. A method of monitoring a therapeutic T cell product for HHV-6B reactivation, the method comprising:

administering a first population of therapeutic T cells from the therapeutic T cell product
5 to a subject in need thereof;

culturing a second population of therapeutic T cells from the therapeutic T cell product,
wherein the culturing comprises culturing the therapeutic T cells during a period
subsequent to administration of the first population of therapeutic T cells to the
subject; and

10 during the period subsequent to administration of the first population of therapeutic T
cells to the subject, monitoring the T cell culture for HHV-6B reactivation.

45. The method according to claim 44, wherein the monitoring is by quantitative nucleic acid
sequencing.

46. The method according to claim 45, wherein the monitoring is by single cell nucleic acid
sequencing.

47. The method according to claim 46, wherein the single cell nucleic acid sequencing is
20 single cell RNA sequencing (scRNA-Seq).

48. The method according to claim 47, wherein the monitoring comprises:
aligning RNA sequence reads of single cells of the therapeutic T cell product to a
reference comprising human genomic DNA sequences and HHV-6B RNA
25 sequences; and
counting the number of RNA sequence reads of single cells mapping to the HHV-6B
RNA sequences of the reference,
wherein HHV-6B reactivation is determined to be present when a threshold number of
RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the
30 reference.

49. A computer-implemented method of monitoring a therapeutic T cell product for HHV-6B
reactivation, the method comprising:

aligning RNA sequence reads of single cells of the therapeutic T cell product to a
35 reference comprising human genomic DNA sequences and HHV-6B RNA
sequences; and

counting the number of RNA sequence reads of single cells mapping to the HHV-6B
RNA sequences of the reference,

wherein HHV-6B reactivation is determined to be present when a threshold number of RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the reference.

5 50. The method according to claim 48 or 49, wherein the threshold number of RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

10 51. The method according to claim 47, wherein the monitoring comprises:
pseudoaligning RNA sequence reads of single cells of the therapeutic T cell product to an HHV-6B reference index;
removing RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and
counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B
15 reference index,
wherein HHV-6B reactivation is determined to be present in a cell of the therapeutic T cell product when a threshold number of remaining RNA sequence reads are pseudoaligned to the HHV-6B RNA reference index.

20 52. A computer-implemented method of monitoring a therapeutic T cell product for HHV-6B reactivation, the method comprising:
pseudoaligning RNA sequence reads of single cells of the therapeutic T cell product to an HHV-6B reference index;
removing RNA sequence reads pseudoaligned to highly homologous regions of the
25 HHV-6B reference index and human T cell transcriptome; and
counting the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index,
wherein HHV-6B reactivation is determined to be present when a threshold number of
remaining RNA sequence reads of a cell of the therapeutic T cell product are
30 pseudoaligned to the HHV-6B RNA reference index.

53. The method according to claim 51 or 52, wherein the highly homologous regions of the HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A.
35

54. The method according to any one of claims 51 to 53, wherein the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

5 55. A method of preventing or mitigating viral reactivation in therapeutic human cells during a therapeutic human cell manufacturing process, the method comprising expanding the therapeutic human cells in the presence of an anti-viral agent at a concentration effective to prevent or mitigate viral reactivation in the expanding therapeutic human cells.

10 56. The method according to claim 55, wherein the therapeutic human cells are therapeutic human T cells.

57. The method according to claim 56, wherein HHV-6A, HHV-6B, and/or HHV-7 viral reactivation is prevented or mitigated.

15

58. The method according to claim 56 or 57, wherein the anti-viral agent is selected from ganciclovir, cidofovir, foscarnet, or any combination thereof.

59. The method according to claim 55, wherein the therapeutic human cells are therapeutic
20 human induced pluripotent stem cells (iPSCs).

60. The method according to claim 59, wherein HSV-1 viral reactivation is prevented or mitigated.

25 61. The method according to any one of claims 55 to 60, wherein the therapeutic human cells are genetically modified.

62. The method according to claim 61, wherein the therapeutic human cells are genetically modified to express an engineered receptor.

30

63. The method according to claim 62, wherein the engineered receptor is a chimeric antigen receptor (CAR), an engineered T cell receptor (TCR), a chimeric cytokine receptor (CCR), a chimeric chemokine receptor, a synthetic notch receptor (synNotch), a Modular Extracellular Sensor Architecture (MESA) receptor, a Tango receptor, a ChaCha receptor, or a
35 generalized extracellular molecule sensor (GEMS) receptor.

64. The method according to claim 62, wherein the engineered receptor is a CAR.

65. The method according to any one of claims 62 to 64, wherein the engineered receptor comprises an extracellular binding domain that binds to a tumor antigen.

66. The method according to any one of claims 55 to 61, wherein the therapeutic human
5 cells are not genetically modified to express an engineered receptor.

67. The method according to any one of claims 55 to 66, further comprising administering the expanded therapeutic human cells to a subject in need thereof.

10 68. One or more non-transitory computer readable media having stored thereon instructions that cause a computer device to:

align RNA sequence reads of single T cells to a reference comprising human genomic
DNA sequences and HHV-6B RNA sequences; and

count the number of RNA sequence reads of single T cells mapping to the HHV-6B

15 RNA sequences of the reference,

wherein HHV-6B reactivation is determined to be present when a threshold number of
RNA sequence reads from a single cell map to the HHV-6B RNA sequences of the
reference.

20 69. The one or more non-transitory computer readable media of claim 68, wherein the threshold number of RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

70. A computer device comprising
25 one or more processors; and
one or more non-transitory computer readable media having stored thereon instructions that cause the computer device to:

align RNA sequence reads of single T cells to a reference comprising human
genomic DNA sequences and HHV-6B RNA sequences; and

30 count the number of RNA sequence reads of single T cells mapping to the HHV-
6B RNA sequences of the reference,

wherein HHV-6B reactivation is determined to be present when a threshold
number of RNA sequence reads from a single cell map to the HHV-6B RNA
sequences of the reference.

35

71. The computer device of claim 70, wherein the threshold number of RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

72. One or more non-transitory computer readable media having stored thereon instructions that cause a computer device to:

pseudoalign RNA sequence reads of single cells of therapeutic T cells to an HHV-6B reference index;

5 remove RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and
count the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index.

10 73. The one or more non-transitory computer readable media of claim 72, wherein the highly homologous regions of the HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A.

15 74. The one or more non-transitory computer readable media of claim 72 or 73, wherein the instructions further cause the computer device to assess the single cells for HHV-6B reactivation, wherein HHV-6B reactivation is determined to be present when a threshold number of remaining RNA sequence reads for a cell of the therapeutic T cells are pseudoaligned to the HHV-6B RNA reference index.

20 75. The one or more non-transitory computer readable media of claim 74, wherein the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

25 76. A computer device comprising
one or more processors; and
one or more non-transitory computer readable media having stored thereon instructions that cause the computer device to:

pseudoalign RNA sequence reads of single cells of candidate therapeutic T cells to an HHV-6B reference index;

30 remove RNA sequence reads pseudoaligned to highly homologous regions of the HHV-6B reference index and human T cell transcriptome; and
count the number of remaining RNA sequence reads pseudoaligned to the HHV-6B reference index.

77. The computer device of claim 76, wherein the highly homologous regions of the HHV-6B reference index and human T cell transcriptome comprise a homologous region between HHV-6B DR1 and human KDM2A.

5 78. The computer device of claim 76 or 77, wherein the instructions further cause the computer device to assess the single cells for HHV-6B reactivation, wherein HHV-6B reactivation is determined to be present when a threshold number of remaining RNA sequence reads of a cell of the candidate therapeutic T cells are pseudoaligned to the HHV-6B RNA reference index.

10

79. The computer device of claim 78, wherein the threshold number of remaining RNA sequence reads is from 5 to 15, 6 to 14, 7 to 13, 8 to 12, 9 to 11, or about 10 RNA sequence reads.

15

80. One or more non-transitory computer readable media having stored thereon instructions that cause a computer device to:

- (i) obtain from a sequencing database of nucleic acid sequencing samples having at least one annotated read from a virus known to infect humans;
- (ii) perform step (i) for one or more viruses known to infect humans;
- 20 (iii) obtain metadata for the annotated biosample that was linked to specific sequencing reads, wherein the metadata comprises sample name, descriptor, biosample ID, derived organism, or any combination thereof; and
- (iv) identify relevant cell types for a specific virus and identifying virus-specific reactivation, or identify viral reactivation for a specific cell type and identifying cell type-specific reactivation.

25

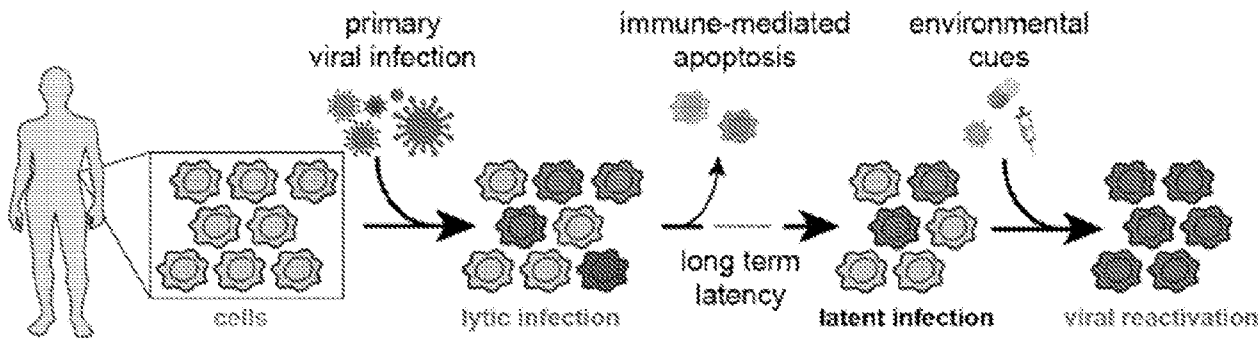
81. A computer device, comprising:
one or more processors; and
one or more non-transitory computer readable media having stored thereon instructions
30 that cause the computer device to:

- (i) obtain from a sequencing database of nucleic acid sequencing samples having at least one annotated read from a virus known to infect humans;
- (ii) perform step (i) for one or more viruses known to infect humans;
- 35 (iii) obtain metadata for the annotated biosample that was linked to specific sequencing reads, wherein the metadata comprises sample name, descriptor, biosample ID, derived organism, or any combination thereof; and

35

- (iv) identify relevant cell types for a specific virus and identifying virus-specific reactivation, or identify viral reactivation for a specific cell type and identifying cell type-specific reactivation.

a



b

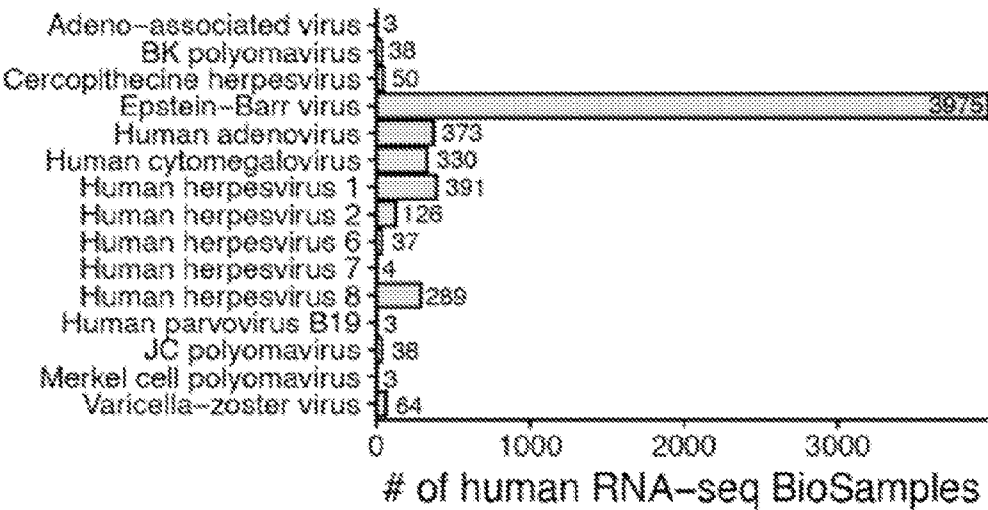


FIG. 1

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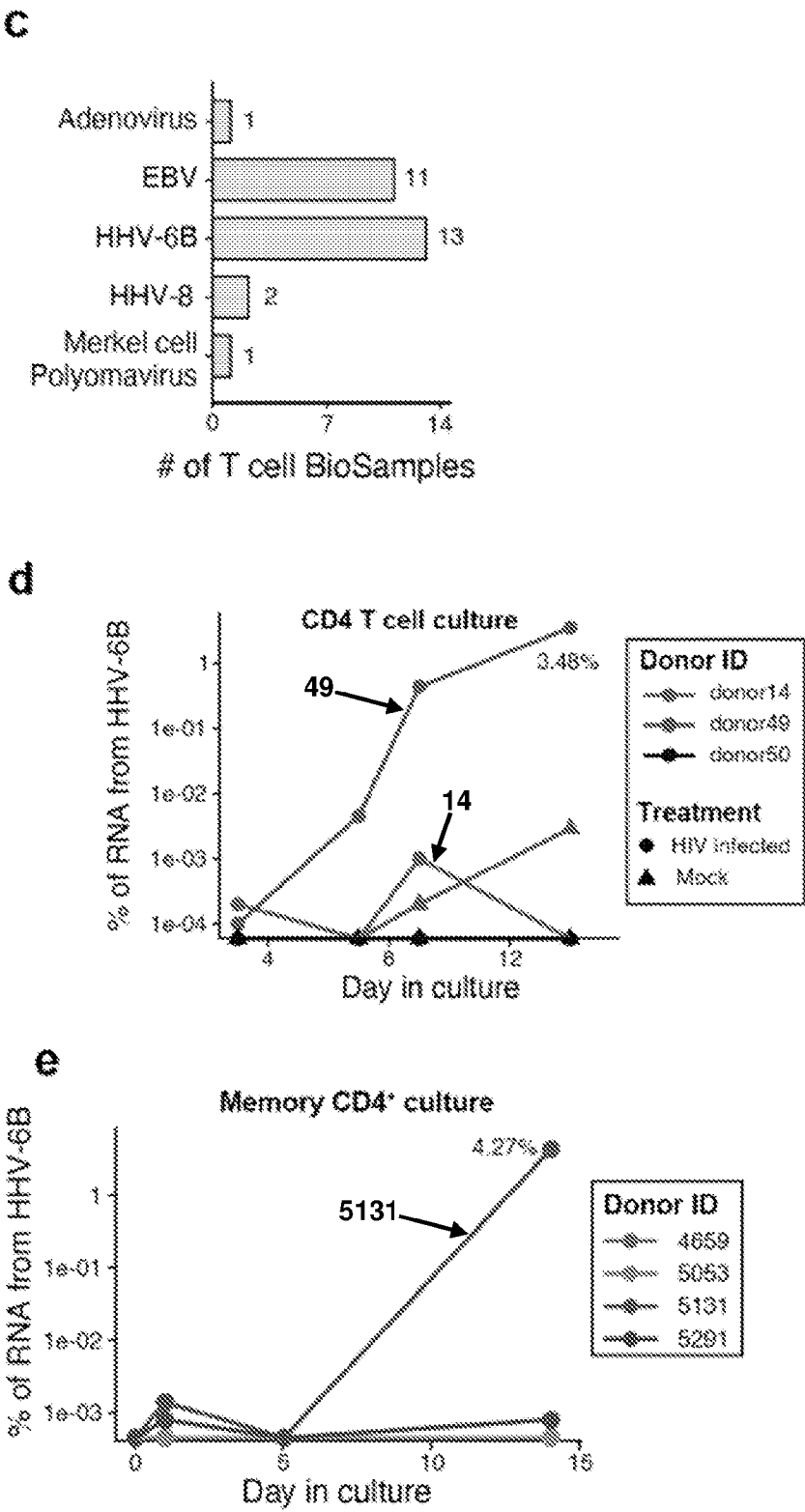


FIG. 1 (cont'd)

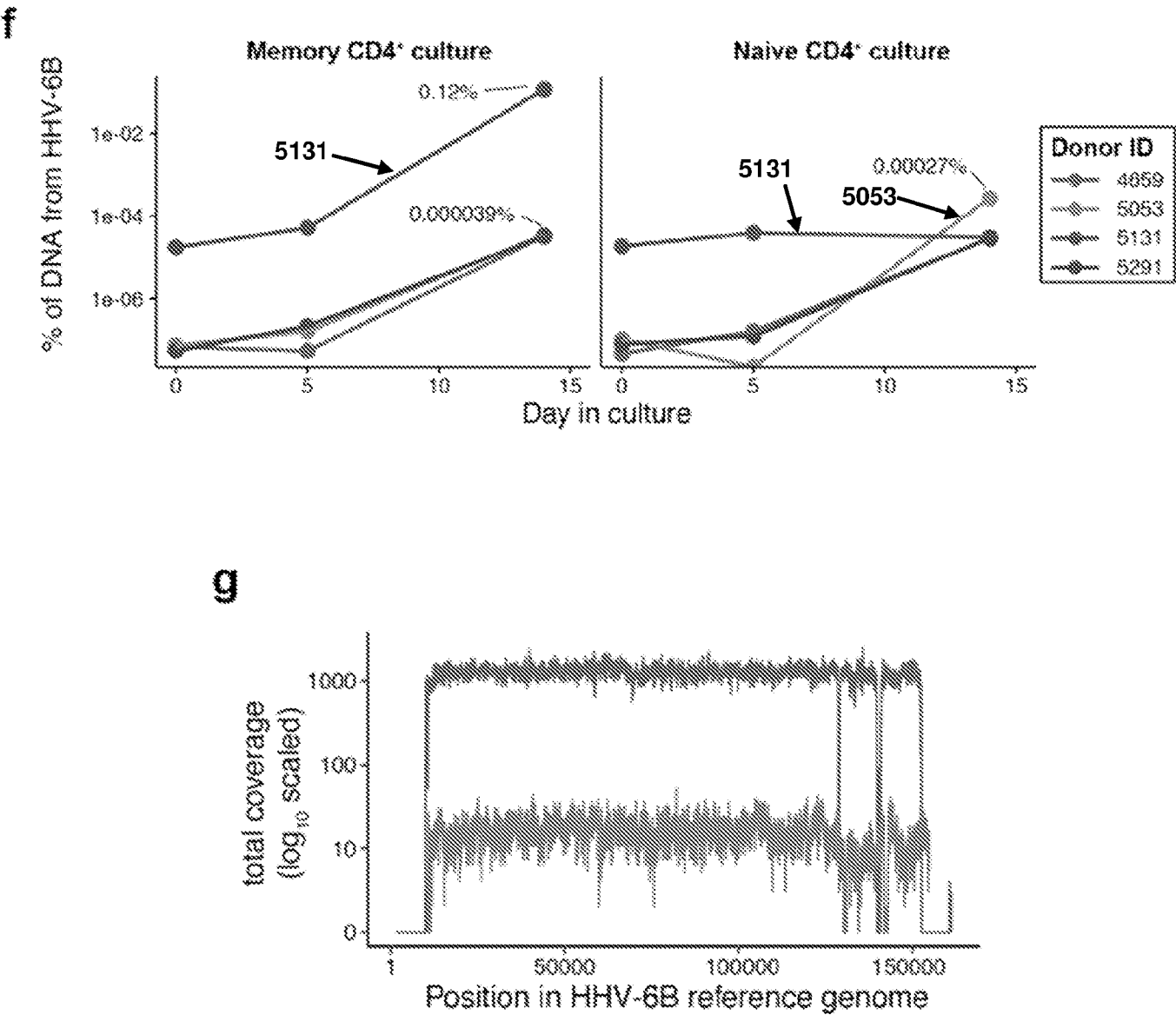


FIG. 1 (cont'd)

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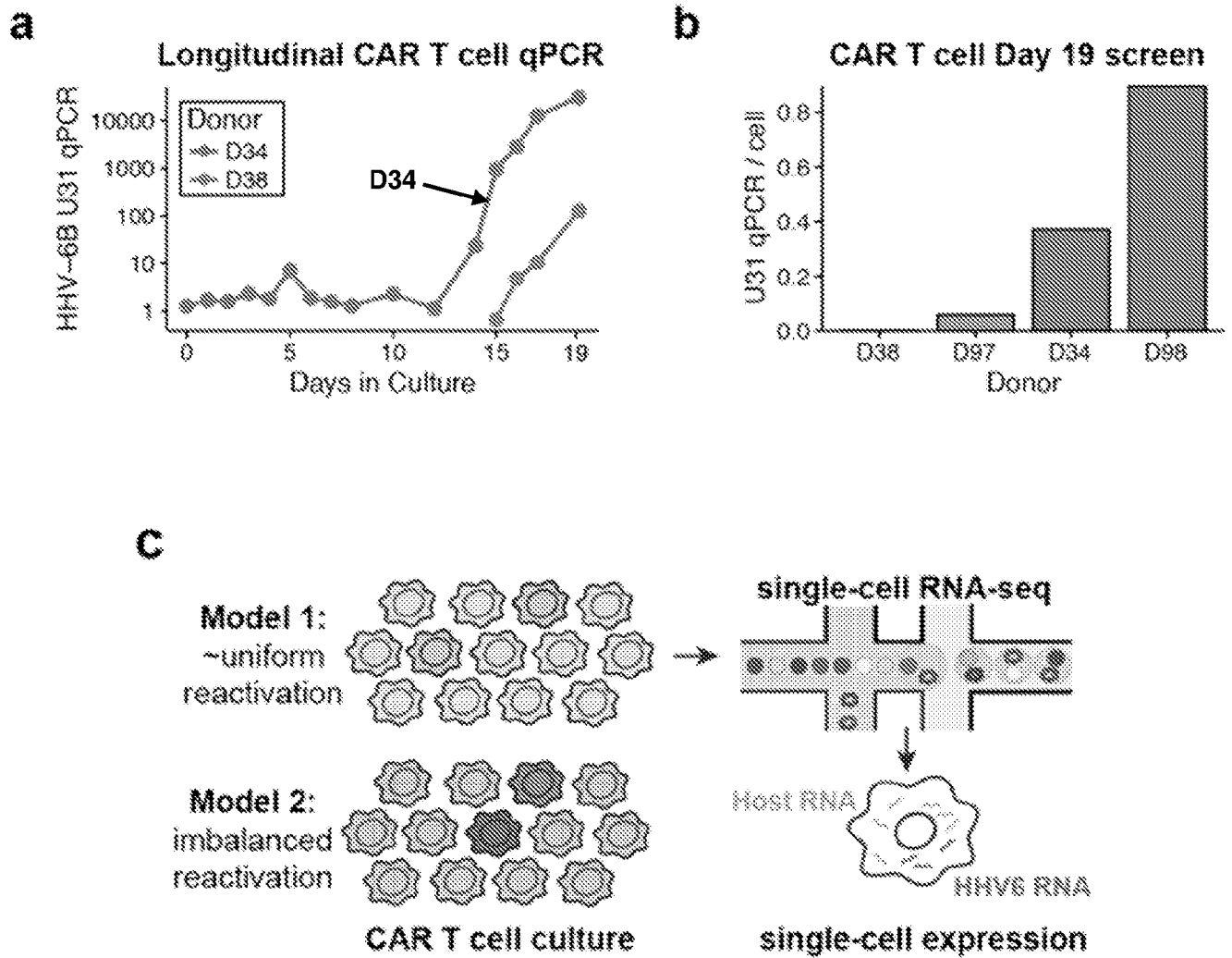


FIG. 2

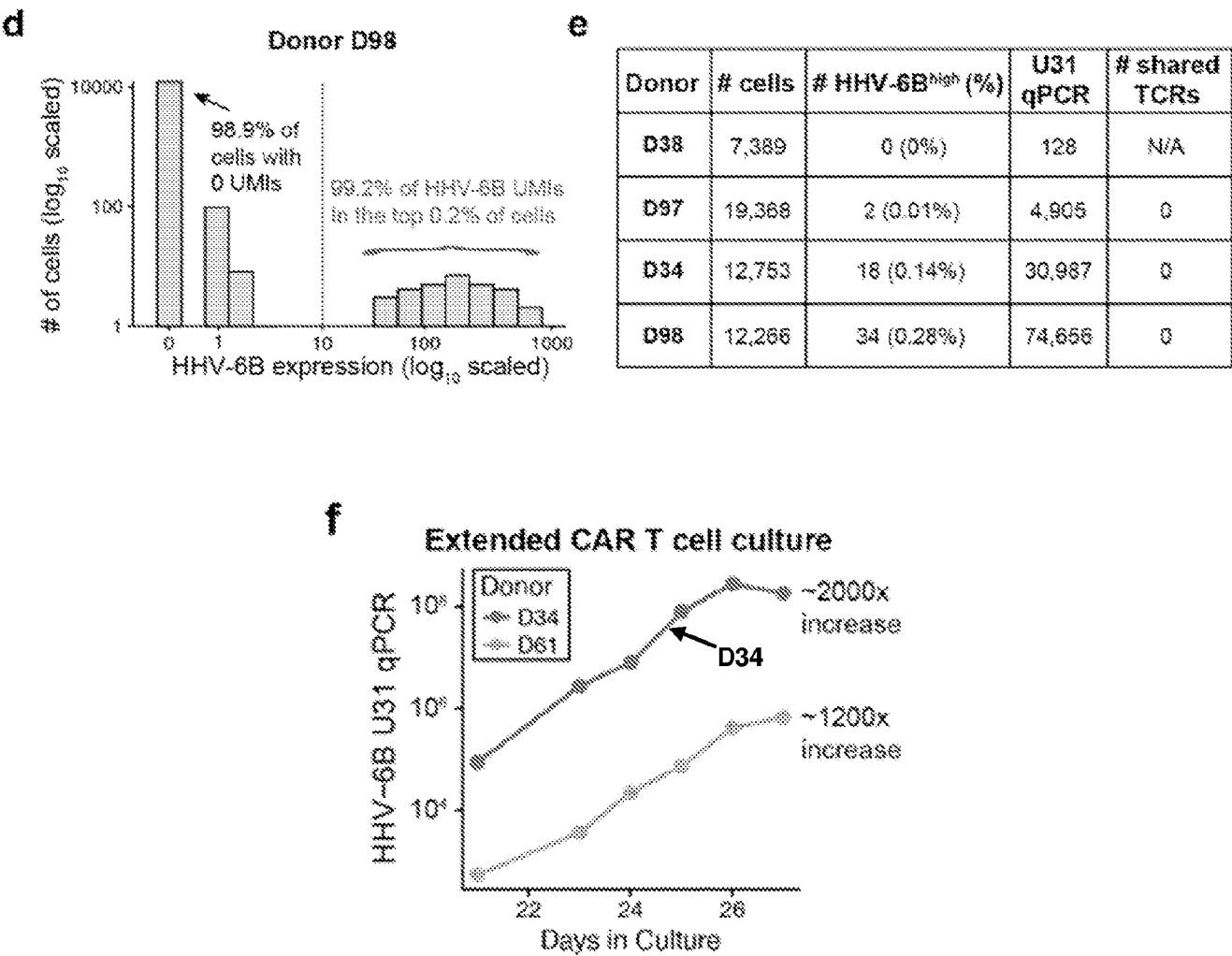


FIG. 2 (Cont'd)

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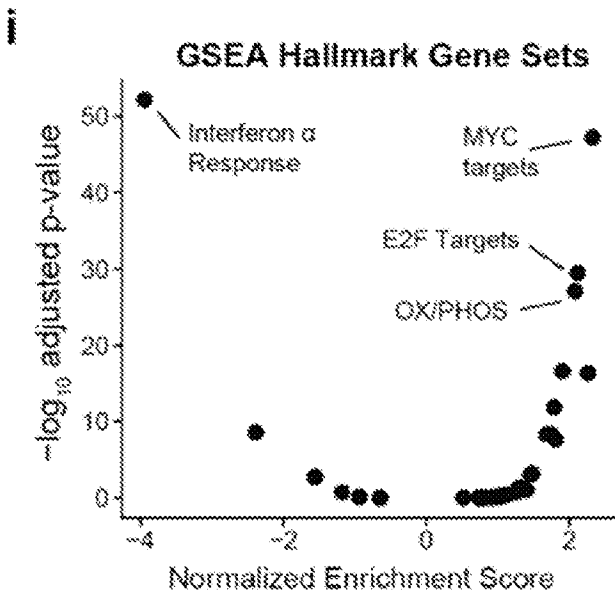
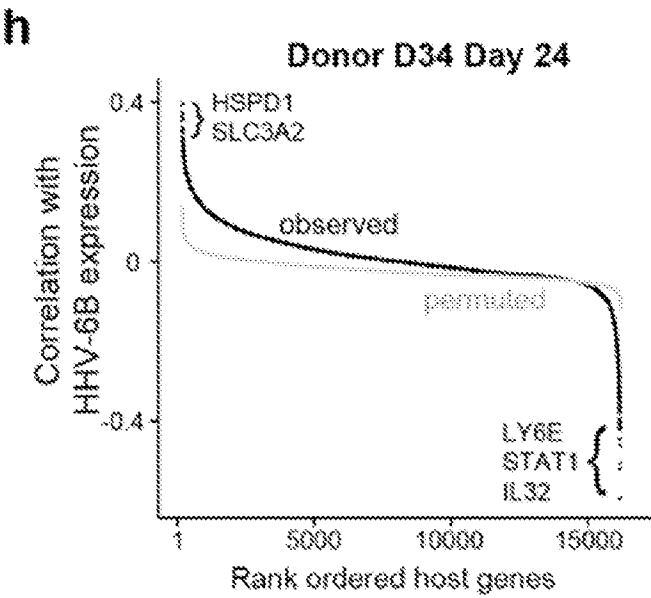
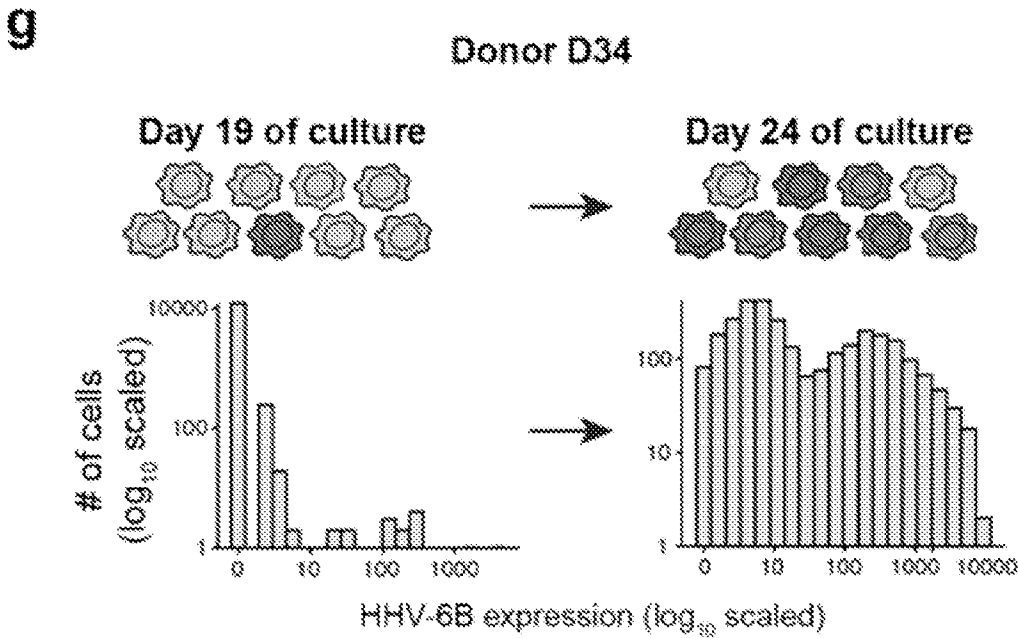


FIG. 2 (Cont'd)

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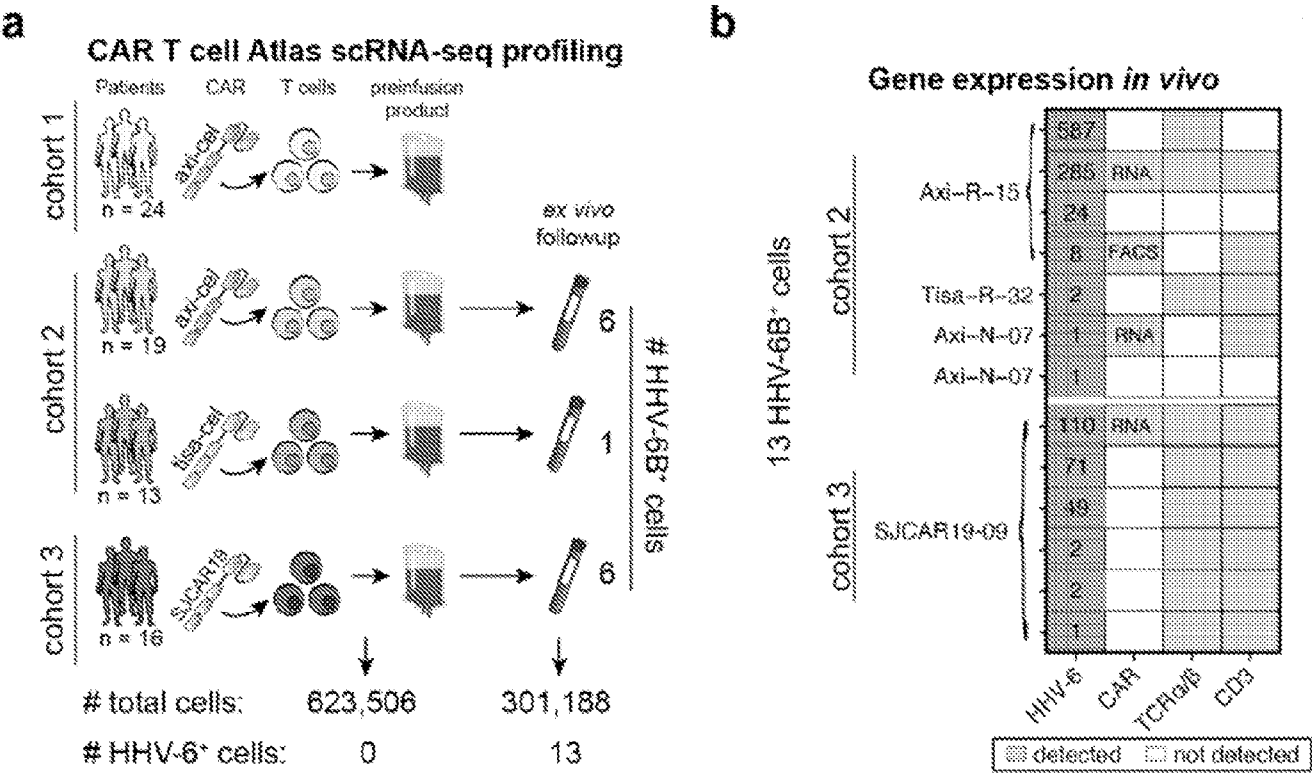


FIG. 3

C

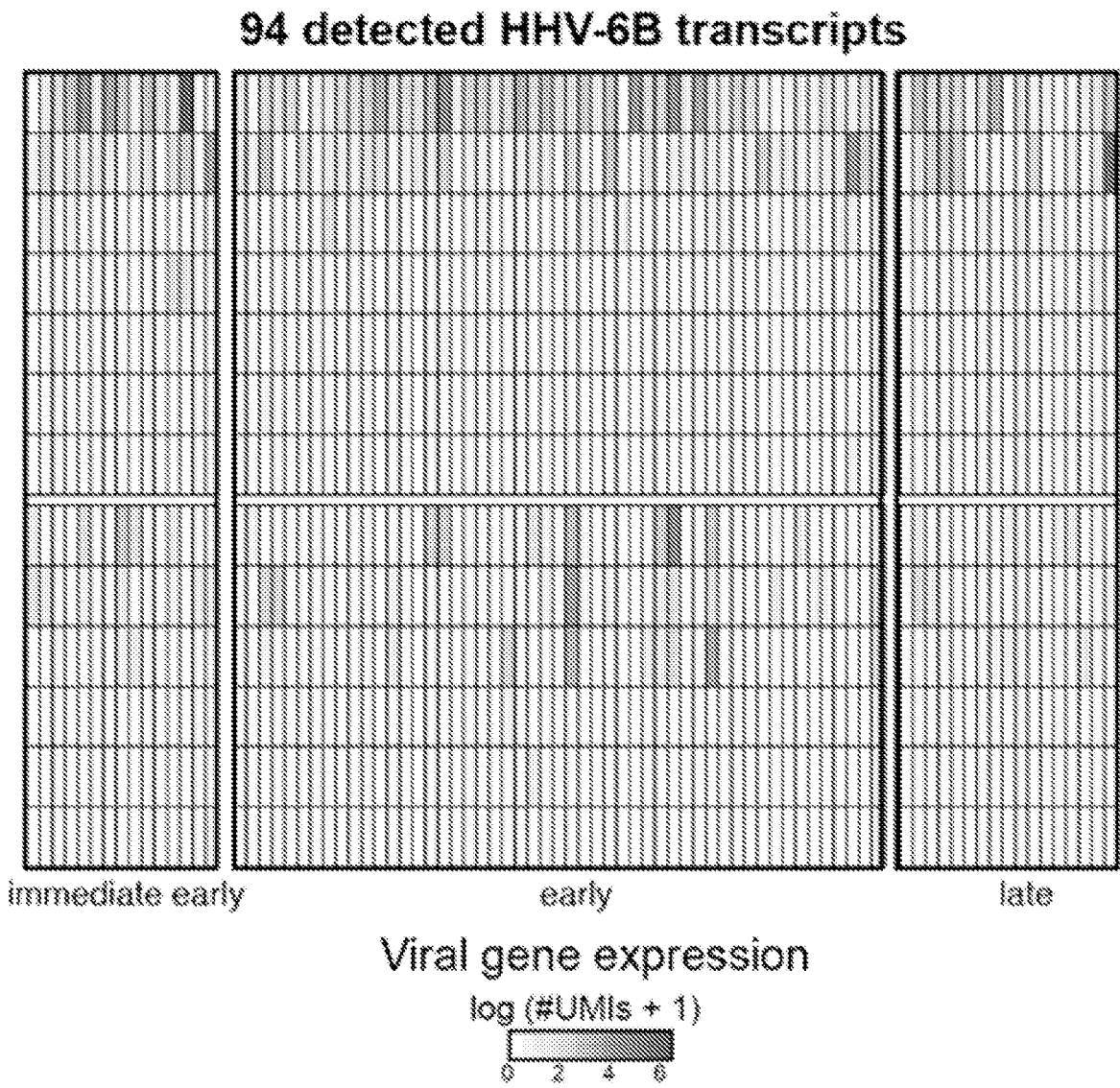


FIG. 3 (Cont'd)

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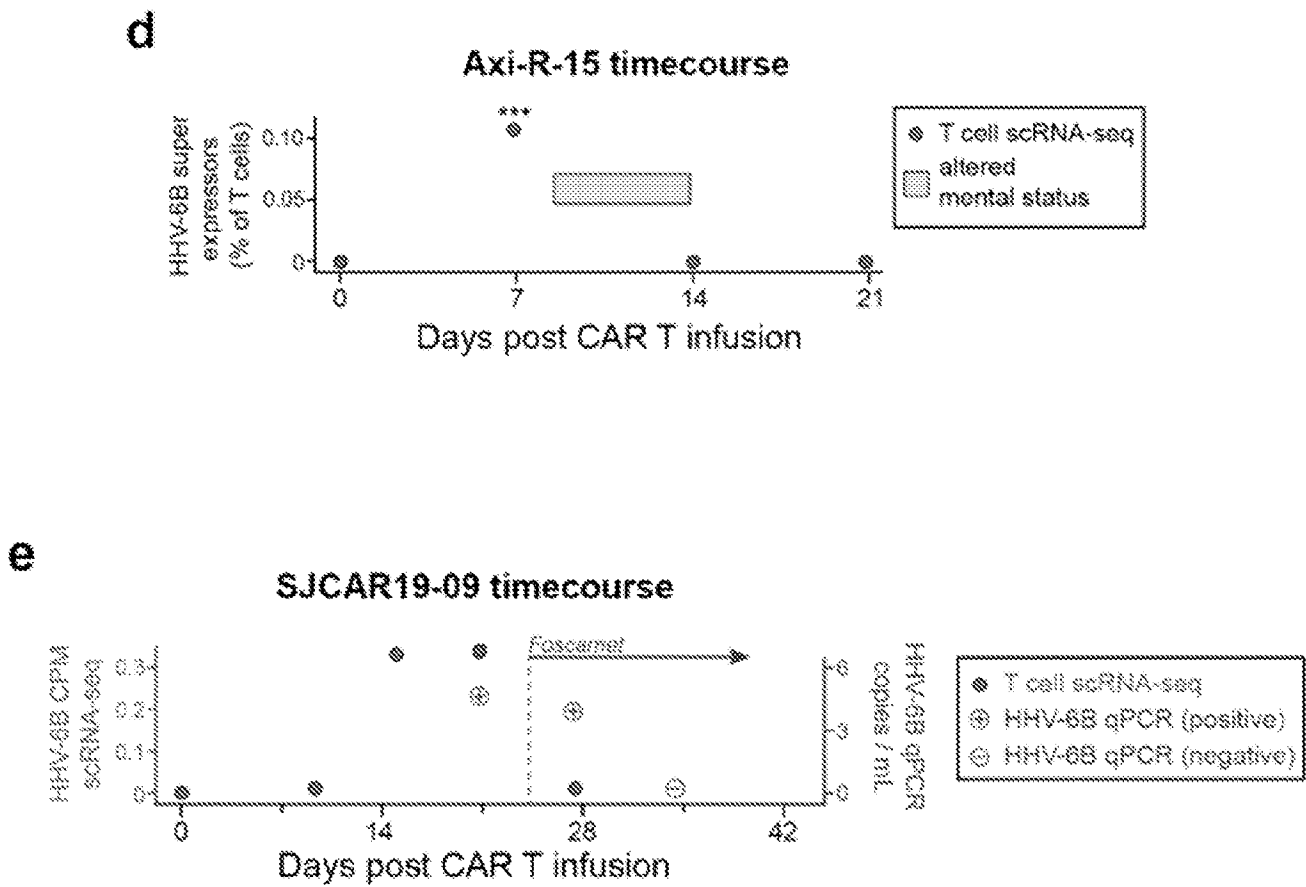
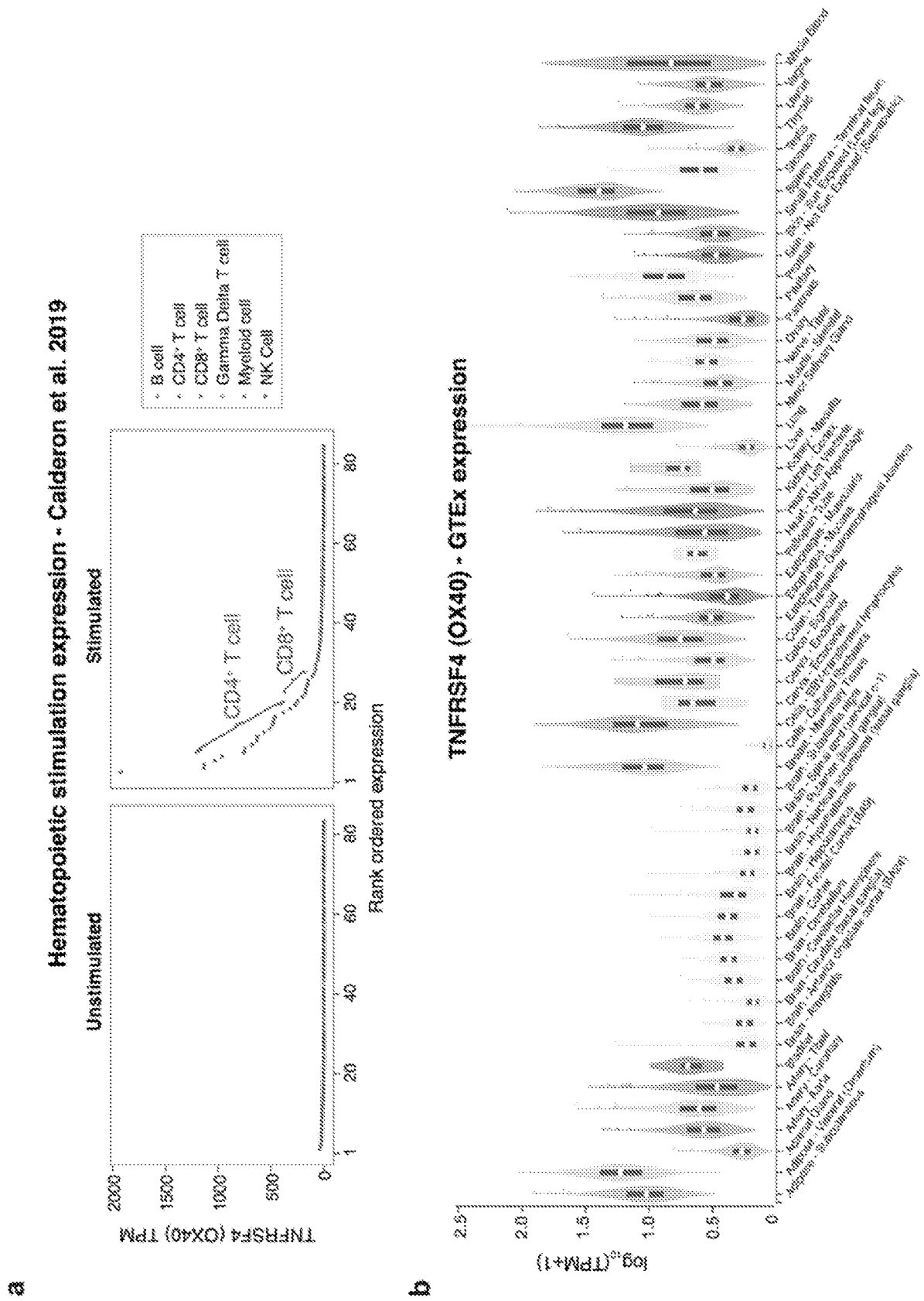


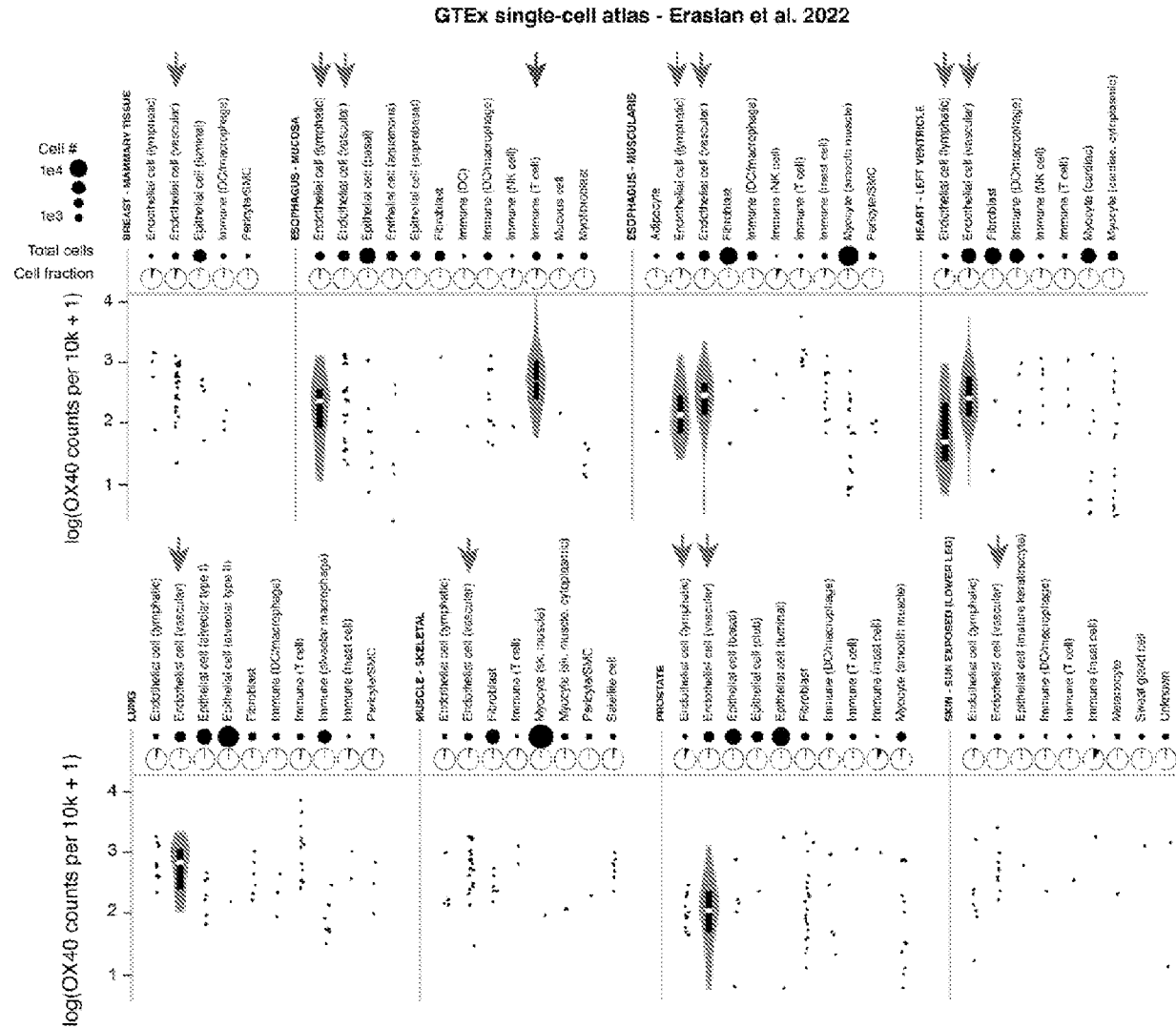
FIG. 3 (Cont'd)

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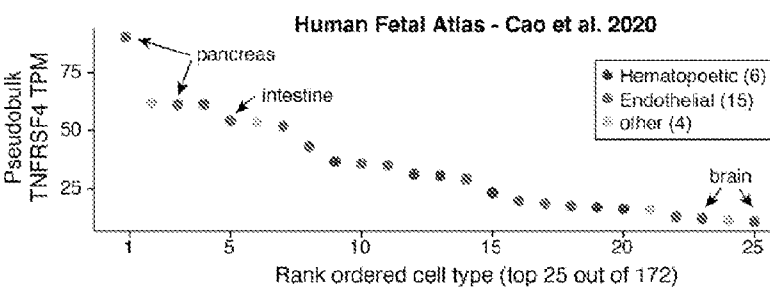


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a



b



c

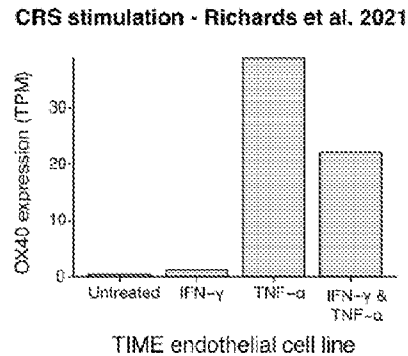


FIG. 5

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a

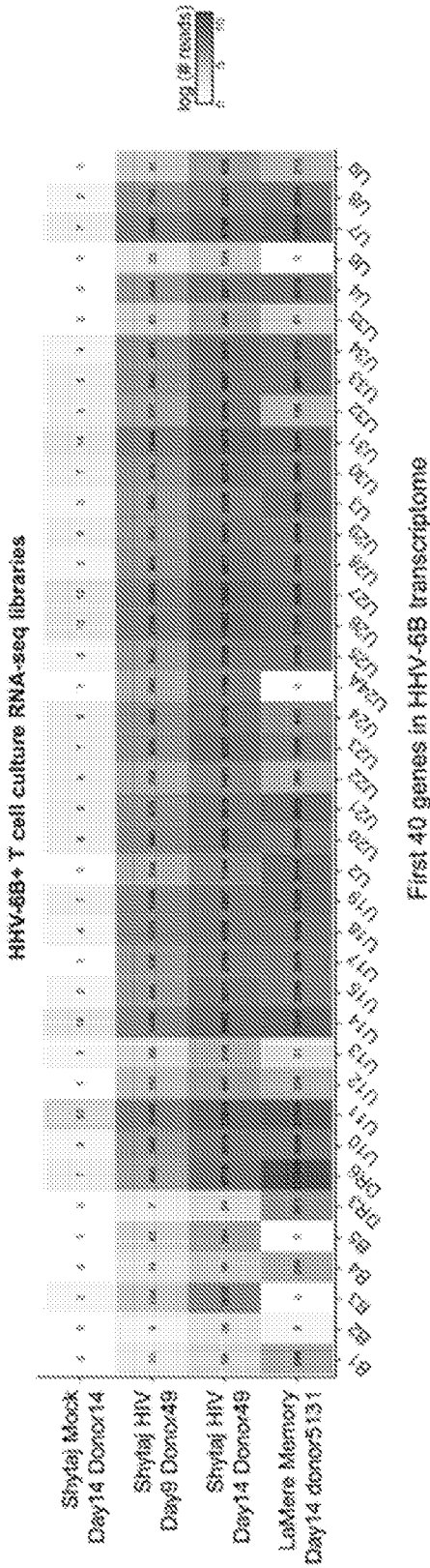


FIG. 6

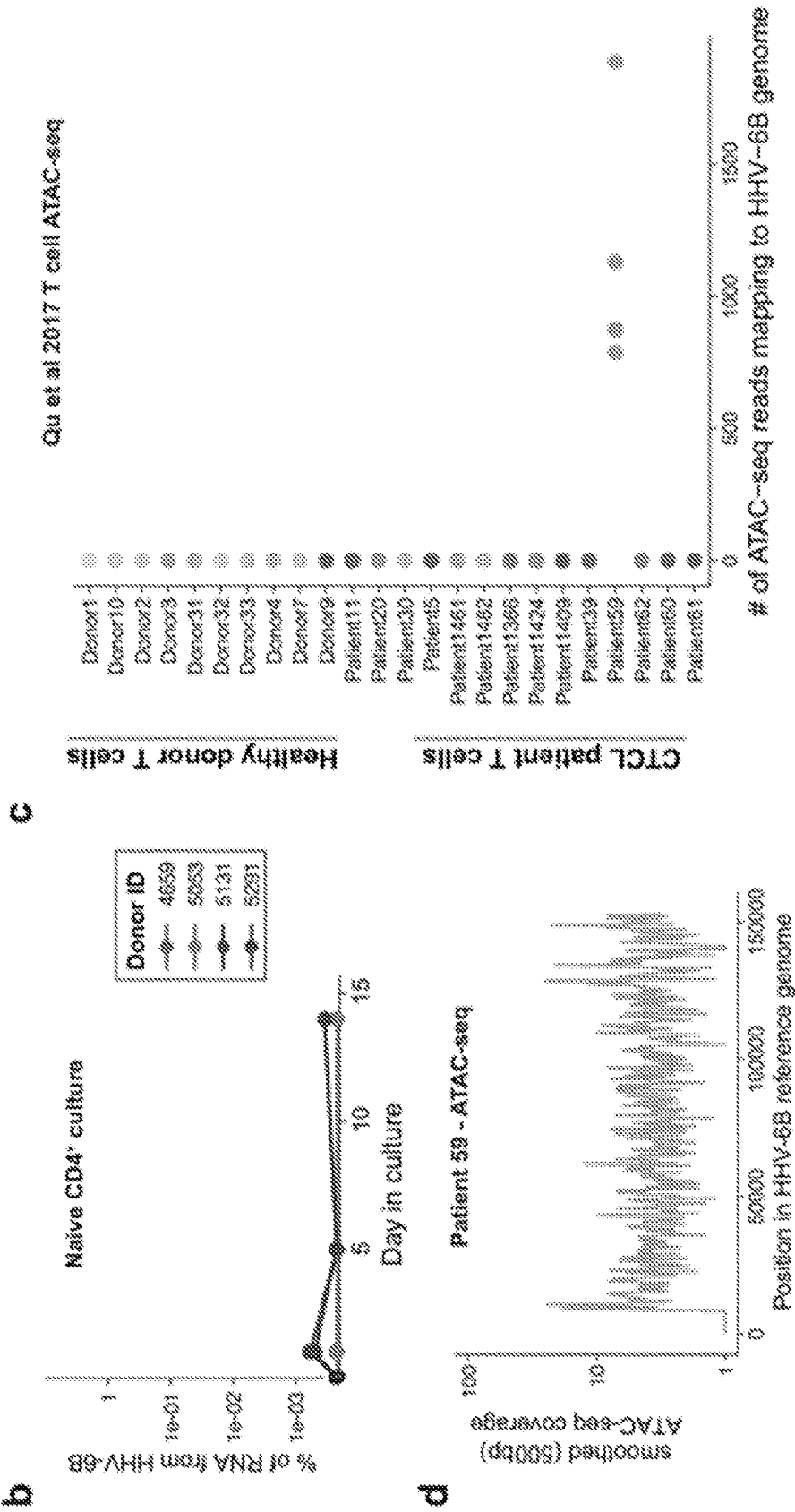
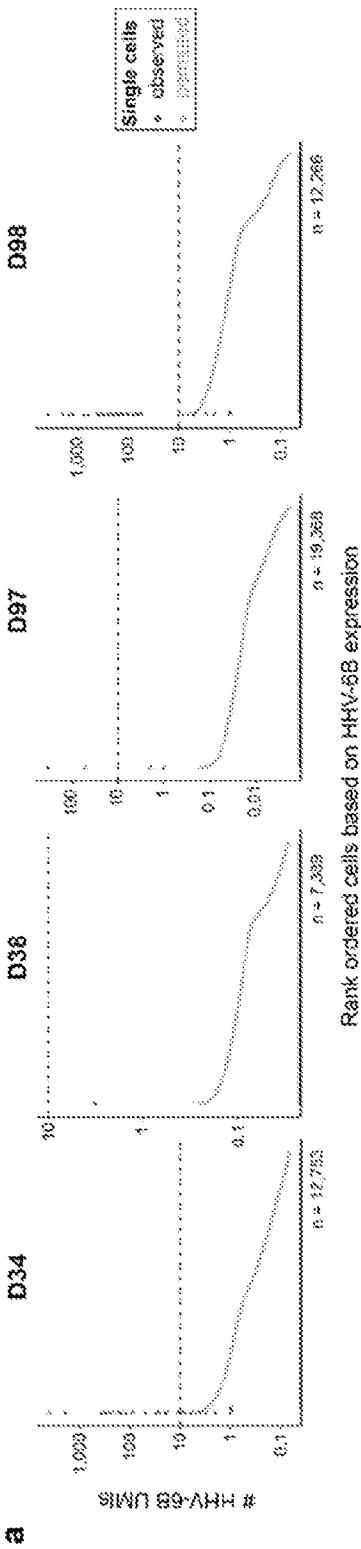


FIG. 6 (Cont'd)



b

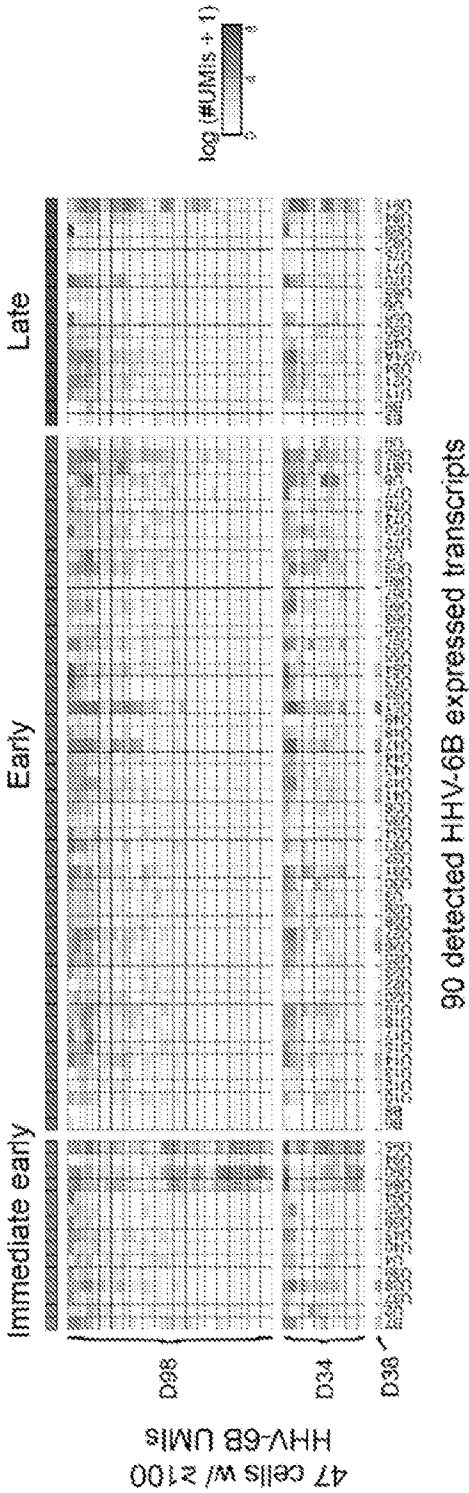


FIG. 7

C

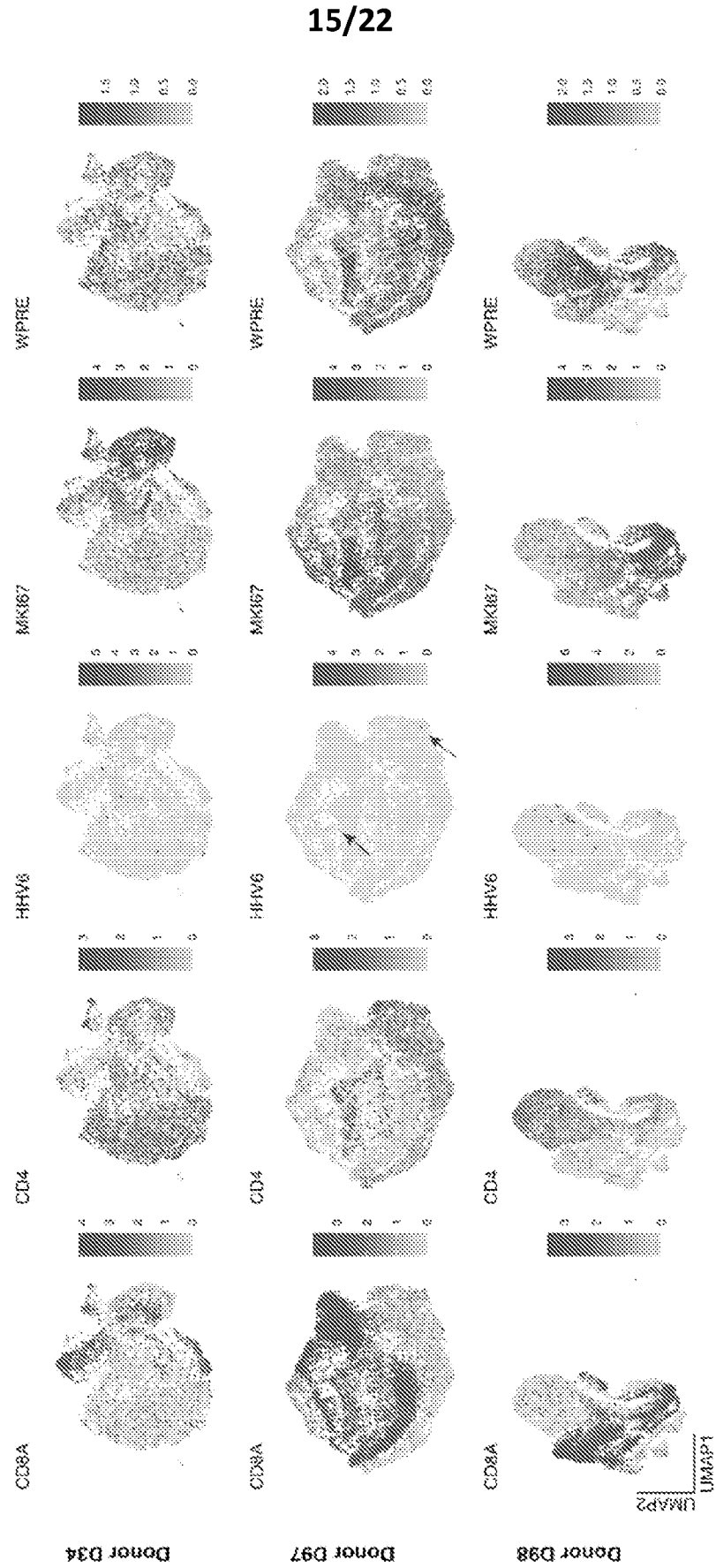


FIG. 7 (Cont'd)

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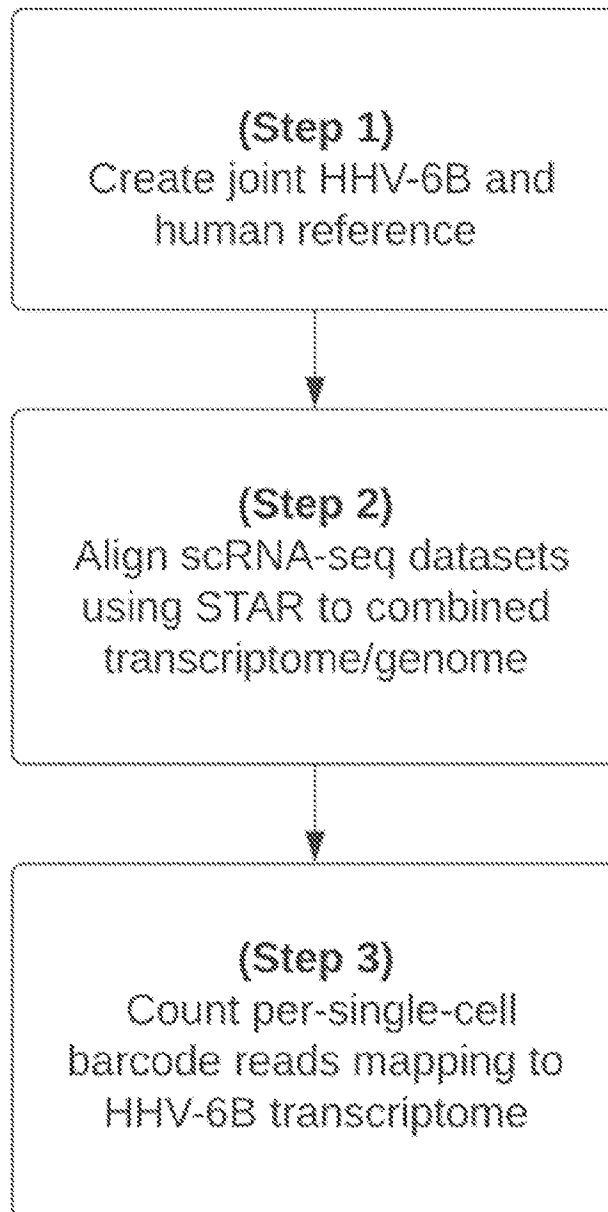


FIG. 8

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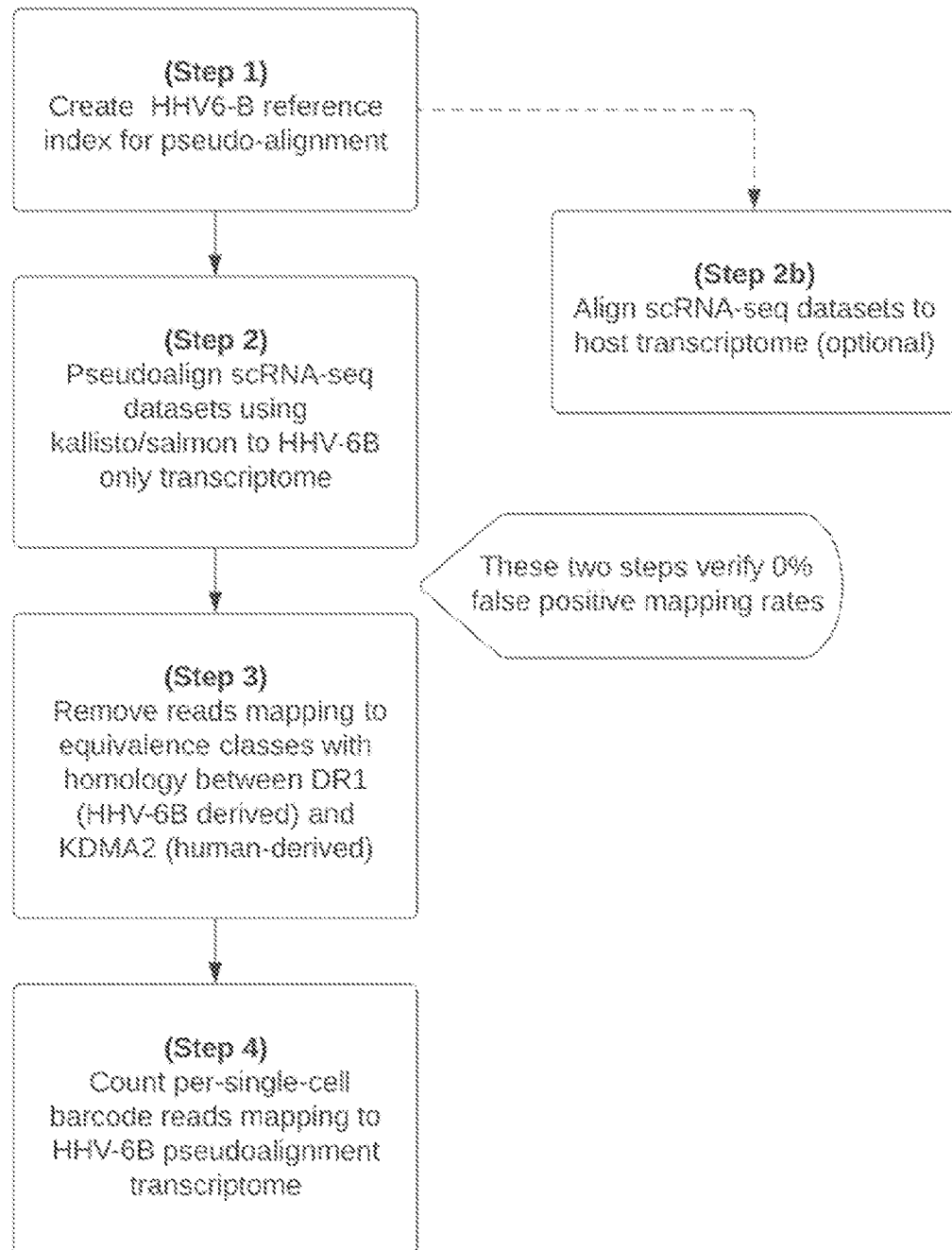


FIG. 9

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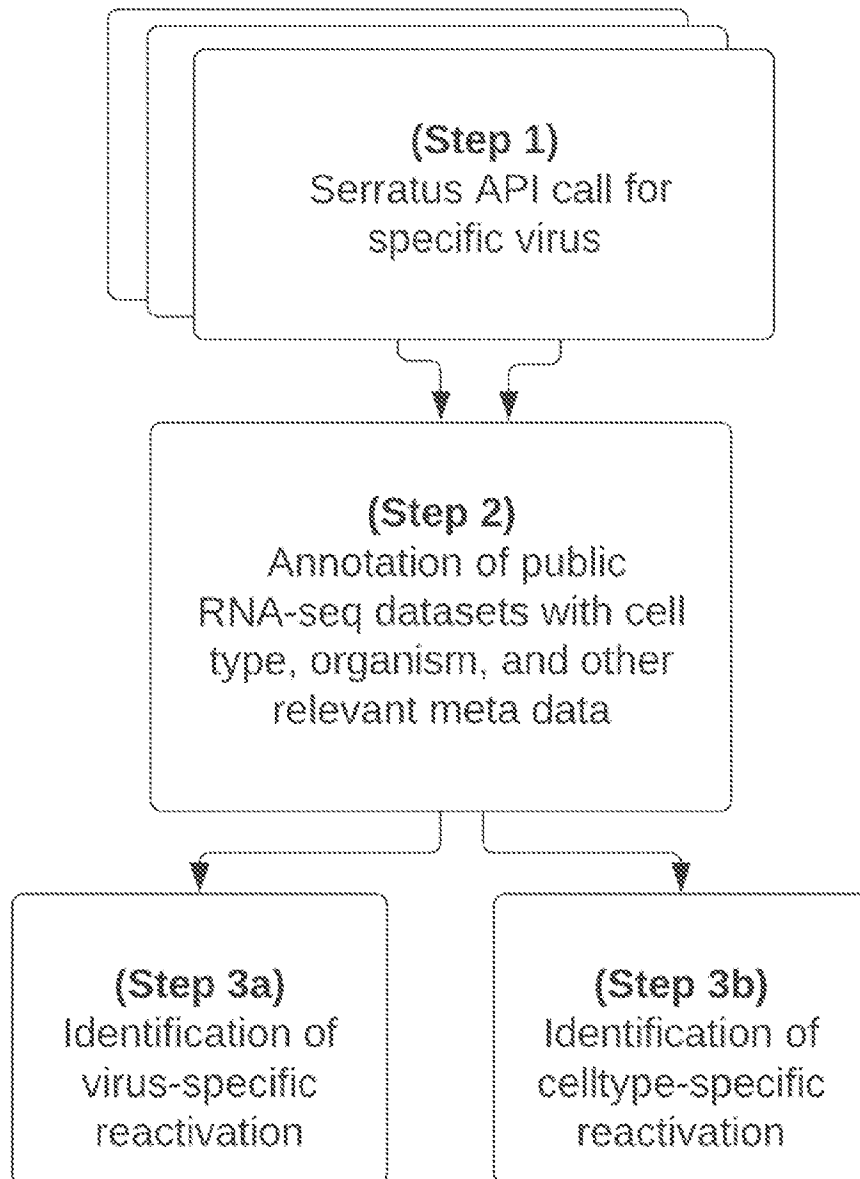
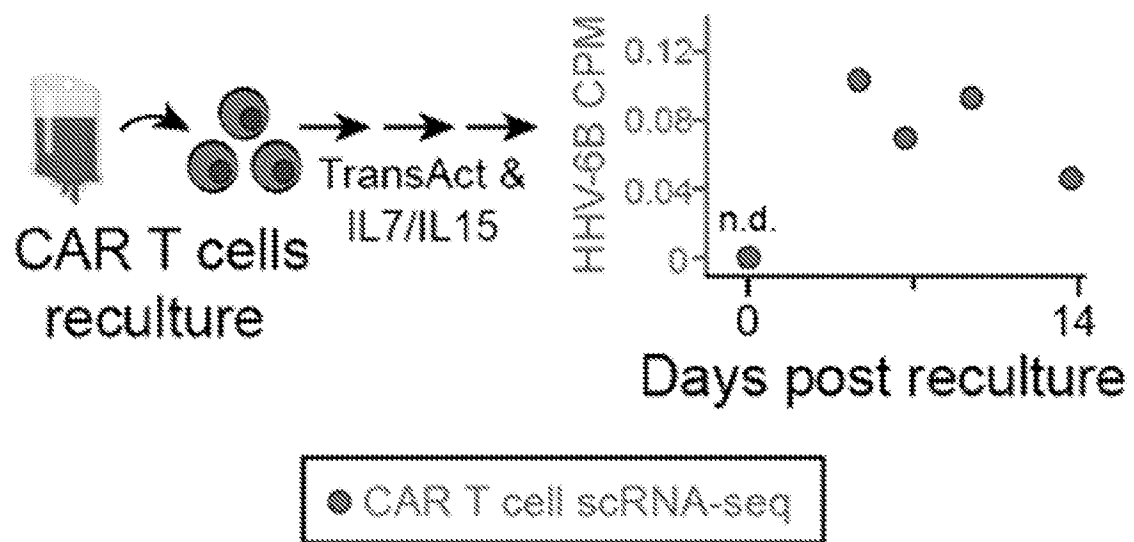


FIG. 10

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SJCAR19-09 *in vitro* timecourse**FIG. 11**

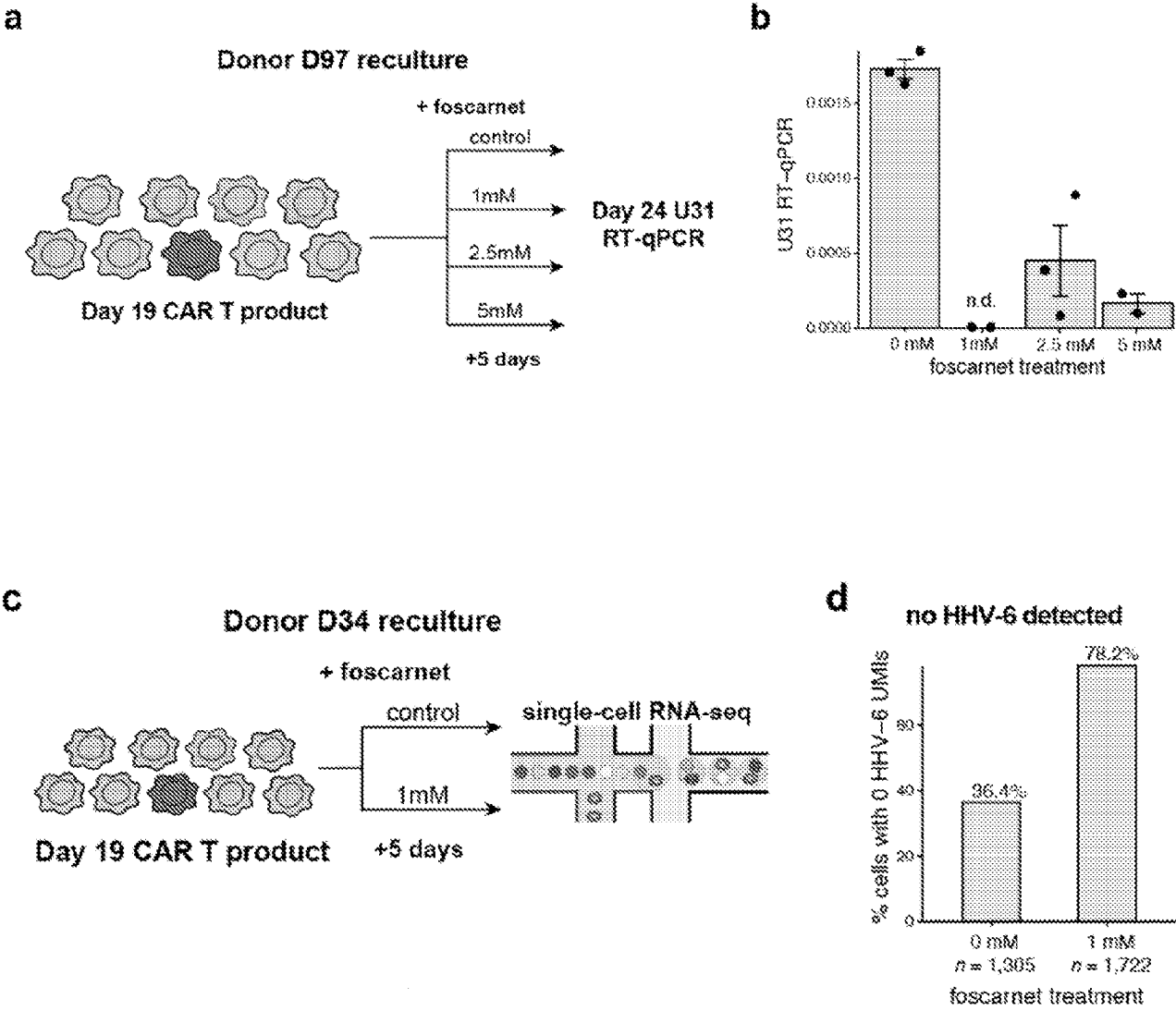


FIG. 12

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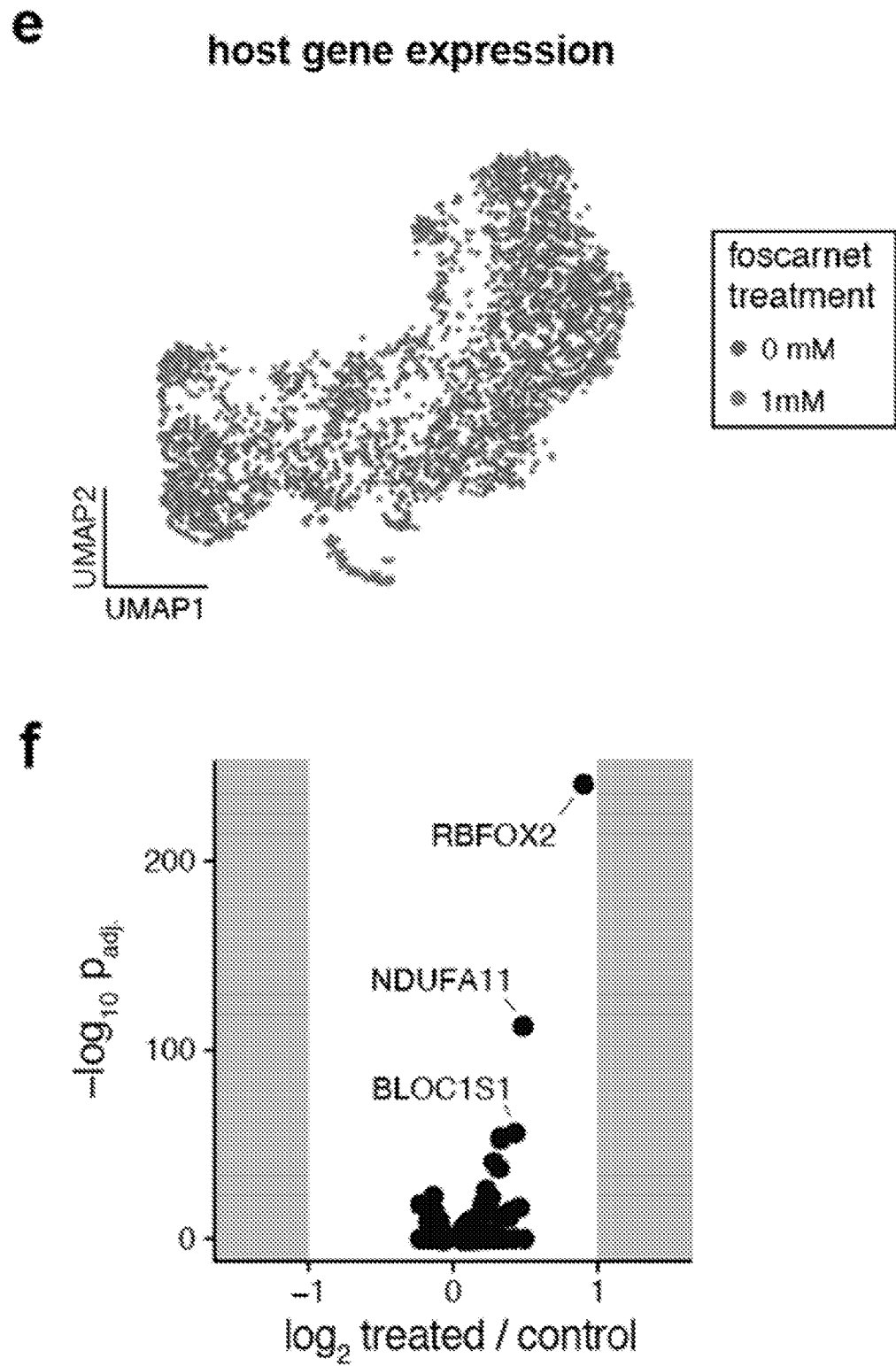


FIG. 12 (Cont'd)