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(54) Title: **TSYN-SEQ: A T CELL SYNAPSE-BASED TUMOR ANTIGEN IDENTIFICATION PLATFORM**

(57) Abstract: Provided herein are chimeric protein molecules that generate detectable signals and exhibit low apoptotic activity. The present invention further discloses cells and cell reporter systems that express the chimeric protein molecule and generate detectable signals upon synapse formation, as well as associated methods of use for identifying T cell receptors and cognate antigens.

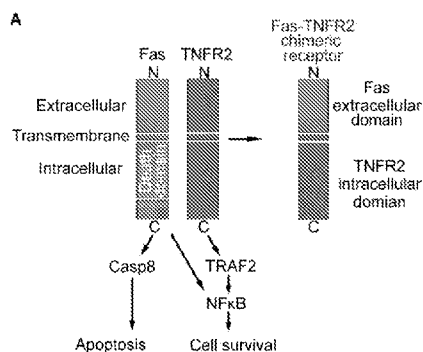


FIG. 1A



TSYN-SEQ: A T CELL SYNAPSE-BASED TUMOR ANTIGEN IDENTIFICATION PLATFORM

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of priority under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 63/524,557, filed June 30, 2023. The disclosure of the prior application is considered part of and is herein incorporated by reference in the disclosure of this application in its entirety.

INCORPORATION OF SEQUENCE LISTING

[0002] The material in the accompanying sequence listing is hereby incorporated by reference into this application. The accompanying sequence listing xml file, name MDA1200-1WO, was created on June 19, 2024, and is 41,066 bytes in size.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0003] The present invention relates generally to engineered cell receptors, and more specifically to engineered tumor necrosis factor receptors (TNFRs) and cell systems that utilize these receptors to generate detectable signals upon MHC-TCR binding.

BACKGROUND INFORMATION

[0004] T cells play important roles in a variety of immune responses, including some that can be beneficial and others that can be harmful. These include providing protection against infections and cancers, but can also include pathological inflammation, such as in settings of autoimmunity or impaired tolerance to environmental or transplanted antigens. TCRs are cell surface molecules that endow T cells with their unique antigen specificities and can recognize a vast pool of antigens that can be foreign- or host-derived and can include intracellular and extracellular proteins. These antigens are presented as peptides by major histocompatibility complexes (pMHC) on the cell surface of target cells. Sequencing TCR α and TCR β genes can identify unique T cell clones and expressing these genes in another T cell can endow the new cell with the same antigen specificity as the original. While the recent advent of single cell sequencing methodologies has increased the

ease and affordability of identifying TCR sequences from samples, identifying which antigens are recognized by T cells continues to be challenging.

[0005] Several well-developed methods are available to identify TCR–pMHC interactions. These include T cell functional assays and peptide-MHC multimers, which allow for evaluation of a priori-generated hypotheses but are generally not readily amenable to high-throughput antigen discovery. More recently, untargeted assays have been developed utilizing antigen libraries presented by baculovirus, yeast or mammalian cell. These methods, while powerful, can also be laborious, often require unusual and non-commercially available reagents and may be technically difficult in some other way. Particularly challenging is the identification of antigens at the genome scale.

[0006] Improving the ease of identifying antigens recognized by T cells is important for advancing knowledge of T cell antigen recognition, as well as accelerating the development of T cell-based therapies for a variety of clinical indications, including infectious diseases, transplantation tolerance, autoimmune disorders and cancer. In the field of cancer immunology, this system could be applied to identify neoantigens for personalized vaccines and could also be utilized as a screening tool in the development of TCR-based therapy leads for evaluating potential off-target reactions that can lead to toxicities.

SUMMARY OF THE INVENTION

[0007] The present invention is based on the seminal discovery that tumor necrosis factor receptors (TNFRs) can be engineered to generate detectable signals without also promoting apoptosis. The engineered TNFRs can be incorporated into cell reporter systems to enable antigen detection. The cell reporter systems can be configured for bidirectional signaling, and can be used to identify novel antigens, antigen-binding proteins, and intercellular synapse formation. In contrast to previous antigen detection platforms, the systems of the present invention do not require prior knowledge of antigen structures or TCR sequences for antigen or TCR identification and are therefore utilized for high-throughput analyses of large antigen and TCR libraries.

[0008] In one embodiment, the invention provides a chimeric protein molecule that includes: a) an extracellular domain selected from a Fas receptor extracellular domain, a CD80 extracellular domain, a PD-L1 extracellular domain, a CD86 extracellular domain, an LT β R extracellular domain, a 4-1BBL extracellular domain, an OX40L extracellular domain, an antibody that binds

to a tumor necrosis factor, or antigen binding fragment that binds to a tumor necrosis factor; b) an intracellular domain selected from a TNFR1 intracellular domain, a TNFR2 intracellular domain, a RANK intracellular domain, a BCMA intracellular domain, or a CD30 intracellular domain; and c) a transmembrane domain linked to the C-terminus of the extracellular domain and the N-terminus of the intracellular domain.

[0009] In one aspect, the extracellular domain includes a Fas receptor extracellular domain. In particular aspects, the intracellular domain is a TNFR2 intracellular domain. In additional aspects, the transmembrane domain is a TNFR2 transmembrane domain. In further aspects, the extracellular domain is a Fas extracellular receptor domain, the transmembrane domain is a TNFR2 transmembrane domain, and the intracellular domain is a TNFR2 intracellular domain.

[0010] In certain aspects, the chimeric protein molecule includes at least 80% sequence identity to SEQ ID NO:1. In particular aspects, the chimeric protein molecule is SEQ ID NO:1.

[0011] In some aspects, the extracellular domain has at least 80% sequence identity to amino acids 1-170 of SEQ ID NO:1; the transmembrane domain has at least 80% sequence identity to amino acids 171-203 of SEQ ID NO:1; the intracellular domain has at least 80% sequence identity to amino acids 204-374 of SEQ ID NO:1.

[0012] In further aspects, the extracellular domain includes at least 80% sequence identity to SEQ ID NO:2. In another aspect, the transmembrane domain includes at least 80% sequence identity to SEQ ID NO:3. In some aspects, the intracellular domain includes at least 80% sequence identity to SEQ ID NO:4.

[0013] In certain aspects, the intracellular domain does not exhibit apoptotic activity. In some aspects, the intracellular domain does not exhibit FADD, Casp8, FAF, or DAXX activation activity.

[0014] In some aspects, the invention provides an isolated cell that includes any of the chimeric protein molecules described herein. In certain aspects, the cell is an antigen-presenting cell (APC). In some aspects, the APC is a macrophage, a regulatory macrophage, an activated macrophage, an M2 macrophage, a B cell, a plasma cell, a memory cell, a dendritic cell, a plasmacytoid dendritic cell, an inflammatory dendritic cell, or a Langerhans cell.

[0015] In certain aspects, the cell produces a detectable signal upon activation of the chimeric protein molecule. In some aspects, the detectable signal is a fluorescence signal. In further aspects, the detectable signal is generated by an inducible reporter system that is activated by the chimeric

protein molecule. In some aspects, the inducible reporter system includes a transcription factor response element. In particular aspects, the transcription factor response element is an NFκB response element or an AP1 response element. In additional aspects, the transcription factor response element is operably coupled to a gene encoding a fluorescent protein, a luminescent protein, a cell surface protein, or an electrochemically detectable protein. In further aspects, the fluorescent protein is green fluorescent protein (GFP).

[0016] In some aspects, the cell expresses an MHC Class I protein or an MHC Class II protein. In some aspects, the cell overexpresses the MHC Class I protein or the MHC Class II protein. In particular aspects, the cell is engineered to express a single MHC allele.

[0017] In some aspects, the cell does not express a native Fas receptor. In some aspects, the cell is a Fas receptor knockout. In certain aspects, the cell expresses an exogenous antigenic peptide. In certain aspects, the cell displays the exogenous antigenic peptide, or a fragment thereof, in an MHC Class I or MHC Class II protein. In particular aspects, the MHC Class I or MHC Class II protein is endogenously expressed. In further aspects, the MHC Class I or MHC Class II protein is encoded by an exogenous nucleic acid.

[0018] In further aspects, the cell is stably or transiently transfected with a vector encoding the exogenous antigenic peptide.

[0019] In some aspects, the cell overexpresses CIITA, LRC5, B2M, RFX5, RFXAP, or RFXANK. In some aspects, the cell overexpresses CIITA.

[0020] In another embodiment, the invention provides a system that includes: a) an antigen-presenting cell (APC) that includes an MHC protein and a first inducible reporter system, and b) an antigen-sensing cell that includes a T cell receptor (TCR) or a chimeric antigen receptor (CAR), and a second inducible reporter system; wherein the first inducible reporter system generates a first detectable signal and the second inducible reporter system generates a second detectable signal upon contact between the MHC protein and the TCR or the CAR.

[0021] In certain aspects, first inducible reporter system and/or the second inducible reporter system includes a transcription factor response element. In particular aspects, the first inducible reporter system includes an NFκB response element. In some aspects, the second inducible reporter system includes an NFAT response element. In some aspects, the first inducible reporter system includes an NFκB response element and the second inducible reporter system includes an NFAT response element. In some aspects, the transcription factor response element is operably coupled

to a gene encoding a fluorescent protein, a luminescent protein, a cell surface protein, or an electrochemically detectable protein.

[0022] In some aspects, the APC expresses a transmembrane protein that activates the first inducible reporter system upon binding to a ligand expressed by the antigen-sensing cell. In some aspects, the ligand is a tumor necrosis factor or an immune checkpoint molecule. In particular aspects, the ligand includes FasL, CTLA-4, PD-1, LT- α , or TNFSF14. In some aspects, the transmembrane protein is a chimeric protein molecule as outlined herein. In some aspects, the TCR or the CAR activates the second inducible reporter system upon contact with the MHC protein.

[0023] In some aspects, the antigen-presenting cell includes a nucleic acid encoding an antigen. In some aspects, the system includes a plurality of the APCs, and the plurality of the APCs collectively comprise a plurality of nucleic acids encoding a plurality of antigens. In some aspects, the APC is an isolated cell as disclosed herein.

[0024] In certain aspects, the antigen-sensing cell is a T cell. In some aspects, the TCR is overexpressed by the antigen-sensing cell. In particular aspects, the antigen-sensing cell expresses a single TCR molecule. In some aspects, the antigen-sensing cell does not express TNF. In some aspects, the first detectable signal comprises at least about 1.5-fold, at least about 2-fold, at least about 2.5-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 12-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, at least about 50-fold, or at least about 100-fold higher signal-to-noise than an identical system in which the antigen-sensing cell expresses TNF.

[0025] In a further embodiment, the invention provides a system that includes: a) an antigen-presenting cell (APC) that includes an MHC protein, an inducible reporter system that includes an NF κ B response element operably coupled to a gene encoding a first fluorescent protein, and a chimeric protein molecule configured to activate the NF κ B response element; and b) an antigen-sensing cell that includes a T cell receptor (TCR) or a chimeric antigen receptor (CAR), an inducible reporter system that is activated by the TCR or CAR and includes an NFAT response element operably coupled to a gene encoding a second fluorescent protein, and a ligand configured to activate the chimeric protein molecule.

[0026] In some aspects, the chimeric protein molecule is a chimeric protein molecule as disclosed herein. In some aspects, the first fluorescent protein is a green fluorescent protein (GFP). In additional aspects, the second fluorescent protein is an mCherry protein.

[0027] In some aspects, the invention provides a method for detecting an antigen recognized by a T cell that includes contacting a system of the invention with an antigen or a nucleic acid encoding the antigen; and detecting a first detectable signal from the first inducible reporter system and a second detectable signal from the second inducible reporter system, wherein the first and/or second detectable signal is indicative of a TCR or CAR that specifically recognizes the antigen.

[0028] In some aspects, the antigen is selected from a tumor antigen, a bacterial antigen, a viral antigen, a cancer antigen, a neoantigen, and a self-antigen. In further aspects, the method further includes identifying the TCR or the CAR that specifically recognizes the antigen. In particular aspects, the identifying includes sequencing a nucleic acid encoding the TCR or the CAR. In some aspects, the nucleic acid is from a cDNA library.

[0029] In certain aspects, the method further includes identifying the antigen recognized by the TCR or CAR. In particular aspects, the identifying includes sequencing the nucleic acid encoding the antigen.

[0030] In one aspect, the method further includes isolating the APC or the antigen-sensing cell. In some aspects, the isolating includes flow cytometry or fluorescence-activated cell sorting (FACS). In a particular aspect, the isolating includes excluding a cell that does not produce the first detectable signal or the second detectable signal. In some aspects, the isolating includes size-selection of an aggregate that includes the APC and the antigen-sensing cell. In further aspects, the isolating includes selecting a cell that produces the first detectable signal or the second detectable signal.

[0031] In some aspects, the antigen-sensing cell is a T cell. In particular aspects, the antigen is present by the APC. In some aspects, the antigen is exogenous to the APC. In some aspects, an antigen is identified by the method.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] FIGS. 1A-1F is a series of schematics and plots showing Fas-TNFR2 structure and activity. FIG. 1A is a schematic of Fas-TNFR2 chimeric receptor. FIG. 1B is a schematic that shows T cell-target cell interactions activate Fas-TNFR2 receptor and results in NFκB-induced

fluorescence. **FIG. 1C** is a schematic that shows generation of Fas-TNFR2 chimeric receptor-overexpressing NFκB reporter cells. **FIG. 1D** is a series of representative flow cytometry plots of GFP expression in 293T reporter cells described in C. Cells were stimulated with anti-Fas antibody (dark) or PBS as untreated groups (light). **FIG. 1E** is a set of bar graphs with apoptosis data for 293T reporter cells after anti-Fas antibody treatment compared to untreated groups. The levels of cleaved PARP in 293T NFκB reporter cells were analyzed by flow cytometry (left panel, $n = 3$) or MTT assay (right panel, $n = 6$). **FIG. 1F** is a plot that shows extracellular Fas expression levels in 293T reporter cells analyzed by flow cytometry for WT-293T cells (top), NFκB-GFP 293T cells (second from top), FasKO NFκB-GFP 293T cells (second from bottom, “KO” is knockout), and FIR-APCs (bottom). Statistical significance was determined by the one-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey test. Error bars indicate SD across replicates.

[0033] **FIGS. 2A-2F** is a series of schematics and fluorescence plots of APC-T cell reporter assays. **FIG. 2A** is a schematic of an antigen peptide pulsation activation assay in which FIR-APCs are labeled with CellTrace Violet (CTV) and pulsed with antigen peptides before co-culturing with αE7-Jurkat cells. **FIG. 2B** is a series of plots of GFP expression in E7-peptide-pulsed FIR-APCs quantified by flow cytometry. E7-peptide-pulsed FIR-APCs were cultured alone or with αE7-Jurkat cells for 1 day. **FIG. 2C** is a time course plot of GFP expression in E7-peptide-pulsed FIR-APCs during coculture with αE7-Jurkat cells. The y-axis depicts fold change in mean fluorescence intensity (MFI) of GFP in FIR-APCs. **FIG. 2D** is a series of plots showing GFP expression in peptide-pulsed FIR-APCs after 2 days of coculture with the indicated TCR-Jurkat cells. Representative flow cytometry plots are shown (left panel) as well as MFI fold change of GFP in FIR-APCs (right panel). Fold change is defined as the ratio of GFP MFI in the cocultured FIR-APCs relative to the mono-cultured FIR-APCs. $n = 3$ independent samples. **FIG. 2E** is a schematic of an antigen overexpression activation assay. **FIG. 2F** is a series of plots of GFP expression in FIR-APCs with overexpressed antigens after 2 days of coculture with TCR-Jurkat cells. $n = 6$ independent samples. Statistical significance was determined by the one-way ANOVA with post-hoc Tukey test. Error bars indicate SD across replicates.

[0034] **FIGS. 3A-3E** is a schematic of an APC-T cell co-culture assay and graphs of APC and T cell activation following the co-culture assay. **FIG. 3A** is a schematic of an APC-T cell co-culture assay in which CTV-labeled FIR-APCs are pulsed with peptides and cocultured with CellTrace Far Red (CTFR) labeled αE7-Jurkat cells for 2 days. **FIG. 3B** is a plot of frequencies of

multiparticle and single particle in CTV and/or CTFR positive populations after co-culturing E7-peptide-pulsed FIR-APCs with Jurkat cells. Multiparticle and single particle gates are generated after visualizing FSC-A and FSC-H. $n = 6$ independent samples. **FIG. 3C** is a plot of frequencies of CTV/CTFR double-positive events after coculture following pulsing with cognate or non-cognate peptide. $n = 3$ independent samples. **FIG. 3D** is a plot of GFP expression in CTV/CTFR single- or double-positive populations in APC-T cell cocultures pulsed with E7-peptide. **FIG. 3E** is a plot of MFI fold change of GFP expression in coculture of Jurkat cells and FIR-APCs pulsed with cognate or non-cognate peptides. $n = 3$ independent samples. Statistical significance of b and c were determined by the one-way ANOVA with post-hoc Tukey test, and statistical significance of e was determined by the t-test. Error bars indicate SD across replicates.

[0035] **FIGS. 4A-4D** is a schematic and plots of fluorescence data from a bidirectional APC-T cell reporter system. **FIG. 4A** is a schematic of an APC that expresses a chimeric Fas receptor and Fas-iNF κ B reporter and a T cell that expresses an NFAT-mCherry reporter. The schematic shows simultaneous activation of the Fas-iNF κ B and NFAT-mCherry reporters from interaction between the two cells. **FIG. 4B** is a plot of frequencies of Fas-iNF κ B reporter positive populations in NFAT-mCherry reporter positive or negative aggregates. E7-peptide-pulsed FIR-APCs are cocultured with α E7-JR-T cells, while NLV-peptide-pulsed FIR-APCs are cocultured with α E7-JR-T cells as control. Gates demarcating mCherry positive (top arrow) and negative (bottom arrow) populations among the CTV/CTFR double-positive aggregates are shown. **FIG. 4C** is a bar graph of GFP intensity for a coculture of JR-T cells and FIR-APCs pulsed with cognate and non-cognate peptides. Statistical significance is determined by the Mann-Whitney U test. Error bars indicate SD across 5 replicates. **FIG. 4D** is a set of ROC curve analyses of dual-reporter signaling in the coculture of **FIG. 4B**. The left ROC curve represents GFP fluorescence intensity of aggregates population. The right ROC curve represents mCherry fluorescence intensity of GFP positive population in aggregates population.

[0036] **FIGS. 5A-5C** is a schematic and plots of fluorescence from a bidirectional APC-T cell reporter system. **FIG. 5A** is a schematic of a APC-T cell reporter system in which non-cognate HLA and TCR genes were removed with gene editing. **FIG. 5B** is a series of representative flow cytometric plots and frequencies of aggregates in the reporting system of **FIG. 5A**, in which α E7-JR-T cells were cocultured with FIR-APCs with or without endogenous E7 antigen expression. **FIG. 5C** is a series of representative flow cytometric plots and frequencies of dual-reporter

positive aggregate events in the reporting system of **FIG. 5A**, in which α E7-JR or α NLV-JR-T cells were cocultured with FIR-APCs with or without endogenous E7 antigen expression. KO, knock out. Statistical significance was determined by the one-way ANOVA with post-hoc Tukey test. Error bars indicate SD across 3 replicates.

[0037] **FIGS. 6A-6F** is a schematic of an antigen screening workflow of a bidirectional APC-T cell reporter system and graphs of fluorescence data obtained using this workflow. **FIG. 6A** is a series of representative flow cytometric plots of NFAT-mCherry reporter activation in α E7-JR-T cells. α E7-JR-T cells were cultured alone or with control 293T cells (HLA-A*02:01-positive, HPV16-negative) or CaSki cells (HLA-A*02:01-positive, HPV16-positive). **FIG. 6B** is a bar graph that summarizes frequencies in **FIG. 6A**. Error bars indicate SD across 6 replicates. **FIG. 6C** is a schematic of an antigen screening workflow of a bidirectional APC-T cell reporter system in which CTV-labeled CaSki-FIR-APCs are cocultured with CTFR-labeled TCR-JR-T cells for 2 days. Interacting CaSki-FIR-APCs and α E7-JR-T cells are sorted as dual-reporter positive CTV/CTFR double-positive aggregates, followed by gDNA isolation and cDNA insert amplification. The amplified product is then evaluated using qPCR as well as deep sequencing. **FIG. 6D** is a set of bar graphs with E7 gene quantification from cDNA inserts enriched following Tsyn-reporter testing with α E7-JR-T cells, quantified by qPCR. Statistical significance is determined by the t-test. Error bars indicate 95% IC across three replicates. **FIG. 6E** is a plot of deep sequencing data of cDNA inserts enriched following Tsyn-reporter testing with α E7-JR-T cells. The genes encoding the cognate E7 antigen are indicated. Each dot represents one gene, with the y-axis plotting its log₁₀ p-value, and the x-axis plotting its effect size. Both p-value and effect sizes are quantified with DESeq2 across 6 biological replicates in the sorted population relative to that in the input library prior to flow-sort enrichment. Genes with p value < 0.05 and effect size > 0 are marked, and genes with p value < 0.05 and effect size < 0 are marked. The size of the dot stands for the abundance of gene. **FIG. 6F** is a graph illustrating E7 gene quantification from cDNA inserts enriched following Tsyn-reporter testing with α E7-JR-T cells and control α NLV-JR-T cells. n = 6 independent samples. Statistical significance is determined using DESeq2.

[0038] **FIG. 7** shows illustrative sequences of the invention as described herein.

[0039] **FIGS. 8A-8D** illustrate the Tsyn-seq triple-reporter system version 3 (V.3). **FIG. 8A** is a schematic of Fas-TNFR2 chimeric receptor. The death domain mediates apoptotic caspase activation, while the intracellular domain of TNFR2 recruits cytoplasmic TNF receptor-associated

factor-2 (TRAF-2) resulting in initiation of NF κ B signaling pathways and AP1 pathways. To prevent death domain-mediated caspase activation, we fused the extracellular domain of Fas (aa 1-170) with TNFR2 transmembrane (aa 258-280) and intracellular domains (aa 281-461). **FIG. 8B** is a schematic of the Tsyn-seq triple-reporter system V.3. Simultaneous activation of Fas-iNF κ B reporter and Fas-iAP1 reporter in FIR-APCs, as well as NFAT-mCherry reporter in JR-T cells through FasL/FasR and TCR/pMHC interactions, respectively. **FIG. 8C** shows representative flow cytometry plots of reporters' activation in FIR-APCs V.3. Cells were stimulated with anti-Fas antibody or PBS as untreated groups. **FIG. 8D** shows the Tsyn-seq system spike-in assay (left panel) and fold enrichment of E7-FIR-APCs with and without the gating of Fas-iAP1 reporter activation (right panel). Schematic of the spike-in assay: CTV-labeled E7-FIR-APCs V.3 are used as a spike-in and mixed with unlabeled E7-negative FIR-APCs V.3 before coculture with α E7-JR-T cells. Fold enrichment is defined as the ratio of frequency of CTV positive cells in triple-reporter positive aggregates relative to the initial input of spike-in (0.4%).

[0040] **FIG. 9** is a set of graphs illustrating the amount of reporter gene measured.

DETAILED DESCRIPTION OF THE INVENTION

[0041] Before the present compositions and methods are described, it is to be understood that this invention is not limited to particular compositions, methods, and experimental conditions described, as such compositions, methods, and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only in the appended claims.

[0042] As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0043] As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items.

[0044] As used herein, the term "about" in association with a numerical value is meant to include any additional numerical value reasonably close to the numerical value indicated. For example,

and based on the context, the value can vary up or down by 5-10%. For example, for a value of about 100, means 90 to 110 (or any value between 90 and 110).

[0045] As used herein and in the claims, the terms “comprising,” “containing,” and “including” are inclusive, open-ended and do not exclude additional unrecited elements, compositional components or method steps. Accordingly, the terms “comprising” and “including” encompass the comparably more restrictive terms “consisting of” and “consisting essentially of.”

[0046] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

[0047] 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 this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, it will be understood that modifications and variations are encompassed within the spirit and scope of the instant disclosure. The preferred methods and materials are now described.

[0048] The present invention is based on the seminal discovery that tumor necrosis factor receptors (TNFRs) can be engineered to generate detectable signals without also causing apoptosis. The engineered TNFRs can be incorporated into cell reporter systems to enable antigen detection. The cell reporter systems can be used to identify novel antigens, antigen-binding proteins, and intercellular synapse formation. One or more cells of the reporter systems disclosed herein can include a chimeric protein molecule configured to generate or induce a detectable signal upon association with another cell. The engineered TNFR can be an engineered receptor protein with extracellular receptor, transmembrane, and intracellular domains from endogenous human proteins, such as naturally occurring tumor necrosis factor receptor proteins. Alternatively, the chimeric protein molecule can include a domain from a non-human or non-naturally occurring protein.

[0049] In one embodiment, the invention provides a chimeric protein molecule that includes: a) an extracellular domain selected from the extracellular domain of a TNF receptor superfamily protein, an antibody that binds to a tumor necrosis factor, or an antigen binding fragment that binds to a tumor necrosis factor; b) an intracellular signaling domain; and c) a transmembrane domain

linked to the C-terminus of the extracellular domain and the N-terminus of the intracellular domain.

[0050] The terms “peptide”, “polypeptide” and “protein” are used interchangeably herein and refer to any chain of at least two amino acids, linked by a covalent chemical bound. As used herein polypeptide can refer to the complete amino acid sequence coding for an entire protein or to a portion thereof. A “protein coding sequence” or a sequence that “encodes” a particular polypeptide or peptide, is a nucleic acid sequence that is transcribed (in the case of DNA) and is translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

[0051] The term “molecule” includes, but is not limited to, small molecules (including small molecules that do not have optimal cell-permeability), lipids, nucleosides, nucleotides, nucleic acids, polynucleotides, oligonucleotides, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, fusion proteins, chimeric proteins, peptides, hormones, carbohydrates, or polyamines. Non-limiting examples of polynucleotides include short interfering nucleic acid (siNA), antisense, enzymatic nucleic acid molecules, 2',5'-oligoadenylate, triplex forming oligonucleotides, aptamers, and decoys. Biologically active molecules include antibodies (*e.g.*, monoclonal, chimeric, humanized etc.), cholesterol, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, allozymes, aptamers, decoys and analogs thereof, and small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), antagomirs, and short hairpin RNA (shRNA) molecules.

[0052] The terms “chimeric protein” as used herein is meant to refer to a biologically active protein including several protein domains covalently linked by recombinant, chemical or other suitable method.

[0053] The chimeric proteins described herein are transmembrane proteins. As used herein the term “transmembrane protein” refers to a type of integral membrane protein that spans the entirety of the cell membrane. This implies that such proteins include, a transmembrane domain, as well as an extracellular domain and an intracellular domain. The peptide sequence that spans the membrane, or the transmembrane segment, is largely hydrophobic. Depending on the number of transmembrane segments, transmembrane proteins can be classified as single-pass membrane proteins, or as multipass membrane proteins.

[0054] “Extracellular domain” or “ectodomain” is the domain of a membrane protein that extends into the extracellular space. Ectodomains are usually the parts of proteins that initiate contact with surfaces, which leads to signal transduction. Ectodomains play a crucial part in the signaling pathways of viruses. Ectodomains also interact with membrane systems inducing vesicle aggregation, lipid mixing and liposome leakage which provides information as to how certain viruses spread infection throughout the cellular domain.

[0055] In some aspects, the extracellular domain is the extracellular domain of the TNF receptor superfamily protein.

[0056] The tumor necrosis factor receptor superfamily (TNFRSF) is a protein superfamily of cytokine receptors characterized by the ability to bind tumor necrosis factors (TNFs) via an extracellular cysteine-rich domain. With the exception of nerve growth factor (NGF), all TNFs are homologous to the archetypal TNF-alpha. In their active form, the majority of TNF receptors form trimeric complexes in the plasma membrane. Accordingly, most TNF receptors contain transmembrane domains (TMDs), although some can be cleaved into soluble forms (*e.g.*, TNFR1), and some lack a TMD entirely (*e.g.*, DcR3). In addition, most TNF receptors require specific adaptor protein such as TRADD, TRAF, RIP and FADD for downstream signaling. TNF receptors are primarily involved in apoptosis and inflammation, but they can also take part in other signal transduction pathways, such as proliferation, survival, and differentiation. TNF receptors are expressed in a wide variety of tissues in mammals, especially in leukocytes. The term death receptor refers to those members of the TNF receptor superfamily that contain a death domain, such as TNFR1, Fas receptor, DR4 and DR5. They were named after the fact that they seemed to play an important role in apoptosis (programmed cell death), although they are now known to play other roles as well. In the strict sense, the term TNF receptor is often used to refer to the archetypal members of the superfamily, namely TNFR1 and TNFR2, which recognize TNF-alpha.

[0057] In some aspects, the TNF receptor superfamily protein is TNFR1, TNFR2, BAFF receptor, B-cell maturation antigen, glucocorticoid-induced TNFR-related, CD18, OX40, CD40, Fas, decoy receptor 1, decoy receptor 2, decoy receptor 3, CD27, CD30, 4-1BB, death receptor 3, death receptor 4, death receptor 5, death receptor 6, ectodysplasin A2 receptor, herpesvirus entry mediator, nerve growth factor receptor, RANK, osteoprotegerin, TWEAK receptor, TACI, or TROY. In some aspects, the TNF receptor superfamily protein is not TNFR1.

[0058] In some aspects, the extracellular domain is selected from a Fas receptor extracellular domain, a CD80 extracellular domain, a CD86 extracellular domain, a PD-L1 extracellular domain, an LT β R extracellular domain, a 4-1BBL extracellular domain, an OX40L extracellular domain, an antibody that binds to a tumor necrosis factor, or antigen binding fragment that binds to a tumor necrosis factor.

[0059] The “intracellular domain” of the chimeric protein described herein refers to the cytoplasmic domain of the protein, which interacts with the interior of the cell or organelle, relaying the signal. The intracellular domain may communicate via protein-protein interactions against effector proteins, which in turn pass a signal to the destination, or with enzyme-linked receptors, when the intracellular domain has enzymatic activity (*e.g.*, tyrosine kinase activity). The enzymatic activity can also be due to an enzyme associated with the intracellular domain.

[0060] In some aspects, the intracellular domain is selected from a TNFR1 intracellular domain, a TNFR2 intracellular domain, a RANK intracellular domain, a BCMA intracellular domain, or a CD30 intracellular domain.

[0061] For example, in one embodiment, the invention provides a chimeric protein molecule that includes: a) an extracellular domain selected from a Fas receptor extracellular domain, a CD80 extracellular domain, a CD86 extracellular domain, a PD-L1 extracellular domain, an LT β R extracellular domain, a 4-1BBL extracellular domain, an OX40L extracellular domain, an antibody that binds to a tumor necrosis factor, or an antigen binding fragment that binds to a tumor necrosis factor; b) an intracellular domain selected from a TNFR1 intracellular domain, a TNFR2 intracellular domain, a RANK intracellular domain, a BCMA intracellular domain, or a CD30 intracellular domain; and c) a transmembrane domain linked to the C-terminus of the extracellular domain and the N-terminus of the intracellular domain.

[0062] In one aspect, the extracellular domain includes a Fas receptor extracellular domain. Fas receptor, which is also known as “Fas”, “FasR”, “apoptosis antigen 1”, “CD95”, and “TNFRSF6”,

is a cell surface receptor that initiates apoptosis upon association with its canonical ligand FasL (“Fas ligand”). As disclosed herein, FasL forms a stable complex with Fas receptor and is prevalent on certain antigen-sensing cell (*e.g.*, certain T cell) surfaces, making it a ligand for signal induction upon synapse formation. While Fas-induced apoptosis has previously served as a barrier to Fas-based synapse detection, substitution of its intracellular and/or transmembrane domains can diminish its apoptotic activity and repurpose the protein for signal generation.

[0063] In some aspects, the intracellular domain is a TNFR2 intracellular domain, the transmembrane domain is a TNFR2 transmembrane domain, or a combination thereof. In particular aspects, the extracellular domain is a Fas extracellular receptor domain, the transmembrane domain is a TNFR2 transmembrane domain, and the intracellular domain is a TNFR2 intracellular domain. In many aspects, the intracellular domain does not exhibit apoptotic activity. In some aspects, the intracellular domain does not exhibit FADD, Casp8, FAF, or DAXX activation activity.

[0064] In certain aspects, the chimeric protein molecule includes at least about 80% sequence identity to SEQ ID NO:1-4.

[0065] The terms “sequence identity” or “percent identity” are used interchangeably herein. To determine the percent identity of two polypeptide molecules or two polynucleotide sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first polypeptide or polynucleotide for optimal alignment with a second polypeptide or polynucleotide sequence). The amino acids or nucleotides at corresponding amino acid or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = number of identical positions / total number of positions (*i.e.*, overlapping positions) x 100). In some embodiments the length of a reference sequence (*e.g.*, SEQ ID NO:1-4) aligned for comparison purposes is at least 80% of the length of the comparison sequence, and in some embodiments is at least 90% or 100%. In an embodiment, the two sequences are the same length.

[0066] Ranges of desired degrees of sequence identity are approximately 80% to 100% and integer values in between. Percent identities between a disclosed sequence and a claimed sequence can be at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least

98%, at least 99%, at least 99.5%, or at least 99.9%. In general, an exact match indicates 100% identity over the length of the reference sequence (*e.g.*, SEQ ID NO:1-4).

[0067] Polypeptides and polynucleotides that are about 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 99.5% or more identical to polypeptides and polynucleotides described herein are embodied within the disclosure.

[0068] For example, a polypeptide can have 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity to SEQ ID NO:1-4.

[0069] Variants of the disclosed sequences also include peptides, or full-length protein, that contain substitutions, deletions, or insertions into the protein backbone, that would still leave at least about 70% homology to the original protein over the corresponding portion. A yet greater degree of departure from homology is allowed if like-amino acids, *i.e.* conservative amino acid substitutions, do not count as a change in the sequence. Examples of conservative substitutions involve amino acids that have the same or similar properties. Illustrative amino acid conservative substitutions include the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine, glutamine, or glutamate; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; valine to isoleucine to leucine.

[0070] In certain aspects, the chimeric protein molecule includes at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% sequence identity to SEQ ID NO:1. In particular aspects, the chimeric protein molecule is SEQ ID NO:1.

[0071] In some aspects, the extracellular domain has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at

least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% sequence identity to amino acids 1-170 of SEQ ID NO:1; the transmembrane domain has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% sequence identity to amino acids 171-203 of SEQ ID NO:1; the intracellular domain has at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% sequence identity to amino acids 204-374 of SEQ ID NO:1; or a combination thereof.

[0072] In further aspects, the extracellular domain includes at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% sequence identity to SEQ ID NO:2.

[0073] In yet further aspects, the transmembrane domain includes at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% sequence identity to SEQ ID NO:3.

[0074] In additional aspects, the intracellular domain includes at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% sequence identity to SEQ ID NO:4.

[0075] In certain aspects, the chimeric protein molecule includes at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, at least about 99.5%, or 100% sequence identity to SEQ ID NO:5. In particular aspects, the chimeric protein molecule is SEQ ID NO:5.

[0076] In particular aspects, the chimeric protein molecule is a chimeric Fas-tumor necrosis factor receptor 2 (TNFR2) receptor that has the extracellular domain of Fas fused to the transmembrane and cytoplasmic domains of TNFR2. This construct avoids death domain-mediated caspase activation, as it does not include the death domain of Fas, and instead includes TNFR2 transmembrane and intracellular domains active in NF κ B signaling. As detailed further herein, when coupled with a nuclear factor- κ B (NF κ B) response element, the chimeric protein molecule can function as a Fas-inducible NF κ B (Fas-iNF κ B) reporter.

[0077] The invention also provides an isolated cell that includes the chimeric protein molecule.

[0078] The chimeric protein molecule of the present invention may be expressed in a host cell to be altered thus allowing expression of the chimeric protein within the cell. A variety of host cells are known in the art and suitable for chimeric proteins expression. Examples of typical cell used for transfection include, but are not limited to, a bacterial cell, a eukaryotic cell, a yeast cell, an insect cell, or a plant cell.

[0079] In certain aspects, the cell is an antigen-presenting cell (APC).

[0080] An antigen-presenting cell (APC) or accessory cell is a cell that displays an antigen bound by major histocompatibility complex (MHC) proteins on its surface; this process is known as antigen presentation. T cells may recognize these complexes using their T cell receptors (TCRs). APCs process antigens and present them to T cells. Almost all cell types can present antigens in some way. They are found in a variety of tissue types. Dedicated antigen-presenting cells, including macrophages, B cells and dendritic cells, present foreign antigens to helper T cells, while virus-infected cells (or cancer cells) can present antigens originating inside the cell to cytotoxic T cells. In addition to the MHC family of proteins, antigen presentation relies on other specialized signaling molecules on the surfaces of both APCs and T cells. Antigen-presenting cells are vital for effective adaptive immune response, as the functioning of both cytotoxic and helper T cells is

dependent on APCs. Antigen presentation allows for specificity of adaptive immunity and can contribute to immune responses against both intracellular and extracellular pathogens. It is also involved in defense against tumors. Some cancer therapies involve the creation of artificial APCs to prime the adaptive immune system to target malignant cells.

[0081] In some aspects, the APC is a macrophage, a regulatory macrophage, an activated macrophage, an M2 macrophage, a B cell, a plasma cell, a memory cell, a dendritic cell, a plasmacytoid dendritic cell, an inflammatory dendritic cell, or a Langerhans cell. In some aspects, the cell is a stem cell, such as a human embryonic kidney cell. In further aspects, the cell is an immortalized cell (*e.g.*, an immortalized cell derived from human or mouse tissue).

[0082] The cell can produce a detectable signal upon activation of the chimeric protein molecule. For example, the cell can be engineered to generate a detectable signal such as a fluorescence signal. In some aspects, the detectable signal is generated by an inducible reporter system that is activated by the chimeric protein molecule. As used herein, “activated by the chimeric protein molecule” can denote direct activation of a molecule or system by the chimeric protein molecule (*e.g.*, the chimeric protein molecule phosphorylates the cleaves the molecule or system) or indirect activation of the molecule (*e.g.*, the chimeric protein molecule initiates a signaling cascade that activates the molecule or system).

[0083] In some aspects, the inducible reporter system includes a transcription factor response element. In some aspects, the transcription factor response element is an NFκB response element or an AP1 response element. In particular aspects, the transcription factor response element is an NFκB response element. In a particular aspect, cell includes an NFκB response element and an AP1 response element. The transcription factor response element can be configured to drive expression of a detectable molecule. For example, the transcription factor response element can be operably coupled to a gene encoding a fluorescent protein, a luminescent protein, a cell surface protein (*e.g.*, Lyt2 or another cell surface protein reporter than can be detected by antibodies), or an electrochemically detectable protein. In further aspects, the fluorescent protein is green fluorescent protein (GFP).

[0084] In some aspects, the cell expresses an MHC Class I protein or an MHC Class II protein. In some aspects, the cell overexpresses the MHC Class I protein or the MHC Class II protein. In particular aspects, the cell is engineered to express a single MHC allele.

[0085] In some aspects, the cell does not express a native Fas receptor. In some aspects, the cell is a Fas receptor knockout. In certain aspects, the cell expresses an exogenous antigenic peptide. In certain aspects, the cell displays the exogenous antigenic peptide, or a fragment thereof, in an MHC Class I or MHC Class II protein. In particular aspects, the MHC Class I or MHC Class II protein is endogenously expressed. In further aspects, the MHC Class I or MHC Class II protein is encoded by an exogenous nucleic acid (*e.g.*, a cDNA molecule).

[0086] The cell can also be stably or transiently transfected with a vector encoding the exogenous antigenic peptide.

[0087] The term “vector”, “expression vector”, or “plasmid DNA” is used herein to refer to a recombinant nucleic acid construct that is manipulated by human intervention. A recombinant nucleic acid construct can contain two or more nucleotide sequences that are linked in a manner such that the product is not found in a cell in nature. In particular, the two or more nucleotide sequences can be operatively linked, such as a gene encoding a protein of interest, one or more protein tags, functional domains and the like. In a specific embodiment the proteins of the present invention include an extracellular domain, an intracellular domain and a transmembrane domain.

[0088] Polynucleotides can be delivered to cells (*e.g.*, a plurality of different cells or cell types including target cells or cell types and/or non-target cell types) in a vector (*e.g.*, an expression vector). Examples of vectors include, but are not limited to, (a) non-viral vectors such as nucleic acid vectors including linear oligonucleotides and circular plasmids; artificial chromosomes such as human artificial chromosomes (HACs), yeast artificial chromosomes (YACs), and bacterial artificial chromosomes (BACs or PACs); episomal vectors; transposons (*e.g.*, PiggyBac); and (b) viral vectors such as retroviral vectors, lentiviral vectors, adenoviral vectors, and AAV vectors. Viral vectors have several advantages for delivery of nucleic acids, including high infectivity and/or tropism for certain target cells or tissues. In some cases, a viral vector can be used to deliver a polynucleotide described herein.

[0089] The term “AAV” is an abbreviation for adeno-associated virus and can be used to refer to the virus itself or a derivative thereof. The term covers all serotypes, subtypes, and both naturally occurring and recombinant forms, except where required otherwise. The abbreviation “rAAV” refers to recombinant adeno-associated virus, also referred to as a recombinant AAV vector (or “rAAV vector”). The term “AAV” includes AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, AAVDJ, rhlO, derivatives and hybrids thereof, avian

AAV, bovine AAV, canine AAV, equine AAV, primate AAV, non-primate AAV, and ovine AAV. Additionally, any engineered or variant derived from ancestral AAV sequence reconstruction can be used as a vector. The genomic sequences of various serotypes of AAV, as well as the sequences of the native terminal repeats (TRs), Rep proteins, and capsid subunits are known in the art.

[0090] Following signal detection (*e.g.*, from an inducible reporter system) and cell collection, the vector encoding the antigen can be sequenced, enabling rapid and accurate antigen identification and highly multiplexed antigen screening.

[0091] The cell can overexpress a molecule that promotes MHC expression. In some aspects, the cell overexpresses CIITA, LRC5, B2M, RFX5, RFXAP, or RFXANK. In some aspects, the cell overexpresses CIITA.

[0092] Further disclosed herein are systems and associated screening methods based on T cell synapse formation. The methods can utilize genetically modified cell lines to functionally report T cell- target cell interactions, such as TCR-HLA binding between a T cell and an antigen-presenting cell. The system and methods can provide monodirectional or bidirectional signaling activity wherein one or more cells generate signals upon association or pairing with a partner cell. The cells can be isolated and sequenced to determine the sequence of a biomolecule of a recovered cell, enabling, among a broad range of applications, rapid and highly multiplexed antigen and TCR screening.

[0093] In some embodiments, the invention provides a system that includes: a) an antigen-presenting cell (APC) that includes an MHC protein and a first inducible reporter system, and b) an antigen-sensing cell that includes a T cell receptor (TCR) or a chimeric antigen receptor (CAR), and a second inducible reporter system; wherein the first inducible reporter system generates a first detectable signal and the second inducible reporter system generates a second detectable signal upon contact between the MHC protein and the TCR or the CAR.

[0094] As detailed further herein, the first inducible reporter system and/or the second inducible reporter system can include a transcription factor response element. In some aspects, the first inducible reporter system includes an NF κ B response element. In further aspects, the second inducible reporter system includes an NFAT response element. In a particular aspect, the first inducible reporter system includes an NF κ B response element and the second inducible reporter system includes an NFAT response element. In some aspects, the transcription factor response

element is operably coupled to a gene encoding a fluorescent protein, a luminescent protein, a cell surface protein, or an electrochemically detectable protein.

[0095] In certain aspects, the antigen-sensing cell is a T cell. In some aspects, the TCR is overexpressed by the antigen-sensing cell. In particular aspects, the antigen-sensing cell expresses a single TCR molecule. In some aspects, the antigen-sensing cell does not express TNF. In particular aspects, the first detectable signal comprises at least about 1.5-fold, at least about 2-fold, at least about 2.5-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 12-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, at least about 50-fold, or at least about 100-fold higher signal-to-noise than an identical system in which the antigen-sensing cell expresses TNF.

[0096] In some embodiments, the system detects when Fas ligation activates NF κ B signaling (Fas-iNF κ B). Fas is a cell surface receptor protein belonging to the TNFR family. Cytotoxic T lymphocytes (CTLs) recognize antigen-expressing targets through TCR-pMHC engagement and in turn mediate killing via mechanisms which include perforin/granzyme B, cytotoxic cytokines including interferon-gamma (IFN γ) and tumor necrosis factor (TNF), and Fas ligand (FasL)/Fas signaling. Fas-signaling has historically been avoided in engineered detection systems, as the interaction between FasL and Fas at the immunological synapse is a predominant method of CTL-induced apoptosis. Fas signaling simultaneously produces NF κ B activation as well as induction of apoptosis via interaction of the ligated conformation of Fas with Fas-Associated Via Death Domain (FADD) (**FIG. 1A**). In some aspects, the transmembrane protein is a chimeric protein molecule as outlined herein, such as a Fas-TNFR2 chimeric construct with diminished apoptotic activity.

[0097] In some aspects, the APC expresses a transmembrane protein that activates the first inducible reporter system upon binding to a ligand expressed by the antigen-sensing cell. In some aspects, the ligand is a tumor necrosis factor or an immune checkpoint molecule. In particular aspects, the ligand includes FasL, CTLA-4, PD-1, LT- α , or TNFSF14. In some aspects, the TCR or the CAR activates the second inducible reporter system upon contact with the MHC protein.

[0098] An important aspect of the systems and methods of the present invention is that they are not limited to already-generated antigen libraries. Instead, as signal induction only requires pairing between an antigen-sensing and antigen-presenting cell, these systems and methods can screen for

previously unidentified antigens and TCRs. This characteristic can be exploited to rapidly screen large libraries of antigens and antigen-binding molecules. For example, the antigen-presenting cell can include a nucleic acid encoding an optionally unknown antigen. More broadly, the system can include a plurality of the APCs, and the plurality of the APCs collectively comprise a plurality of nucleic acids encoding a plurality of optionally unknown antigens. For example, the systems can be capable of enriching antigens that are endogenously processed and presented by a reporter cell line after introducing a cDNA library generated from a subject (*e.g.*, a biopsy sample) or by using commercially available kits. Because a cDNA library can be produced from cells or tissues of interest, such as a patient-derived tumor sample, tools that can utilize a cDNA library have the potential advantage of including personal neoantigens.

[0099] In a further embodiment, the invention provides a system that includes: a) an antigen-presenting cell (APC) that includes an MHC protein, an inducible reporter system that includes an NFκB response element operably coupled to a gene encoding a first fluorescent protein, and a chimeric protein molecule configured to activate the NFκB response element; and b) an antigen-sensing cell that includes a T cell receptor (TCR) or a chimeric antigen receptor (CAR), an inducible reporter system that is activated by the TCR or CAR and includes an NFAT response element operably coupled to a gene encoding a second fluorescent protein, and a ligand configured to activate the chimeric protein molecule.

[0100] Association between the APC and antigen-sensing cell (*e.g.*, synapse formation driven by TCR or CAR recognition of an antigen displayed by the APC) can generate fluorescence signals in both cells by activating expression of the first and second fluorescent proteins. Upon association between the APC and the antigen-sensing cell, stimulation of the chimeric protein molecule by its cognate ligand can cause the inducible reporter system of the APC to drive expression of the first fluorescent protein. Similarly, activation of the TCR or CAR upon complexation to an MHC-displayed antigen of the antigen-presenting cell can activate the inducible reporter system of the antigen-sensing cell to drive expression of the second fluorescent protein.

[0101] In some aspects, the chimeric protein molecule is a chimeric protein molecule as disclosed herein. In some aspects, the first fluorescent protein is a green fluorescent protein (GFP). In additional aspects, the second fluorescent protein is an mCherry protein.

[0102] In some aspects, the invention provides a method for detecting an antigen recognized by a T cell that includes contacting a system of the invention with an antigen or a nucleic acid

encoding the antigen; and detecting a first detectable signal from the first inducible reporter system and a second detectable signal from the second inducible reporter system, wherein the first and/or second detectable signal is indicative of a TCR or CAR that specifically recognizes the antigen.

[0103] An “antigen” according to the invention covers any substance that will elicit an immune response. In particular, an “antigen” relates to any substance, preferably a peptide or protein, that reacts specifically with antibodies or T-lymphocytes (T cells). According to the present invention, the term “antigen” comprises any molecule which comprises at least one epitope. Preferably, an antigen in the context of the present invention is a molecule which, optionally after processing, induces an immune reaction. According to the present invention, any suitable antigen may be used, which is a candidate for an immune reaction, wherein the immune reaction is preferably a cellular immune reaction. In the context of the embodiments of the present invention, the antigen is preferably presented by a cell, preferably by an antigen presenting cell which includes a diseased cell, in particular a cancer cell, in the context of MHC molecules, which results in an immune reaction against the antigen. An antigen is preferably a product which corresponds to or is derived from a naturally occurring antigen. Such naturally occurring antigens include tumor antigens, bacterial antigens, viral antigens and self-antigens.

[0104] In part due to its library screening capabilities, the disclosed methods are less labor intensive than previous antigen-identification platforms. As a non-limiting example, a system of the present invention can include a coculture of T cells and target cells transduced with a cDNA library in a single flask. The system can be incubated for less than 12 hours, less than 18 hours, less than 24 hours, less than 36 hours, less than 42 hours, less than 48 hours, less than 60 hours, less than 72 hours, or less than 80 hours prior to flow-sorting, DNA isolation, and amplicon sequencing. For example, it was demonstrated herein that an ACP-T cell bidirectional reporter system can correctly identify the cognate HPV16 E7 antigen from among the antigens recognized by an HPV16 E7-specific TCR, as well as a novel neoantigen formed by a previously not yet described mutation in DDR1.

[0105] The methods of the present invention are applicable to diverse antigen types, including viral, bacterial, and neoantigens. In some aspects, the antigen is selected from a tumor antigen, a bacterial antigen, a viral antigen, a cancer antigen, a neoantigen, and a self-antigen. In some aspects, the antigen is expressed by the APC. In other aspects, the antigen is contacted to the APC.

[0106] The methods can include identifying the TCR or the CAR that specifically recognizes the antigen. The antigen-sensing cell can optionally be captured (*e.g.*, with flow cytometry) so that a nucleic acid encoding the TCR or CAR can be collected or sequenced. In some aspects, the nucleic acid is from a cDNA library.

[0107] In certain aspects, the method further includes identifying the antigen recognized by the TCR or CAR. In particular aspects, the identifying optionally includes capturing the antigen-sensing cell and sequencing the nucleic acid encoding the antigen.

[0108] In one aspect, the method further includes isolating the APC or the antigen-sensing cell. In some aspects, the isolating includes flow cytometry or fluorescence-activated cell sorting (FACS). In a particular aspect, the isolating includes excluding a cell that does not produce the first detectable signal or the second detectable signal. In some aspects, the isolating includes size-selection of an aggregate that includes the APC and the antigen-sensing cell. In further aspects, the isolating includes selecting a cell that produces the first detectable signal or the second detectable signal.

[0109] In some aspects, the antigen-sensing cell is a T cell. In particular aspects, the antigen is present by the APC. In some aspects, the antigen is exogenous to the APC. In some aspects, the method includes identifying the antigen.

[0110] The following examples are provided to further illustrate the embodiments of the present invention but are not intended to limit the scope of the invention. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

EXAMPLES

EXAMPLE 1

MATERIALS AND METHODS

Cell Culture

[0111] 293T (#CRL-3216), Jurkat RRID:CVCL_0367, clone E6-1, Cat#TIB-152), and CaSki cells (#CRM-CRL-1550) were obtained from ATCC. Cell Line Authentication was done by ATCC and our institution. 293T and CaSki cells were cultured in DMEM (Corning) with 10% (v/v) FBS (Hyclone), and 1X Penicillin-Streptomycin-Glutamine (100X) (Gibco). Jurkat cells were cultured in RPMI1640 (Corning) with 10% (v/v) FBS (Hyclone), and 1X Penicillin-Streptomycin-

Glutamine (100X) (Gibco). Human cell lines were sourced ethically, and their research use was in accord with the terms of the informed consents under an IBC-approved protocol.

Antibodies and flow cytometry

[0112] Human TruStain FcX (Fc Receptor Blocking Solution, Biolegend cat#422301) and all of the following fluorochrome-labeled antibodies (all for human unless otherwise indicated) were obtained from BioLegend: CD178 (Fas ligand)-PE (NOK-1, RRID:AB_314603), CD95 (Fas)-PE (DX2), anti-mouse TCR β -APC (H57-597), HLA-ABC-APC (W6/32), HLA-A2-APC (BB7.2), TCR α/β -PE (IP26), Cleaved PARP (Asp214)-PE (QA17A17), TCR α/β -APC (T10B9.1A-31), HLA-B-PE (YTH 76.3.rMAb) and HLA-C-PE (DT-9) were obtained from BD Biosciences. HLA-BC-APC (B1.23.2) was obtained from eBioscience. CellTrace Violet (CTV) and CellTrace Far Red (CTFR) were obtained from Thermo Fisher Scientific and cell staining was performed per the manufacturer's instructions. Cells were acquired on a BD LSRFortessa X-20 Cell Analyzer (BD Biosciences) and a BD FACSAria Cell Sorter (BD Biosciences) with BD FACSDiva software (RRID:SCR_001456, 8.0.1). Data were analyzed with FlowJo (RRID:SCR_008520).

Retroviral Production

[0113] 293T cells were transiently transfected with retroviral expression vectors, pUMVC (Addgene#8449) and pCMV-VSV-G plasmids (RRID:Addgene_8454), or RD114 and PegPam plasmids (kindly provided by Dr. Maksim Mamonkin's lab) using Lipofectamine 2000 (Thermo Fisher Scientific). Retroviral supernatant was harvested 2 days later and concentrated using PEG-it (SBI).

Lentiviral Production

[0114] Approximately 80% confluent 293T cells in a 10 cm dish were transiently transfected with 10 μ g transfer plasmid, 5 μ g PsPAX2 plasmid (RRID:Addgene_12260), and 3 μ g PMD2.G plasmid (Addgene#12259) using Lipofectamine 2000 (Thermo Fisher Scientific). Lentiviral supernatant was harvested 2 days after transfection, filtered (0.45 μ m) and concentrated using PEG-it (System Biosciences).

Generation of NF κ B-GFP Reporter

[0115] The DNA sequence encoding GFP was amplified by PCR using the following primers (all primers synthesized by IDT): GFP-F: 5'-GCCCCCATGGTGAGCAAGGGCGAGGAG-3' (SEQ ID NO:6) and GFP-R: 5'-AAGTCATATGTTACTTGTACAGCTCGTCC-3' (SEQ ID NO:7). Luciferase and Ubc promoter regions in pHAGE NF κ B-TA-LUC-UBC-GFP-W plasmid

(Addgene#49343) were replaced with eGFP using the NcoI and NdeI sites to generate the pHAGE NFκB-GFP plasmid. The resulting vector was introduced into 293T cells by lentiviral transduction. Cells were incubated with 1 μg/ml rabbit Fas antibody overnight (Sigma, clone CH11) and then NFκB-GFP 293T cells were sorted by flow cytometry to produce NFκB-GFP reporter positive cells.

Knockout of endogenous Fas gene

[0116] pLentiCRISPR V2 vector (RRID:Addgene_169885) with the CRISPR/Cas9 guide RNA (gRNA, 5'-GTGTAACATACCTGGAGGAC-3' (SEQ ID NO:8)) directed to cleave the Fas gene was introduced into NFκB-GFP 293T cells (all the CRISPR/Cas9 gRNA vectors were purchased from GenScript). After transduction, Fas-knockout (FasKO) NFκB-GFP 293T cells were flow-sorted after staining with anti-Fas-PE antibody (Biolegend, Cat#305608).

Generation of Fas-TNFR2 Chimeric Receptor

[0117] The transmembrane domain (amino acid 258-280) and cytoplasmic domain (amino acid 281-461) of TNFR2 were amplified together by PCR from TNFR2-GFP plasmid (RRID:Addgene_111207) using the following primers:

TNFR2-F: 5'-GCCCCGATCCTTCGCTCTTCCAGTTGGACTG-3' (SEQ ID NO:9) and

TNFR2-R: 5'-AAGTGATATCACTGGGCTTCATCCCAGCATC-3' (SEQ ID NO:10).

[0118] Amplified TNFR2 was then cloned into Fas_PLX307 lentiviral vector (Addgene#98334) using BamHI and EcoRV sites to fuse with the C terminus of the extracellular region of Fas (amino acids 1-170). This Fas-TNFR2_PLX307 plasmid was introduced into FasKO NFκB-GFP 293T cells by lentiviral transduction. Fas-TNFR2 receptor-expressing FasKO NFκB-GFP 293T cells (FIR-APCs) were selected with 2 μg/ml puromycin. Clones were grown up from single cells and activation of NFκB reporter was verified by flow cytometry.

MTT Assay

[0119] Cell viability was assessed with the MTT cell proliferation assay (ATCC) according to the manufacturer's protocol. Absorbance was measured at 570 nm using a BioTek Synergy HTX plate reader.

Knockout of Endogenous HLA Genes in FIT-APCs

[0120] CRISPR/Cas9 gRNAs directed to cleave the HLA-A locus were amplified using the following primers and cloned into pLenti-eCas9 vector (Addgene#140237):

HLA-A CRISPR 1: CGTCCTGCCGGTACCCGCGG (SEQ ID NO:11),

CRISPR HLA class I F1: 5'-CACCGAGGTCAGTGTGATCTCCGCA-3' (SEQ ID NO:12),

CRISPR HLA class I R1: 5'-AAACTGCGGAGATCACACTGACCTC-3' (SEQ ID NO:13),

HLA-A CRISPR 2: TACCGGCAGGACGCCTACGA (SEQ ID NO:14),

CRISPR HLA class I F2: 5'-CACCGCGGCTACTACAACCAGAGCG-3' (SEQ ID NO:15), and

CRISPR HLA class I R2: 5'-AAACCGCTCTGGTTGTAGTAGCCGC-3' (SEQ ID NO:16).

[0121] The following CRISPR/Cas9 gRNAs were directed to cleave the HLA-B and HLA-C loci:

HLA-B CRISPR: 5'-GGATGGCGAGGACCAAACCTC-3' (SEQ ID NO:17), and

HLA-C CRISPR: 5'-GACACAGAAGTACAAGCGCC-3' (SEQ ID NO:18).

[0122] Vectors were introduced into FIR-APCs by lentiviral transduction. After transduction, HLA-negative (HLA-KO) FIR-APCs were sorted using anti-HLA-ABC, anti-HLA-BC, anti-HLA-B, anti-HLA-C antibodies and clones were grown up from single cells.

*Overexpression of HLA-A*02:01 in HLA-KO FIR-APCs*

[0123] The gene expressing HLA-A*02:01 was amplified from pMP71-HLA-A0201-His plasmid (RRID:Addgene_108214) using the following primers: HLA-A2-F: 5'-GCCCATCGATATGGCCGTCATGGCGCCCCGAAC-3' (SEQ ID NO:19) and HLA-A2-R: 5'-AAGTGATATCCACTTTACAAGCTGTGAGAGAC-3' (SEQ ID NO:20). Amplified HLA-A*02:01 was then cloned into the Fas_PLX307 lentiviral vector (Addgene#98334) using ClaI and EcoRV sites to replace the Fas region. The resulting HLA-A2 _PLX307 plasmid was introduced into HLA-KO FIR-APCs by lentiviral transduction. After transduction, HLA-A*02:01 positive HLA-KO FIR-APCs were enriched by flow-sorting after staining with an anti-HLA-A2 antibody.

Generation of NFAT Reporter in T Cells

[0124] DNA encoding mCherry was amplified using the following primers: mCherryF: 5'-GCACAGATCTCGCCACCATGGTGAGCAAGGGCG-3' (SEQ ID NO:21) and mCherryR: 5'-TTCAGCTCGAGCTACTTGTACAGCTCG-3' (SEQ ID NO:22). Then, mCherry was cloned into the 8xNFAT-ZsGreen-hCD8 plasmid (Addgene#153417) to replace the ZsGreen region using BglII and XhoI sites to generate an 8xNFAT-mCherry-hCD8 plasmid. To remove the hCD8 region from the 8xNFAT-mCherry-hCD8 plasmid, we replaced it with the following DNA sequence (synthesized by IDT) encoding a random sequence (MVSKGGGGGS (SEQ ID NO:23)) using AgeI and HindIII sites: 5'-GCACAGATCTCGCCACCATGGTGAGCAAGGGCGGTGGTGGTGGTTCTTAGCTCGAGTGAA-3' (SEQ ID NO:24). The resulting 8xNFAT-mCherry

plasmid was introduced into Jurkat cells using retroviral transduction. JR-T cells were enriched by flow-sorting for mCherry expression after PMA and ionomycin (Biolegend) treatment. Clones were grown up from single cells and expression of reporter was verified by flow cytometry.

Overexpression of TCRs

[0125] Retroviral expression plasmids encoding α HPV16E6₂₉₋₃₈ TCR genes (α and β) and α HPV16E7₁₁₋₁₉ TCR genes were obtained from Addgene (#122727 and #122728). A gene expressing α CMVpp65₄₉₅₋₅₀₃ TCR genes was synthesized (IDT) and cloned into the MSGV1 retroviral vector (Addgene #122727). To avoid mispairing between the introduced TCR genes with the endogenous TCR genes, the constant regions of each TCR chain were exchanged for their murine counterparts. After transduction, JR-T cells with TCR expression (TCR-JR-T cells) were enriched by flow-sorting after staining with an anti-mouse TCR β antibody.

Knockout of endogenous TCR genes

[0126] The CRISPR/Cas9 gRNA vector CRISPR_TRAC (Addgene Plasmid#164993) was directed to edit the human TCR α constant (TRAC) locus. The CRISPR/Cas9 gRNA directed to edit the human TCR β constant 1 (TRBC1) locus was amplified using the following primers and cloned into pLenti-eCas9 vector (Addgene#140237):

CRISPR TCR β F: 5'-CACCGCGTAGAACTGGACTTGACAG-3' (SEQ ID NO:25) and

CRISPR TCR β R: 5'-AAACCTGTCAAGTCCAGTTCTACGC-3' (SEQ ID NO:26).

[0127] The resulting vectors were introduced into JR-T cells by lentiviral transduction. TCR-negative (TCR-KO) JR cell clones were grown up from single cells. Absence of TCR was verified by flow cytometry using an anti-TCR α/β antibody and further confirmed by western blot using anti-TCR α (Santa Cruz Biotechnology, #H-1) and anti-TCR β (Cell Signaling, #77046).

[0128] *Fas-iNFB Reporter Activation Assays With Peptides*

[0129] HPV16E6 antigen peptide (TIHDIILECV (SEQ ID NO:27)), HPV16E7 antigen peptide (YMLDLQPET (SEQ ID NO:28)), and CMVpp65 antigen peptide (NLVPMVATV (SEQ ID NO:29)) were synthesized with purity greater than 95% by Genemed Synthesis. CTV-labeled FIR-APCs were pulsed using 1 μ M of each peptide and seeded at 3×10^4 cells/well in round-bottom 96 well plates, with CTR-labeled T cells in T cell media at 9×10^4 cells/well. Cells were cocultured for 2 days and then harvested for flow cytometry analysis.

Fas-iNFB Reporter Activation Assays With Endogenously Expressed Antigens

[0130] Full-length HPV16E6 and HPV16E7 DNA sequences were amplified from pLXSN16E6E7 plasmid (RRID:Addgene_52394), and pp65CMV 9-mer (NLVPMVATV (SEQ ID NO:30)) was amplified from PresentER-NLVPMVATV plasmid (RRID:Addgene_102947) using the following primers:

E6-F: 5'-GCCCCGGATCCCACCATGGACCAAAAGAGAACTGCA-3' (SEQ ID NO:31),

E6-R: 5'-AAGTGTCGACTTATGGTTTCTGAGAACAGATGGGGC-3' (SEQ ID NO:32),

E7-F: 5'-GCCCCGGATCCCACCATGGATGGAGATACACCTACA-3' (SEQ ID NO:33),

E7-R: 5'-AAGTGTCGACTTATGGTTTCTGAGAACAGATGGGGC-3' (SEQ ID NO:34),

CMV_F65: 5'-

GCCCCGGATCCACCATGGTGAACCTGGTGCCCATGGTGGCCACCGTGTGAGTCGACTGAA-3' (SEQ ID NO:35), and

CMV_R65: 5'-

TTCAGTCGACTCACACGGTGGCCACCATGGGACACAGGTTCCACCATGGTGGATCCGGGC-3' (SEQ ID NO:36).

[0131] The amplified antigens were then cloned into the pLenti-CMV-GFP-Zeo lentiviral vector (Addgene#17449) using BamHI and SalI sites. The resulting vectors were introduced into FIR-APCs by lentiviral transduction. FIR-APCs expressing each antigen (E6-FIR-APCs, E7-FIR-APCs, NLV-FIR-APCs) were selected using 500 µg/ml zeocin (Invivogen). FIR-APCs expressing antigens or CaSki cells were labeled and cocultured under the same conditions as above without peptide pulsation.

ROC Analysis

[0132] Fluorescence intensity of reporters in individual cells was analyzed using the FlowCore R package. An ROC curve and the corresponding AUC value were calculated for the fluorescence intensity of reporters as predictive of the target antigen expression in the FIR-APCs.

Construction of cDNA Library

[0133] mRNA was extracted from 1×10^7 CaSki cells using Magnetic mRNA Isolation Kit (NEB). Gateway system-compatible cDNA library was generated from 1 µg of mRNA using CloneMiner II cDNA Library Construction Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Constructed cDNA libraries were cloned into pLenti CMV Hygro DEST vector (RRID:Addgene_17454) and transduced into FIR-APCs. FIR-APCs expressing the CaSki cDNA library (CaSki-FIR-APCs) were selected using 200 µg/ml hygromycin.

CaSki cDNA Library Screen

[0134] CTV-labeled CaSki-FIR-APCs were cocultured with CTFR-labeled TCR-JR-T cells at a ratio of 1:3 in a 25 cm² flask for 2 days, after which CTV+CTFR+/mCherry+(highest 10%)/GFP+(highest 10%) aggregates were selected by flow-sorting. Unsorted cocultured cells were collected as a control.

gDNA purification and adaptor PCR

[0135] gDNA was isolated from flow-sorted dual-reporter positive aggregates using the GeneJET gDNA purification kit (Thermo Fisher Scientific). cDNA inserts were amplified from the isolated gDNA by PCR using PrimeSTAR HS DNA polymerase (TaKaRa) and the following Gateway attB adaptor primers: attB1 primer: 5'-TGGTGGAATTCTGCAGATATCAACAAG-3' (SEQ ID NO:37) and attB2 primer: 5'-CTGTGCTGGATATCAACCACTTTGT-3' (SEQ ID NO:38). The amplified cDNA inserts were purified using QIAquick PCR Purification Kit (QIAGEN) for subsequent qPCR and amplicon library construction.

Quantitative PCR

[0136] The abundance of E7 gene copies in amplified cDNA inserts was quantified by qPCR using KAPA SYBR FAST qPCR Master Mix (2X) Kit (Sigma-Aldrich) with the following primers and was normalized to the abundance of β -actin:

E7-qPCR-F: 5'-ATGCATGGAGATACACCTACATTGC-3' (SEQ ID NO:39),

E7-qPCR-R: 5'-GATTATGGTTTCTGAGAACAGATGGGGC-3' (SEQ ID NO:40),

β -actin-F: 5'-GCGCGGATCCGCGGACTATGACTTAGTTGCG-3' (SEQ ID NO:41) and

β -actin-R: 5'-GCGCGCGGCCGCCACATTGTGAACCTTGGGGG-3' (SEQ ID NO:42).

Amplicon Deep Sequencing

[0137] An amplicon library was prepared according to the Illumina DNA Prep workflow. The quality of each library was assessed using a 4200 TapeStation system (Agilent). Pooled libraries were sequenced on the Illumina NovaSeq platform with a 2x150 base pair paired-end protocol, resulting in more than ~5 Gb per sample. Raw DNA reads were filtered using VSEARCH 2.17.1 with a quality score below 15. Remaining reads were aligned to the human GCRh38 database and HPV database (NC_001526.4) using Diamond ver 0.9.24. 4819 genes were detected in all the flow-sorted samples. Genes were considered if 80% of samples had at least a relative abundance of 10^{-5} . Differential gene abundance testing comparing flow-sorted and unsorted samples was performed using DESeq2.

Neoantigen Prediction

[0138] Amplicon deep sequencing reads were evaluated for somatic mutation calls using MuTect2, and binding affinities with HLA-A*02:01 molecules of all possible 9-mer and 10-mer peptides spanning mutations were evaluated with the NetMHCpan-4.1 algorithm. Candidate peptides were considered HLA binders when $IC_{50} < 500$ nmol/L, with high-affinity binders presenting an $IC_{50} < 50$ nmol/L.

Statistical Analysis

[0139] Data were checked for normality and similar variances, and the t test was used for comparing values between two groups. Mann-Whitney U test was used to compare data between two groups when the data did not follow a normal distribution. One-way ANOVA with post-hoc Tukey test was used to compare data between multiple groups. Analyses were performed using R software version 4.1.2 and GraphPad Prism (RRID:SCR_002798, version 9.0). P values of <0.05 were considered statistically significant.

EXAMPLE 2**A FAS-INDUCIBLE NFkB REPORTER DETECTS ACTIVATED TARGET CELLS**

[0140] In this example, APCs enriched from T cell association assays were evaluated with amplicon-based deep sequencing of cDNA library subsets expressed by artificial APCs. APCs were generated by introducing an NFkB response element driving expression of enhanced green fluorescent protein (GFP) into human embryonic kidney T293 cells (**FIG. 1C**). Ligation of Fas with an agonist antibody produced expression of GFP in lentivirally transduced 293T cells at 18 hours (**FIG. 1D**). Fas ligation, however, also induced apoptosis, quantified by activity of cleaved Poly (ADP-ribose) polymerase (PARP), as well as by reduced metabolic activity, quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (**FIG. 1E**).

[0141] To reduce Fas ligation-induced apoptosis in APC reporter cells, CRISPR/Cas9 gene editing was used to eliminate expression of Fas (**FIGs. 1C, 1F**). As expected, anti-Fas antibody treatment of Fas-deficient cells showed no changes in activity of cleaved PARP or metabolic activity (**FIG. 1E**), but also showed no induction of GFP (**FIG. 1D**).

[0142] To restore NFkB signaling with Fas ligation, a novel chimeric receptor was generated by fusing the extracellular domain of Fas with the transmembrane and intracellular domain of TNFR2, (referred to herein as Fas-TNFR2, **FIG. 1A**). TNFR2 importantly lacks a death domain but

maintains interactions with TRAF2 which can activate NF κ B. It was found that introducing Fas-TNFR2 into Fas-deficient cells (**FIG. 1C**) restored NF κ B signaling in response to Fas ligation (**FIG. 1D**) without inducing apoptosis (**FIG. 1E**). Fas-TNFR2 expressing APC reporter cells are referred to as “Fas-iNF κ B reporter APCs” (FIR-APCs) herein.

EXAMPLE 3

JURKAT T CELL TARGETING INDUCES SIGNAL GENERATION IN FIR-APCs

[0143] HPV16 E6 (E6), HPV16 E7 (E7), and CMV pp65 (NLV) were used as model antigens to evaluate how FIR-APCs respond to being targeted by T cells. All three antigens contain immunogenic peptides presented by HLA-A*02:01, which is a common HLA-A allele in the United States and globally. FIR-APCs with either E6, E7 or NLV peptide antigens were cocultured with human Jurkat T cell lines overexpressing TCR α and TCR β genes recognizing E6, E7 or NLV peptide antigens (α E6-, α E7- and α NLV-Jurkat cells). The results of these analyses are shown in **FIG. 2A**.

[0144] The kinetics of GFP induction in FIR-APCs were quantified. Increased GFP was detectable in E7 peptide-pulsed FIR-APCs after 1 day of coculture with α E7-Jurkat cells (**FIG. 2B**), with maximal reporter signaling seen at 2 days (**FIG. 2C**). Importantly, it was found that FIR-APCs generated signals when targeted by Jurkat cells in a manner that was peptide and TCR specific, with activation seen only in the presence of the correct cognate peptide antigen and its matching TCR (**FIG. 2D**). To further optimize this system, FIR-APCs underwent single-cell flow-sorting and selection. A clone with low baseline NF κ B activity as well as high upregulation of GFP upon Fas ligation was selected and utilized for further experiments.

[0145] FIR-APCs were further tested for the ability to report in the setting of stable antigen expression, contrasting the analyses performed with peptide pulsing. For these analyses, FIR-APCs were stably transduced with constructs overexpressing whole HPV16 E7 protein, HPV16 E6 protein or the CMV pp65 NLV minigene to produce E7/E6/NLV-expression FIR-APCs (E7-FIR-APCs, E6-FIR-APCs and NLV-FIR-APCs). These cells upregulated GFP in an antigen-specific manner when cocultured with Jurkat cells overexpressing the appropriate cognate TCRs (**FIGs. 2E-2F**). Overexpression of antigen by FIR-APCs produced less GFP upregulation compared with peptide pulsing, likely due to effects of differences in the density of HLA molecules presenting cognate antigen peptide. Overall, these results indicated that FIR-APCs can report when

being targeted by antigen-specific T cells, both after peptide pulsing as well as when forced to express a protein antigen.

EXAMPLE 4

FIR-APCs – T CELL AGGREGATION AND INCREASED FAS SIGNALING

[0146] Crosstalk between T cells and APCs occurs in the setting of an immunological synapse, with an interface characterized by a central TCR-pMHC cluster surrounded by interacting co-stimulatory molecules. It has been demonstrated that T cell-APC pairs can form aggregates detectable by flow cytometry. To evaluate Jurkat cell-FIR cell aggregates, Jurkat cells and FIR-APCs were labeled with distinct cell surface dyes, which allowed detection of cell aggregates with fluorescence from both dyes (FIG. 3A). Following coculture of labeled E7-FIR-APCs with labeled α E7-Jurkat cells, the majority of the dual-labeled events detected by flow cytometry were multiparticle rather than single particle (FIG. 3B). Furthermore, the percentages of dual-labeled events increased in the setting of matching TCRs and cognate antigens (FIG. 3C).

[0147] The degree of activation of FIR-APCs was compared between FIR-APCs suspended as single particles and FIR-APCs aggregated with Jurkat cells. Interestingly, reporter activity was substantially higher in the fraction of FIR-APCs found in aggregates with Jurkat cells (FIG. 3D). Furthermore, reporter activity increased in an antigen-specific manner (FIG. 3E). These results showed that a gating strategy incorporating selection of FIR-Jurkat cell aggregates, followed by selection of activated FIR-APCs within these aggregates, could allow for improved enrichment of the subset of FIR-APCs that are being targeted by Jurkat cells.

EXAMPLE 5

ADDITION OF A TCR SIGNALING REPORTER SYSTEM TO T CELLS FURTHER ENRICHES HIGHLY T CELL-TARGETED FIR-APCs

[0148] This example demonstrates that TCR signal quantitation can be used to identify highly interacting FIR-cell/Jurkat cell aggregates. At the T cell-APC immunological synapse, signaling occurs bidirectionally, with signals on the T cell side mediated by the TCR complex as well as by various co-stimulatory molecules. To evaluate this interaction, a construct that expresses mCherry fluorescent protein driven by an NFAT response element was introduced into Jurkat cells (T cells), with these resulting cells termed “Jurkat reporter T cells” (JR-T cells) (FIG. 4A).

[0149] It was found that treatment of JR-T cells with phorbol-myristate-acetate (PMA) and ionomycin, which activates T cells, led to expression of mCherry. Similarly, co-culturing E7 peptide-pulsed FIR-APCs with E7-specific JR-T cells (α E7-JR-T cells) demonstrated antigen-specific reporting by JR-T cells. When evaluating FIR-cell/JR-cell aggregates, it was found that those with JR-T cells that were NFAT-activated demonstrated increased NF κ B activation in FIR-APCs (**FIGs. 4B,4C**), indicating that highly interacting FIR-cell/JR-cell aggregates tended to have both increased NFAT and NF κ B activation. To identify an optimal culture duration, the kinetics of synapse formation and reporter signals we quantified using positive and negative control FIR-APCs and JR-T cells. It was found that aggregation was not increased in culture conditions where cognate TCRs and antigens were present, either at 24 or 48 hours. In contrast, reporter activity in both FIR-APCs and JR-T cells was significantly higher in cognate culture conditions and was higher at 48 than at 24 hours. Therefore, a 48-hour co-culture was utilized for this system. This system of quantifying aggregates with dual FIR-APC/JR-T cell reporter activity was termed “T-synapse reporting system” (Tsyn reporting system).

[0150] The Tsyn reporter system was able to correctly identify from a population of FIR-APCs those that expressed the correct cognate antigen (E7) that were present as a small minority (1%) mixed with a large majority of antigen-negative FIR-APCs. The enrichment rate was quantified by comparing the percentage of E7-FIR-APCs before and after gating for Tsyn reporter activity. As less heterogeneous enrichment resulted when cells were cocultured in a flask compared to a 96-well U-bottom plate, flask coculturing was incorporated this into the Tsyn protocol.

[0151] Next, two different FIR-cell to JR-cell ratios, 1:3 and 1:10, were compared and observed similar results. Based on these results, a 1:3 ratio was adopted for the Tsyn protocol.

[0152] To further test the sensitivity of this system, an even smaller minority of E7-FIR-APCs (0.1%) was tested. The results of these analyses showed that the percentage of these cells would be approximately 2% in the sorted population, representing a roughly 20-fold enrichment.

[0153] The sensitivity and specificity of the system for correctly identifying antigen-expressing FIR-APCs across all possible GFP fluorescence thresholds was tested to characterize receiver operating characteristics (ROC) of the Tsyn system (**FIG. 4D**). After an optimal GFP threshold was identified, all possible mCherry thresholds were then tested. Notably, the area under the ROC curve (AUC) of the Tsyn reporting system increased from 0.73 (for NF κ B-GFP fluorescence data alone) to 0.92 with the addition of NFAT-mCherry fluorescence data to NF κ B-GFP fluorescence

data, surprisingly demonstrating that bidirectional signaling systems can increase the sensitivity and accuracy of reporter systems. A GFP gating strategy targeting the highest 10% of the population with respect to GFP expression was established and a substantial increase in the proportion of CTV-labeled spike-in E7-FIR-APCs within this gate was observed, as compared to the control conditions with α NLV-JR-T cells. This demonstrated that applying a strategy of selecting the highest GFP-expressing FIR-APCs after co-culture should substantially enrich for FIR-APCs expressing a cognate antigen recognized by JR-T cells.

[0154] Finally, a series of CRISPR/Cas9 gene editing procedures to remove MHC class I genes (HLA-A, B and C) from FIR-APCs were performed to eliminate the possibility of antigen presentation by more than one MHC class I molecule at a time. Similarly, TCR genes (TCR α and TCR β) were removed from JR-T cells to remove the possibility of detecting TCR-pMHC interactions mediated by the endogenous TCR present in Jurkat cells rather than by the expressed TCR of interest. After removing these MHC class I genes from FIR-APCs, HLA-A*02:01 was re-expressed in these cells. These “cleaned-up” versions of reporter cells were named “Tsyn reporting system version 2” (Tsyn V.2) (**FIG. 5A**).

[0155] The performance from the Tsyn V.2 system was evaluated with peptide-pulsed FIR-APCs. Evaluating the combined system including aggregation and dual reporting, V.2 showed similar Tsyn reporting in non-cognate antigen settings and increased Tsyn reporting in the cognate antigen setting (3.1% versus 6.7%). These indicated that in the setting of peptide pulsing, which saturates p-MHC complexes with the peptide antigen of interest, Tsyn V.2 showed slightly improved performance compared to Tsyn V.1.

[0156] The performance of Tsyn V.1 and V.2 was compared using FIR-APCs overexpressing protein antigens. Coculture of E7-expressing FIR-APCs with cognate α E7-JR-T cells demonstrated that Tsyn V.2 resulted in a higher percentage of aggregates (**FIG. 5B**) and an increased population of dual-reporter positive events (**FIG. 5C**). At the same time, with Tsyn V.2, in control conditions where cognate TCR-pMHC interactions are absent, proportions of aggregates and dual-reporter positive populations were significantly reduced (**FIG. 5C**). In summary, it was found that gene editing to remove extraneous MHC class I and TCR genes resulted in substantial improvements in the performance of the Tsyn reporter system. In the following examples, all experiments were performed using V.2 reporter cells.

EXAMPLE 6

GENOME-WIDE SCREENING OF ANTIGENS FROM A TUMOR cDNA LIBRARY

[0157] This example addresses whether, among the various antigens expressed by a tumor cell line, the Tsyn reporter system could allow enrichment of a cognate antigen recognized by a TCR of interest. The HPV16-positive CaSki cervical cancer cell line, which is known to harbor E6 and E7 genes, was utilized for these analyses. As expected, it was found that CaSki cells, when cultured with E6-specific JR-T cells (α E6-JR-T cells) or α E7-JR-T cells, resulted in NFAT activation, indicating expression of each of these antigens (**FIGs. 6A, 6B**). mRNA was harvested from CaSki cells and performed reverse transcription to generate cDNA. A CaSki cDNA-derived lentiviral expression library with extensive genome coverage (3×10^6 primary clones) and an average clone length of ~1.5 Kb was constructed using Gateway technology. This library was then introduced into FIR-APCs to create a CaSki-FIR-APCs library and confirmed by quantitative PCR (qPCR) the presence of the E7 gene within the mixed genomic DNA (gDNA) of the CaSki-FIR-APCs library.

[0158] α E7-JR-T cells were cocultured with the CaSki-FIR-APCs library, followed by flow-sort enrichment for aggregates that were dual-reporter positive (**FIG. 6C**). Lentiviral cDNA inserts from extracted gDNA were amplified, and Copies of E7 genes were quantified by qPCR to quantify the enrichment rate produced by Tsyn reporter enrichment. Tsyn V.1 and V.2 were compared with this method. It was found that Tsyn V.1 produced a median enrichment rate of 2.4, while Tsyn V.2 achieved a statistically superior enrichment rate of 12.1 (**FIG. 6D**). Tsyn V.2 was also evaluated in coculture with non-cognate NLV-specific JR-T cells (α NLV-JR-T) as a control. Interestingly, no enrichment of E7 was observed. In fact, the flow-sorted sample exhibited a significant reduction in gene copies of E7.

[0159] In addition to qPCR for E7, cDNA inserts in gDNA from Tsyn V.2 enriched CaSki-FIR-APCs library cells were quantitatively profiled by performing amplicon deep sequencing (Tsyn-seq). Sequencing read counts were compared with control groups to identify genes enriched by the Tsyn reporter system. The cognate E7 antigen was identified as the most enriched gene among abundant and statistically significant genes, while there was no enrichment for E7 after sorting CaSki-FIR-APCs co-cultured with control α NLV-JR-T cells (**FIGs. 6E, 6F**). These results

demonstrated that Tsyn-seq can enrich cognate antigens recognized by an HPV16 E7-specific TCR from the transcriptome of a cervical cancer cDNA library.

[0160] Given that several genes in addition to E7 were enriched, it was next investigated whether the α E7-TCR could be reacting to some of these antigens. The sequences of these genes were examined for mutations that could produce neoantigens that were predicted to be presented by HLA-A*02:01. This approach identified five epitopes: one from discoidin domain-containing receptor 1 (DDR1), three from antigen peptide transporter 2 (TAP2), and one from coiled-coil alpha-helical rod protein 1 (CCHCR1). FIR-APCs were then pulsed with each of these epitopes. It was found that one peptide, FLKEVKIML (SEQ ID NO:43), a neoantigen derived from DDR1, increased the percentage of dual-reporter positive aggregates when cocultured with JR-T cells expressing α E7-TCR, in contrast to control JR-T cells expressing α NLV-TCR (FIG. 6F). Six non-mutated high-scoring peptides derived from CCHCR1, which is known to be mainly expressed in the testis, were further evaluated. None of these increased the reactivity of FIR-APCs when cocultured with JR-T cells expressing α E7-TCR. In summary, these experiments demonstrated that Tsyn-seq can identify both known and unknown targets of a TCR of interest from a tumor-derived cDNA library.

[0161] DDR1 is thought to play a role in cancer progression for several cancer types. We examined the Cancer Genome Atlas cervical squamous cell carcinoma and endocervical adenocarcinoma (TCGA-CESC) database and identified mutations of DDR1 in 5% of cervical cancer patients. Each of these mutations was found in the kinase domain of DDR1, similar to the mutation we identified in CaSki cells, though there were no instances of common mutations between cases. There was no clear association of DDR1 mutation status with survival rates in either all cancer patients or the subset of cervical cancers. Thus, a novel DDR1 neoantigen within CaSki cells was identified.

EXAMPLE 7

DISCUSSION

[0162] Tsyn-seq is a high-throughput screening platform for discovering antigens recognized by TCRs of interest. It relies on identification of functional immune synapses formed between T cells and target cells. This is achieved through a three-part strategy: 1) the detection of aggregated T cells and target cells, 2) quantifying T cell to target cell signaling and 3) quantifying target cell to

T-cell signaling. Interestingly, it was found that signaling reporter activity was increased in aggregates of cognate APCs and T cells, which is consistent with previous observations that physically interacting cells show increased immune-activation.

[0163] A major strength of this system, in contrast to other TCR antigen screening methods that rely on quantifying TCR-MHC-peptide binding or are purely *in silico*, is the use of T cell functionality as a screening mechanism. Tsyn-reporting quantifies T cell effector cytotoxicity, which requires close cell-cell interactions. The reliance on high-throughput functionality as the primary readout has potential advantages in those genes that are enriched, which have a higher likelihood of being identified as true functional targets, hence enhancing the efficiency of the workflow.

[0164] In contrast to prior functional T cell antigen screening tools which detect granzyme B or effector cytokines, Tsyn-seq relies on detecting Fas pathway signaling, which requires interactions between two cell-surface molecules, unlike perforin-granzyme granules or cytokines which are released by the T cell. To enable detection of Fas signaling, a novel construct was developed by replacing the apoptosis-inducing intracellular death domain of Fas with the intracellular domain of TNFR2, which also signals via NF κ B but does not induce apoptosis. Signaling within T cells was simultaneously measured by quantifying NFAT activity. This combined bidirectional signaling criteria markedly improved the performance of this enrichment strategy, as quantified by a ROC curve analysis and enrichment indices.

[0165] To reduce potential cross-reactivity mediated by other HLA alleles expressed by the target cell line and the endogenous TCR expressed by the T cell line, CRISPR/Cas9 gene editing was used to prevent expression of these genes. Interestingly, these changes reduced the amount of background reporting seen in the setting of T cells cultured with non-cognate antigen-expressing target cells, likely a result of reducing the chances of detecting reporting due to off-target TCR–pMHC interactions.

[0166] An important potential advantage of Tsyn-seq is that it does not depend on already-generated antigen libraries. Instead, Tsyn-seq can screen for antigens present in a cDNA library that can be custom-generated from individual samples using commercially available kits. Hence this system, in contrast to previous APC library screening approaches, can offer increased ease of screening for antigens present in specific tissue samples, such as samples derived from genetically less characterized species (*e.g.*, novel viruses), as well as samples for which commercially

available genome libraries are not available. One particular application could be identifying antigens present in patient-derived tumor samples, where personal neoantigens or viral antigens could likely be of high interest. Indeed, it was found that Tsyn-seq can correctly identify the cognate HPV16 E7 antigen as the most enriched antigen of those recognized by an HPV16 E7-specific TCR. Had the E7 antigen not been included in a pre-existing antigen library, this finding could have been likely missed.

[0167] Another advantage of this approach, in contrast to traditional approaches to identify antigens from cDNA libraries, is the increased ease and reduced costs associated with performing the screen. Prior methods that screened genome-wide libraries have utilized individual microcultures of independent transfections to screen thousands of unique constructs. Tsyn-seq is considerably less labor-intensive, requiring a co-culture performed using T cells and a mixture of target cells transduced with a cDNA library in a single flask, followed 2 days later by flow-sorting, DNA isolation, and amplicon sequencing.

[0168] While unexplored in the current study, this system could be utilized to screen for potential unexpected targeting of self-antigens by TCR-based therapies. This could provide a convenient method to quantify the therapeutic potential and safety profile of novel TCR-based candidate therapies for further development.

[0169] *Limitations and future directions.* An important future direction will be demonstrating that this system can identify antigens recognized by novel TCRs where the cognate antigen is not yet known. Another current limitation includes the ability to only screen a single TCR at a time expressed in Jurkat cells, in contrast to allowing the use of polyclonal primary T cells. Additionally, it has currently only been established that Tsyn-seq can function for human CD8 T cell TCRs that recognize peptides presented by HLA-A*02:01. It will be important to validate this system for other MHC-I HLA molecules, and to explore if the system could also function with murine CD8 cells, or MHC-II molecules presenting to human or murine CD4-derived TCRs. Finally, it is also important to consider that the reliance on 293T cell lines as APCs could produce biases in the results, due to inherent properties with respect to non-canonical transcription, post-translational modifications, or proteasomal behavior, all of which can have downstream impacts on peptide antigen presentation. Thus, validation of any identified antigens using other sources of APCs will be important to include in the workflow.

EXAMPLE 8**IMPROVED REPORTER SYSTEM**

[0170] By fusing the extracellular domain of Fas with the transmembrane and intracellular domains of TNFR2, the interaction between FasL and Fas at the immunological synapse not only produced NF κ B activation, but also activated Activator Protein-1 (AP1) (**FIG. 8A**). This enabled to further improve the system by introducing an AP1 response element driving the expression of *lyt2* (a truncated form of murine CD8), which we named Fas-induced AP-1 (Fas-iAP1) reporter, to FIR-APCs (**FIG. 8B**). It was found that ligation of Fas with an agonist antibody produced *lyt2* expression in FIR-APCs with Fas-iAP1 reporter. These cells were named FIR-APCs version 3 (V.3). It was observed a substantial increase in the proportion of CTV-labeled spike-in E7-FIR-APCs V.3 among the APC and T cell aggregates with activated Fas-iAP1 reporter, as compared to the control conditions without gating of the Fas-iAP1 reporter activation. This demonstrated that applying Fas-iAP1 reporter improved the performance of the reporter system.

EXAMPLE 9**MURINE VERSION OF THE TSYN REPORTER SYSTEM**

[0171] A mouse version of the Tsyn reporter system was generated by knocking out the HLA genes and overexpressed H2Kd in FIR-APC V.3. To evaluate how mouse FIR-APCs respond to targeting by T cells, G6PC2 (VYLKTNVFL (SEQ ID NO:44)) was evaluated as a model antigen. G6PC2 is a major target of autoreactive CD8 T cells (mTCR8.3) in the non-obese diabetic (NOD) mouse. It was found mouse FIR-APCs reported when being targeted by antigen-specific T cells both after peptide pulsing and when forced to express a protein antigen. This demonstrates that Tsyn-seq also works for murine MHC I molecule (**FIG. 9**).

[0172] Sequences:

Description	SEQ ID NO:	Sequence
Chimeric protein	SEQ ID NO:1	MLGIWTLPLVLTSVARLSSKSVNAQVTDINSK GLELRKTVTTVETQNLEGLHHDGQFCHKPCPP GERKARDCTVNGDEPDCVPCQEGKEYTDKAHF SSKCRRCRLCDEGHGLEVEINCTRTQNTKCRCK PNFFCNSTVCEHCDPCTKCEHGHIECTLTSTNK CKEEGSFALPVGLIVGVTALGLLIIGVVNCVIMT QVKKKPLCLQREAKVPHLPAGKARGTQGPEQQ HLLITAPSSSSSSLESSASALDRRAPTRNQPPAP GVEASGAGEARASTGSSDSSPGHGTQVNVTCI

		VNVCSSSDHSSQCSSQASSTMGDTDSSPSESPK DEQVPFSKEECAFRSQLETPETLLGSTEEKPLPL GVPDAGMKPS
Extracellular domain	SEQ ID NO:2	MLGIWTLLPLVLTSVARLSSKSVNAQVTDINSK GLELRKTVTTVETQNLEGLHHDGQFCHKPCPP GERKARDCTVNGDEPDCVPCQEGKEYTDKAHF SSKCRRCRLCDEGHGLEVEINCTRQTQNTKCRCK PNFFCNSTVCEHCDPCTKCEHGHKECTLTSTNTK CKEEGS
Transmembrane domain	SEQ ID NO:3	FALPVGLIVGV TALGLLIIGVVN
Intracellular domain	SEQ ID NO:4	CVIMTQVKKKPLCLQREAKVPHLPAGKARGTQ GPEQQHLLITAPSSSSSSLESSASALDRRAPTRN QPQAPGVEASGAGEARASTGSSDSSPGGHGTQ VNVTCIVNVCSSSDHSSQCSSQASSTMGDTDSS PSESPKDEQVPFSKEECAFRSQLETPETLLGST EKLPLPLGVPDAGMKPS
Chimeric protein	SEQ ID NO:5	STSLYKKVGMLGIWTLLPLVLTSVARLSSKSVN AQVTDINSKGLELRKTVTTVETQNLEGLHHDG QFCHKPCPPGERKARDCTVNGDEPDCVPCQEG KEYTDKAHFSSKCRRCRLCDEGHGLEVEINCTR TQNTKCRCKPNFFCNSTVCEHCDPCTKCEHGH KECTLTSTNTKCKEEGSFALPVGLIVGV TALGLLI IGVVNCVIMTQVKKKPLCLQREAKVPHLPAGK ARGTQGPEQQHLLITAPSSSSSSLESSASALDRR APTRNQPQAPGVEASGAGEARASTGSDSSPGG HGTQVNVTCIVNVCSSSDHSSQCSSQASSTMGD TDSSPSESPKDEQVPFSKEECAFRSQLETPETLL GSTEEKPLPLGVPDAGMKPSDIGKPIPNPLLGLD ST
GFP-F	SEQ ID NO:6	GCCCCCATGGTGAGCAAGGGCGAGGAG
GFP-R	SEQ ID NO:7	AAGTCATATGTTACTTGTACAGCTCGTCC
gRNA	SEQ ID NO:8	GTGTAACATACCTGGAGGAC
TNFR2-F	SEQ ID NO:9	GCCCGGATCCTTCGCTCTTCCAGTTGGACTG
TNFR2-R	SEQ ID NO:10	AAGTGATATCACTGGGCTTCATCCCAGCATC
HLA-A CRISPR 1	SEQ ID NO:11	CGTCCTGCCGGTACCCGCGG
CRISPR HLA class I F1	SEQ ID NO:12	CACCGAGGTCAGTGTGATCTCCGCA
CRISPR HLA class I R1	SEQ ID NO:13	AAACTGCGGAGATCACACTGACCTC
HLA-A CRISPR 2	SEQ ID NO:14	TACCGGCAGGACGCCTACGA
CRISPR HLA class I F2	SEQ ID NO:15	CACCGCGGCTACTACAACCAGAGCG
CRISPR HLA class I R2	SEQ ID NO:16	AAACCGCTCTGGTTGTAGTAGCCGC

HLA-B CRISPR	SEQ ID NO:17	GGATGGCGAGGACCAAACCTC
HLA-C CRISPR	SEQ ID NO:18	GACACAGAAGTACAAGCGCC
HLA-A2-F	SEQ ID NO:19	GCCCATCGATATGGCCGTCATGGCGCCCCGA AC
HLA-A2-R	SEQ ID NO:20	AAGTGATATCCACTTTACAAGCTGTGAGAGA C
mCherryF	SEQ ID NO:21	GCACAGATCTCGCCACCATGGTGAGCAAGGG CG
mCherryR	SEQ ID NO:22	TTCATCGAGCTACTTGTACAGCTCG
random sequence	SEQ ID NO:23	MVSKGGGGGS
	SEQ ID NO:24	GCACAGATCTCGCCACCATGGTGAGCAAGGG CGGTGGTGGTGGTTCTTAGCTCGAGTGAA
CRISPR TCR β F	SEQ ID NO:25	CACCGCGTAGAACTGGACTTGACAG
CRISPR TCR β R	SEQ ID NO:26	AAACCTGTCAAGTCCAGTTCTACGC
HPV16E6 antigen peptide	SEQ ID NO:27	TIHDIILECV
HPV16E7 antigen peptide	SEQ ID NO:28	YMLDLQPET
CMVpp65 antigen peptide	SEQ ID NO:29	NLVPMVATV
pp65CMV 9-mer	SEQ ID NO:30	NLVPMVATV
E6-F	SEQ ID NO:31	GCCCGGATCCCACCATGGACCAAAGAGAACT TGCA
E6-R	SEQ ID NO:32	AAGTGTCGACTTATGGTTTCTGAGAACAGAT GGGGC
E7-F	SEQ ID NO:33	GCCCGGATCCCACCATGGATGGAGATACACC TACA
E7-R	SEQ ID NO:34	AAGTGTCGACTTATGGTTTCTGAGAACAGAT GGGGC
CMV_F65	SEQ ID NO:35	GCCCGGATCCCACCATGGTGAACCTGGTGCCC ATGGTGGCCACCGTGTGAGTCGACTGAA
CMV_R65	SEQ ID NO:36	TTCAGTCGACTCACACGGTGGCCACCATGGG CACCAGGTTACCATGGTGGATCCGGGC
attB1 primer	SEQ ID NO:37	TGGTGGAAATTCTGCAGATATCAACAAG
attB2 primer	SEQ ID NO:38	CTGTGCTGGATATCAACCACTTTGT
E7-qPCR-F	SEQ ID NO:39	ATGCATGGAGATACACCTACATTGC
E7-qPCR-R	SEQ ID NO:40	GATTATGGTTTCTGAGAACAGATGGGGC
β -actin-F	SEQ ID NO:41	GCGCGGATCCGCGGACTATGACTTAGTTGCG
β -actin-R	SEQ ID NO:42	GCGCGCGGCCGCCACATTGTGAACTTTGGG GG
DDR1 derived neoantigen	SEQ ID NO:43	FLKEVKIML
G6PC2 model antigen	SEQ ID NO:44	VYLKTNVFL

[0173] Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A chimeric protein molecule comprising:
 - a) an extracellular domain selected from a Fas receptor extracellular domain, a CD80 extracellular domain, a PD-L1 extracellular domain, a CD86 extracellular domain, an LT β R extracellular domain, a 4-1BBL extracellular domain, an OX40L extracellular domain, an antibody that binds to a tumor necrosis factor, or antigen binding fragment that binds to a tumor necrosis factor;
 - b) an intracellular domain selected from a TNFR1 intracellular domain, a TNFR2 intracellular domain, a RANK intracellular domain, a BCMA intracellular domain, or a CD30 intracellular domain; and
 - c) a transmembrane domain linked to the C-terminus of the extracellular domain and the N-terminus of the intracellular domain.
2. The chimeric protein molecule of claim 1, wherein the extracellular domain comprises a Fas receptor extracellular domain.
3. The chimeric protein molecule of claim 1, wherein the intracellular domain is a TNFR2 intracellular domain.
4. The chimeric protein molecule of claim 1, wherein the transmembrane domain is a TNFR2 transmembrane domain.
5. The chimeric protein molecule of claim 1, wherein the extracellular domain is a Fas extracellular receptor domain, the transmembrane domain is a TNFR2 transmembrane domain, and the intracellular domain is a TNFR2 intracellular domain.
6. The chimeric protein molecule of claim 4, wherein the chimeric protein molecule comprises at least 80% sequence identity to SEQ ID NO:1.
7. The chimeric protein molecule of claim 6, wherein the chimeric protein molecule is SEQ ID NO:1.
8. The chimeric protein molecule of claim 1, wherein:
 - a) the extracellular domain comprises at least 80% sequence identity to amino acids 1-170 of SEQ ID NO:1;

b) the transmembrane domain comprises at least 80% sequence identity to amino acids 171-203 of SEQ ID NO:1;

c) the intracellular domain comprises at least 80% sequence identity to amino acids 204-374 of SEQ ID NO:1; or

d) a combination thereof.

9. The chimeric protein molecule of claim 2, wherein the extracellular domain comprises at least 80% sequence identity to SEQ ID NO:2.

10. The chimeric protein molecule of claim 3, wherein the intracellular domain comprises at least 80% sequence identity to SEQ ID NO:4.

11. The chimeric protein molecule of claim 4, wherein the transmembrane domain comprises at least 80% sequence identity to SEQ ID NO:3.

12. The chimeric protein molecule of claim 1, wherein the intracellular domain does not exhibit apoptotic activity.

13. The chimeric protein molecule of claim 1, wherein the intracellular domain does not exhibit FADD, Casp8, FAF, or DAXX activation activity.

14. An isolated cell comprising the chimeric protein molecule of any of claims 1-13.

15. The isolated cell of claim 14, wherein the cell is an antigen-presenting cell (APC).

16. The isolated cell of claim 15, wherein the APC is a macrophage, a regulatory macrophage, an activated macrophage, an M2 macrophage, a B cell, a plasma cell, a memory cell, a dendritic cell, a plasmacytoid dendritic cell, an inflammatory dendritic cell, or a Langerhans cell.

17. The isolated cell of claim 14, wherein the cell produces a detectable signal upon activation of the chimeric protein molecule.

18. The isolated cell of claim 17, wherein the detectable signal is a fluorescence signal.

19. The isolated cell of claim 17, wherein the detectable signal is generated by an inducible reporter system that is activated by the chimeric protein molecule.

20. The isolated cell of claim 19, wherein the inducible reporter system comprises a transcription factor response element.
21. The isolated cell of claim 20, wherein the transcription factor response element is an NF κ B response element or an AP1 response element.
22. The isolated cell of claim 20, wherein the transcription factor response element is operably coupled to a gene encoding a fluorescent protein, a luminescent protein, a cell surface protein, or an electrochemically detectable protein.
23. The isolated cell of claim 22, wherein the fluorescent protein is green fluorescent protein (GFP).
24. The isolated cell of claim 14, wherein the cell expresses an MHC Class I protein or an MHC Class II protein.
25. The isolated cell of claim 24, wherein the cell overexpresses the MHC Class I protein or the MHC Class II protein.
26. The isolated cell of claim 24, wherein the cell is engineered to express a single MHC allele.
27. The isolated cell of claim 14, wherein the cell does not express a native Fas receptor.
28. The isolated cell of claim 14, wherein the cell is a Fas receptor knockout.
29. The isolated cell of claim 14, wherein the cell expresses an exogenous antigenic peptide.
30. The isolated cell of claim 29, wherein the cell displays the exogenous antigenic peptide, or a fragment thereof, in an MHC Class I or MHC Class II protein.
31. The isolated cell of claim 29, wherein the cell is stably or transiently transfected with a vector encoding the exogenous antigenic peptide.
32. The isolated cell of claim 14, wherein the cell overexpresses CIITA, LRC5, B2M, RFX5, RFXAP, or RFXANK.
33. The cell of claim 32, wherein the cell overexpresses CIITA.

34. A system comprising:

- a) an antigen-presenting cell (APC) comprising an MHC protein and a first inducible reporter system, and
- b) an antigen-sensing cell comprising a T cell receptor (TCR) or a chimeric antigen receptor (CAR), and a second inducible reporter system;

wherein the first inducible reporter system generates a first detectable signal and the second inducible reporter system generates a second detectable signal upon contact between the MHC protein and the TCR or the CAR.

35. The system of claim 34, wherein the first inducible reporter system and/or the second inducible reporter system comprises a transcription factor response element.

36. The system of claim 35, wherein the first inducible reporter system comprises an NF κ B response element.

37. The system of claim 35, wherein the second inducible reporter system comprises an NFAT response element.

38. The system of claim 35, wherein the first inducible reporter system comprises an NF κ B response element, and the second inducible reporter system comprises an NFAT response element.

39. The system of claim 35, wherein the transcription factor response element is operably coupled to a gene encoding a fluorescent protein, a luminescent protein, a cell surface protein, or an electrochemically detectable protein.

40. The system of claim 34, wherein the APC expresses a transmembrane protein that activates the first inducible reporter system upon binding to a ligand expressed by the antigen-sensing cell.

41. The system of claim 40, wherein the ligand is a tumor necrosis factor or an immune checkpoint molecule.

42. The system of claim 41, wherein the ligand comprises FasL, CTLA-4, PD-1, LT- α , or TNFSF14.

43. The system of claim 40, wherein the transmembrane protein is the chimeric protein molecule of any of claims 1-13.
44. The system of claim 34, wherein the TCR or the CAR activates the second inducible reporter system upon contact with the MHC protein.
45. The system of claim 34, wherein the antigen-presenting cell comprises a nucleic acid encoding an antigen.
46. The system of claim 34, wherein the system comprises a plurality of the APCs, and wherein the plurality of the APCs collectively comprises a plurality of nucleic acids encoding a plurality of antigens.
47. The system of claim 34, wherein the APC is an isolated cell of any of claims 14-33.
48. The system of claim 34, wherein the antigen-sensing cell is a T cell.
49. The system of claim 34, wherein the TCR is overexpressed by the antigen-sensing cell.
50. The system of claim 34, wherein the TCR or CAR binds an antigen displayed by the MHC protein.
51. The system of claim 34, wherein the antigen-sensing cell expresses a single TCR molecule.
52. The system of claim 34, wherein the antigen-sensing cell does not express a native TCR.
53. The system of claim 34, with the proviso that the antigen-sensing cell does not express TNF.
54. The system of claim 53, wherein the first detectable signal comprises at least about 1.5-fold, at least about 2-fold, at least about 2.5-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, at least about 10-fold, at least about 12-fold, at least about 15-fold, at least about 20-fold, at least about 25-fold, at least about 50-fold, or at least about 100-fold higher signal-to-noise than an identical system in which the antigen-sensing cell expresses TNF.

55. A system comprising:

- a) an antigen-presenting cell (APC) comprising an MHC protein, an inducible reporter system comprising an NF κ B response element operably coupled to a gene encoding a first fluorescent protein, and a chimeric protein molecule configured to activate the NF κ B response element; and
- b) an antigen-sensing cell comprising a T cell receptor (TCR) or a chimeric antigen receptor (CAR), an inducible reporter system that is activated by the TCR or CAR and comprises an NFAT response element operably coupled to a gene encoding a second fluorescent protein, and a ligand configured to activate the chimeric protein molecule.

56. The system of claim 55, wherein the chimeric protein molecule is the chimeric protein molecule of any of claims 1-13.

57. The system of claim 55, wherein the first fluorescent protein is green fluorescent protein (GFP).

58. The system of claim 55, wherein the second fluorescent protein is mCherry.

59. A method for detecting an antigen recognized by a T cell comprising:

- a) contacting the system of any of claims 34-55 with an antigen or a nucleic acid encoding the antigen; and
- b) detecting a first detectable signal from the first inducible reporter system and a second detectable signal from the second inducible reporter system, wherein the first and/or second detectable signal is indicative of a TCR or CAR that specifically recognizes the antigen, thereby detecting the antigen recognized by a T cell.

60. The method of claim 59, wherein the antigen is selected from a tumor antigen, a bacterial antigen, a viral antigen, a cancer antigen, a neoantigen, and a self-antigen.

61. The method of claim 59, further comprising identifying the TCR or the CAR that specifically recognizes the antigen.

62. The method of claim 51, wherein the identifying comprising sequencing a nucleic acid encoding the TCR or the CAR.

63. The method of claim 62, wherein the nucleic acid is from a cDNA library.
64. The method of claim 59, further comprising identifying the antigen recognized by the TCR or CAR.
65. The method of claim 64, wherein the identifying comprises sequencing the nucleic acid encoding the antigen.
66. The method of claim 59, further comprising isolating the APC or the antigen-sensing cell.
67. The method of claim 66, wherein the isolating comprises flow cytometry or fluorescence-activated cell sorting (FACS).
68. The method of claim 66, wherein the isolating comprises excluding a cell that does not produce the first detectable signal or the second detectable signal.
69. The method of claim 66, wherein the isolating comprises size-selection of an aggregate comprising the APC and the antigen-sensing cell.
70. The method of claim 66, wherein the isolating comprises selecting a cell that produces the first detectable signal or the second detectable signal.
71. The method of claim 59, wherein the antigen-sensing cell is a T cell.
72. The method of claim 59, wherein the antigen is presented by the APC.
73. The method of claim 59, wherein the antigen is exogenous to the APC.
74. A TCR, CAR, or antigen identified by the method of any of claims 59-73.

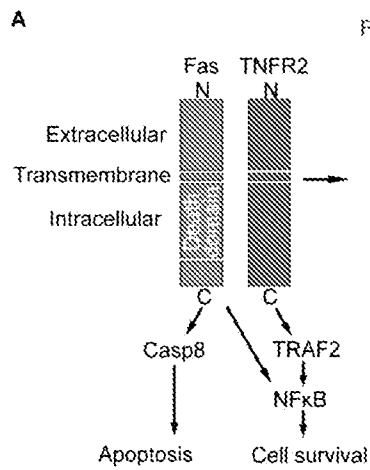


FIG. 1A

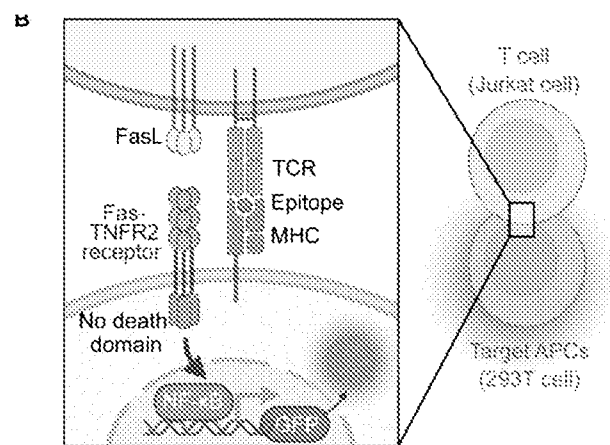


FIG. 1B

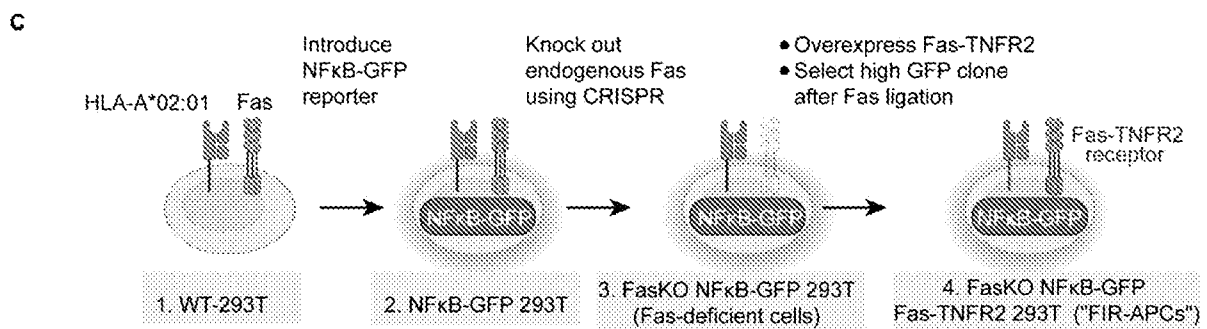


FIG. 1C

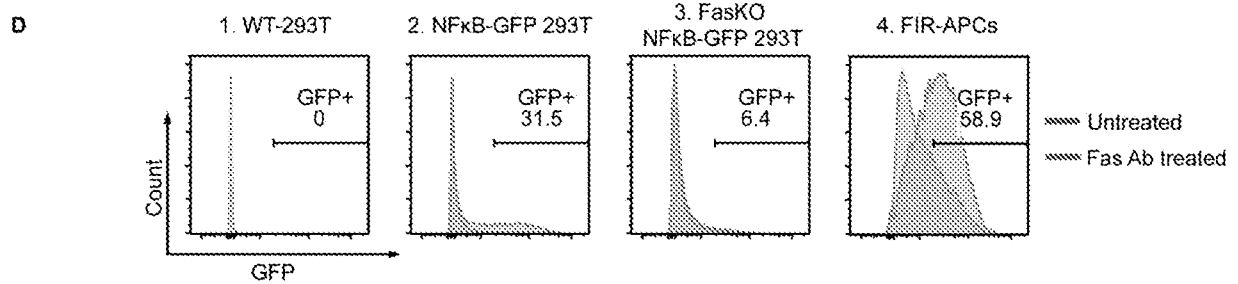


FIG. 1D

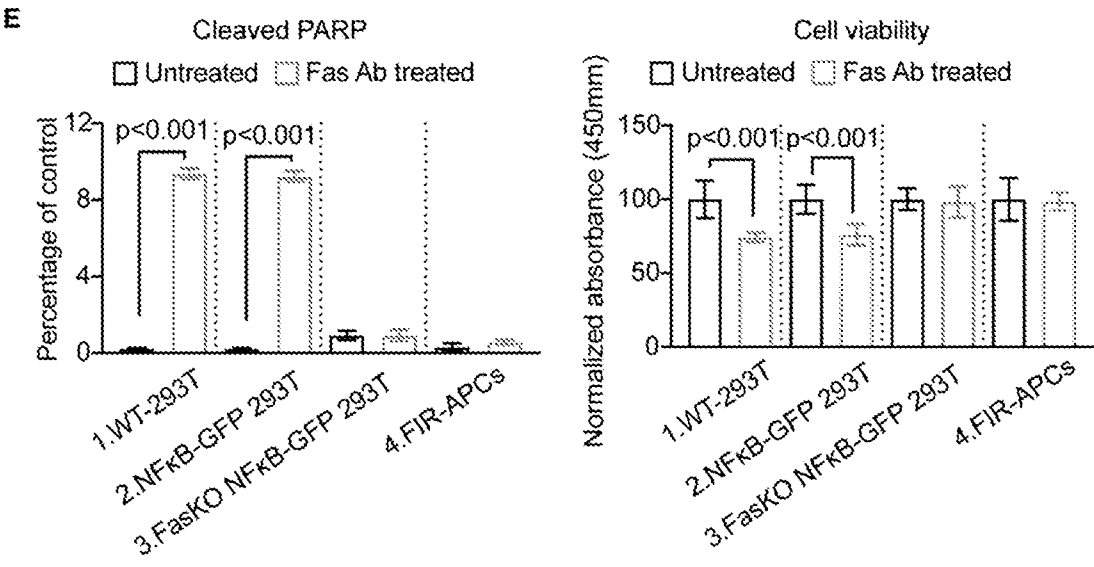


FIG. 1E

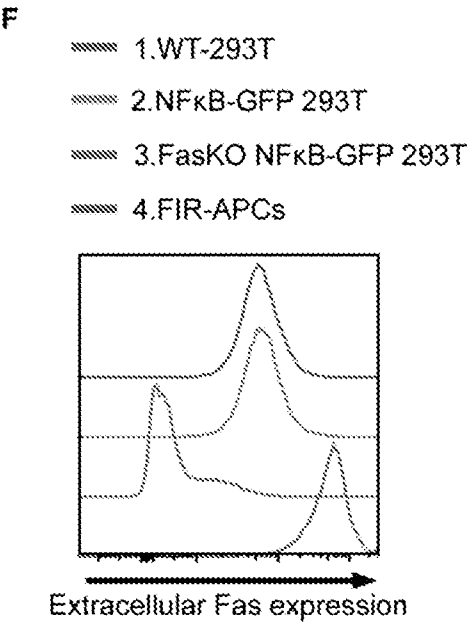


FIG. 1F

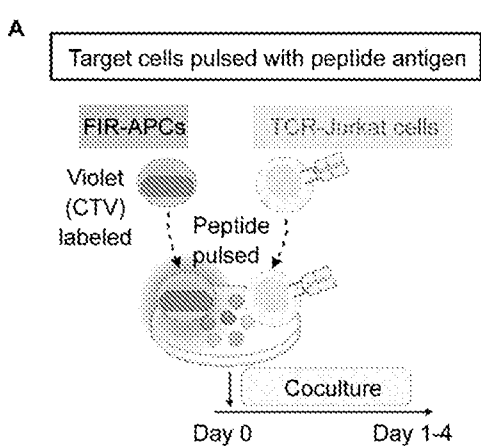


FIG. 2A

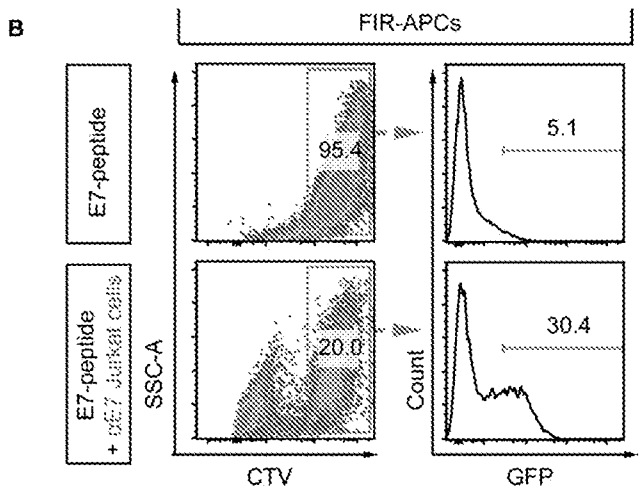


FIG. 2B

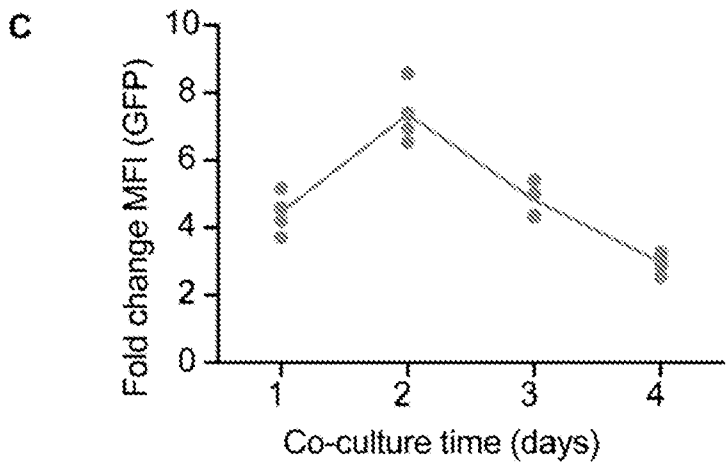


FIG. 2C

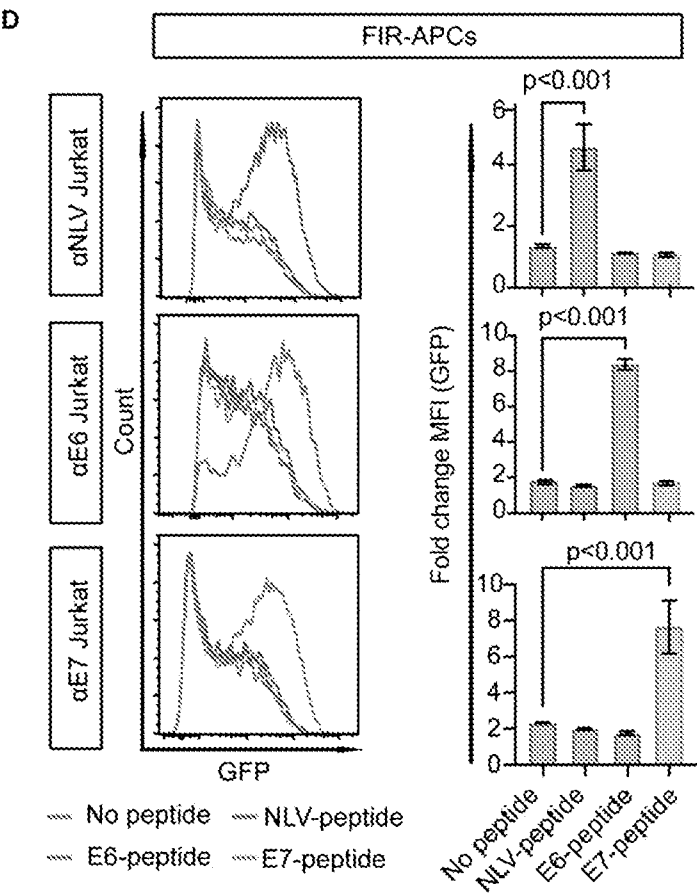


FIG. 2D

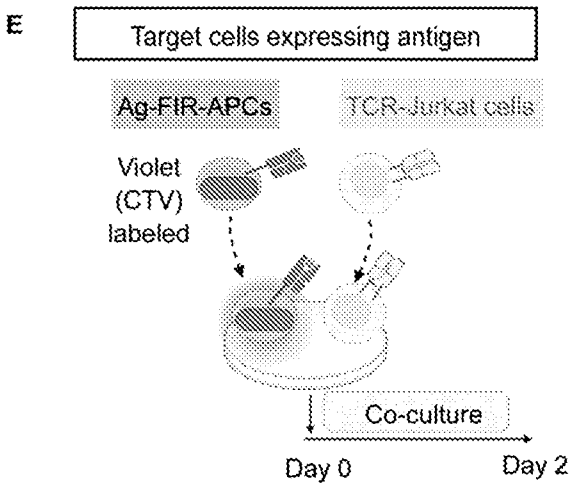


FIG. 2E

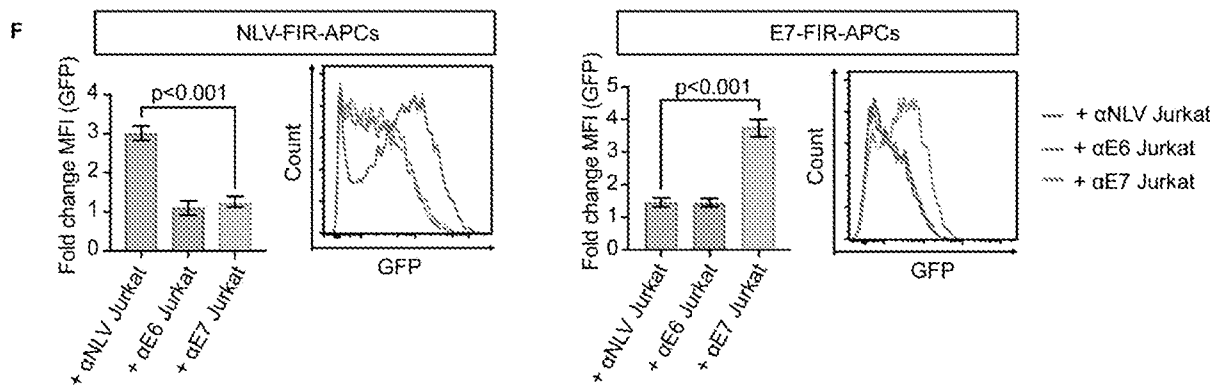


FIG. 2F

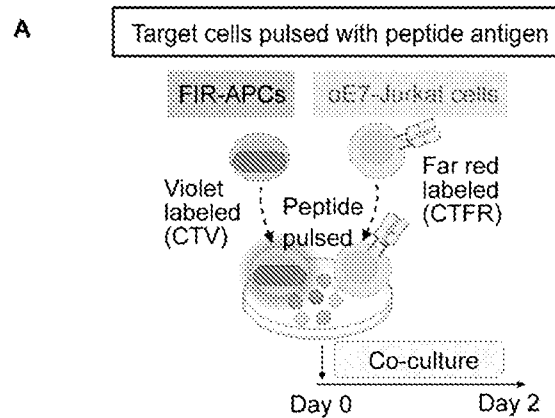


FIG. 3A

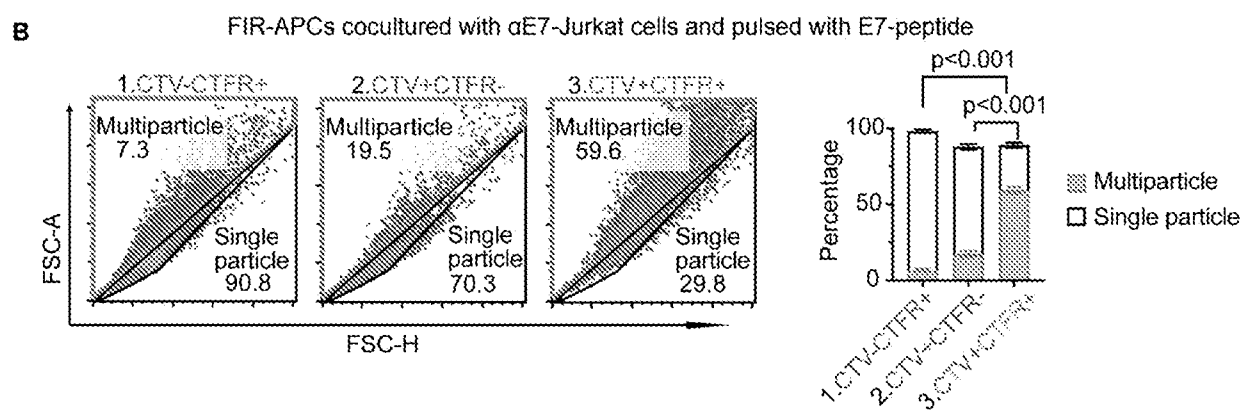


FIG. 3B

C FIR-APCs cocultured with α E7-Jurkat cells
and pulsed with peptide antigen

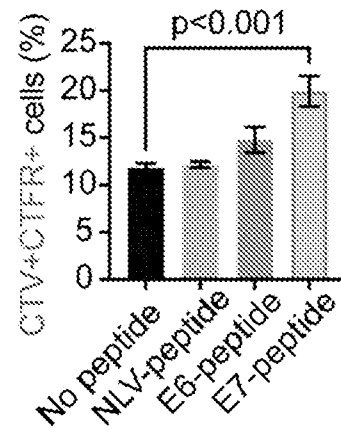


FIG. 3C

D FIR-APCs cocultured with α E7-Jurkat cells and pulsed with E7-peptide

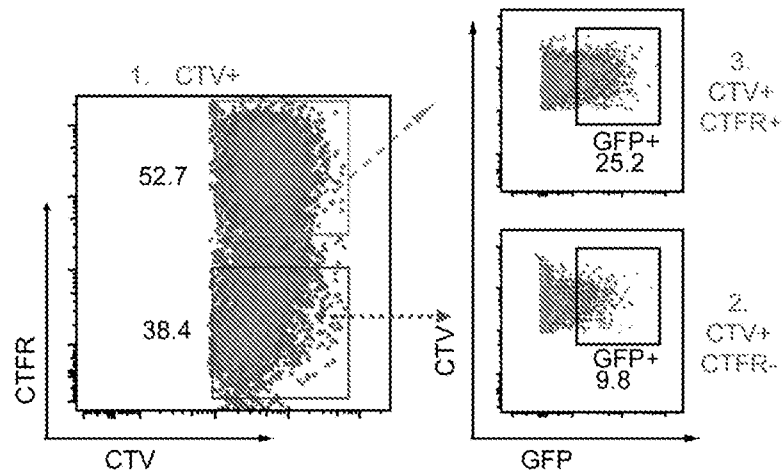


FIG. 3D

E FIR-APCs cocultured with αE7-Jurkat cells and pulsed with peptide antigen

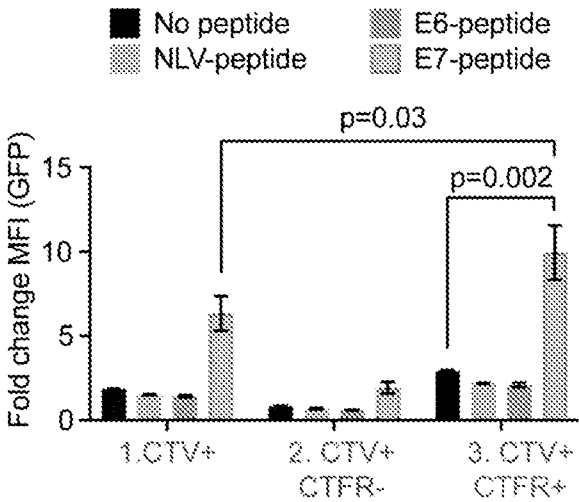


FIG. 3E

A

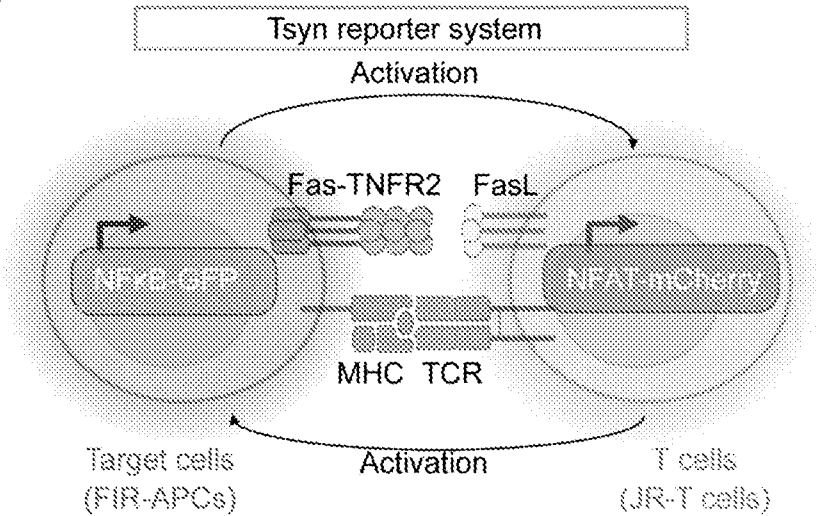


FIG. 4A

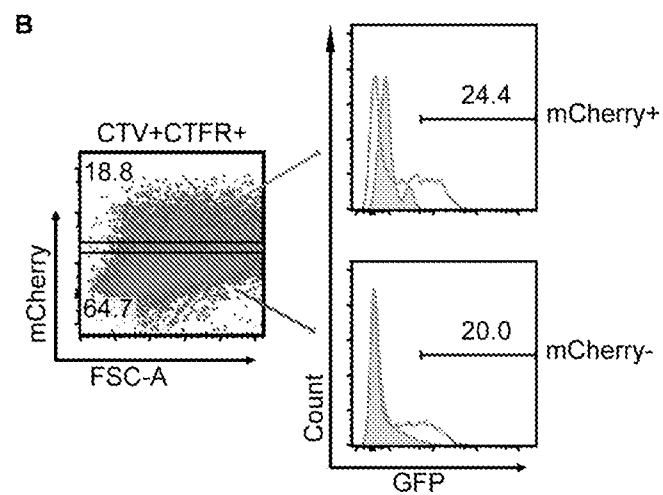


FIG. 4B

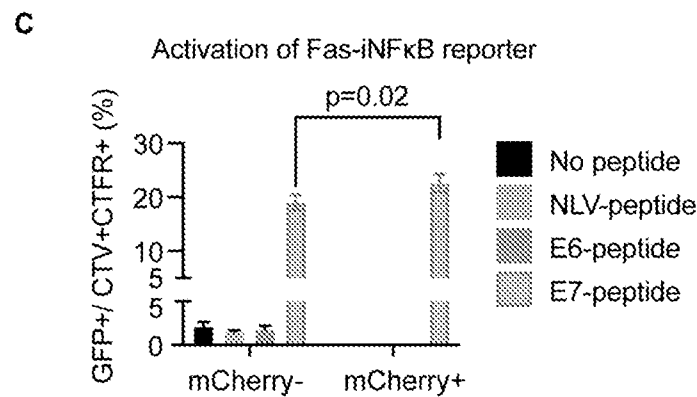


FIG. 4C

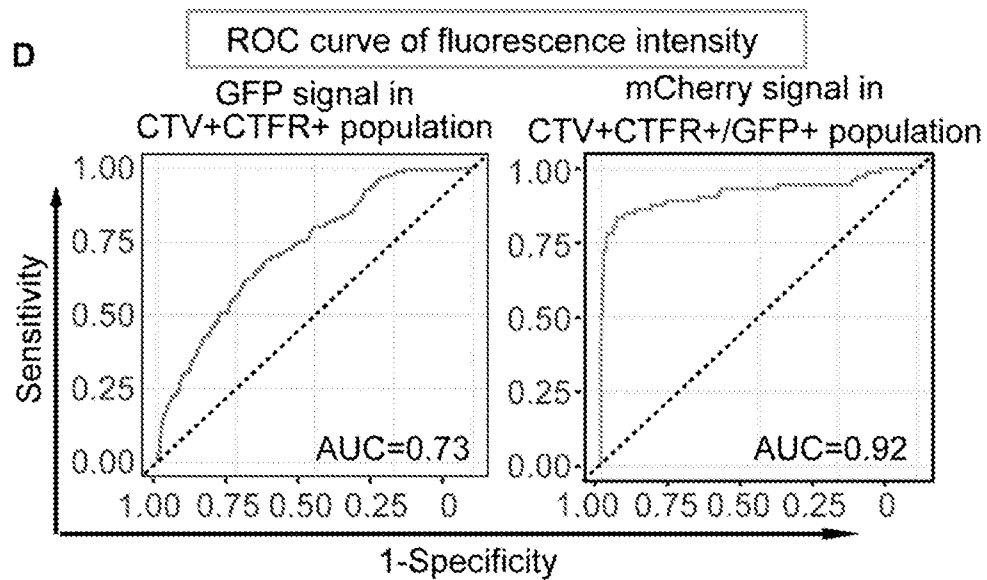


FIG. 4D

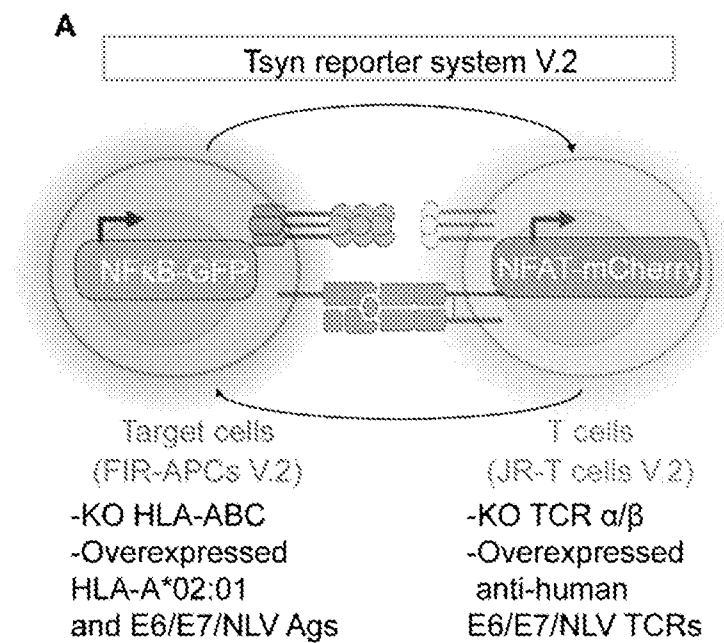


FIG. 5A

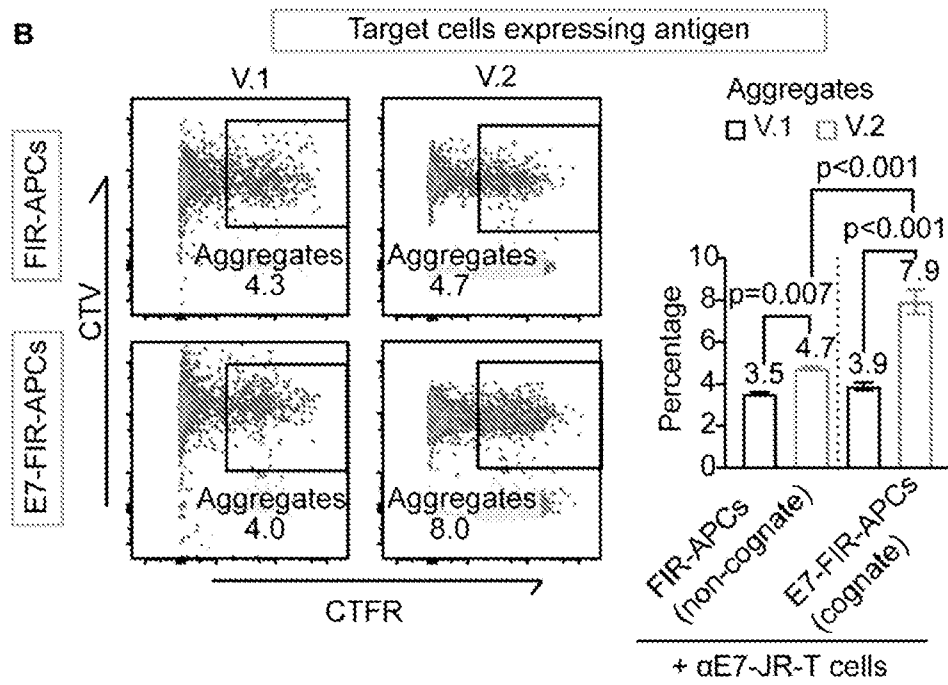


FIG. 5B

c

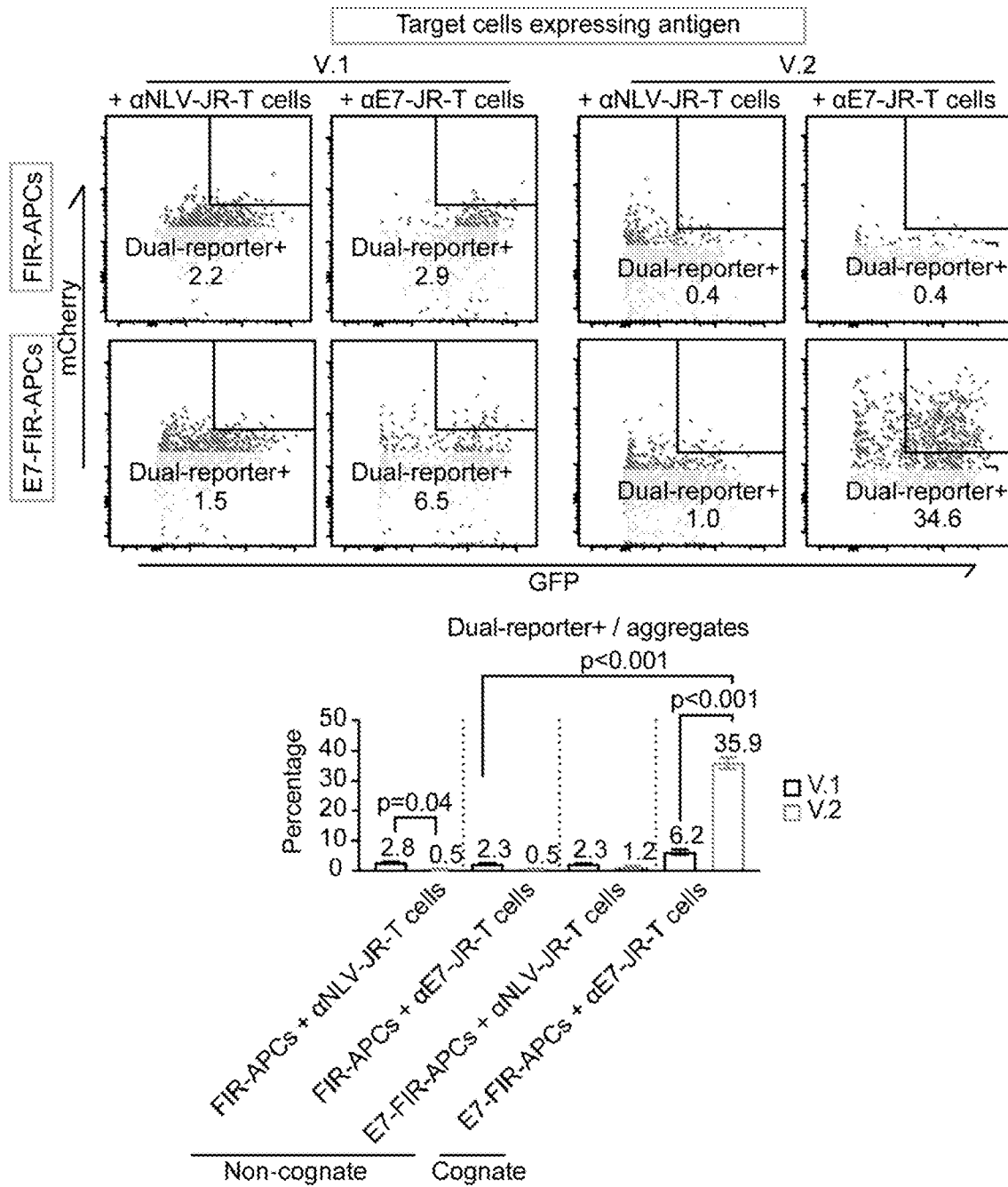


FIG. 5C

A

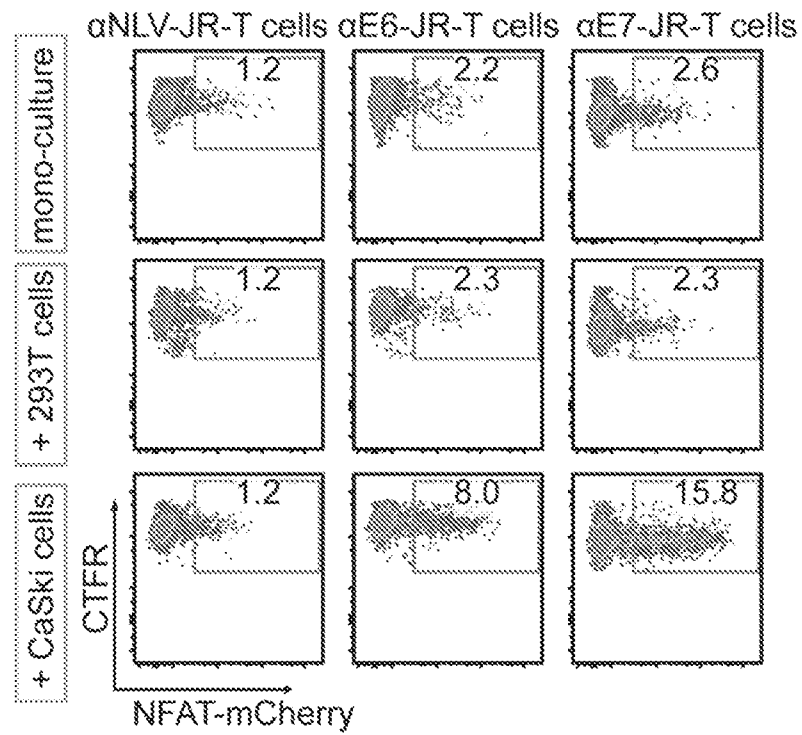


FIG. 6A

B

Activation of NFAT-mCherry reporter

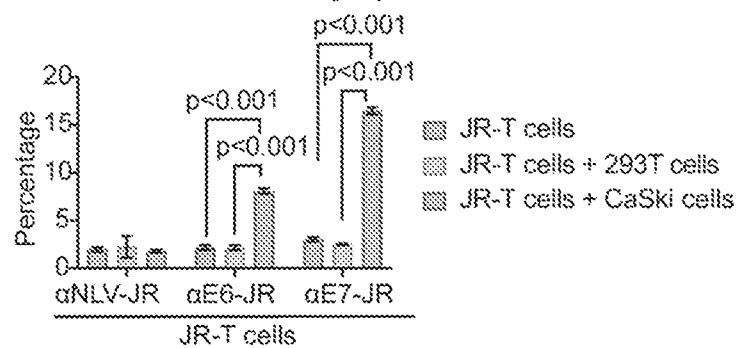


FIG. 6B

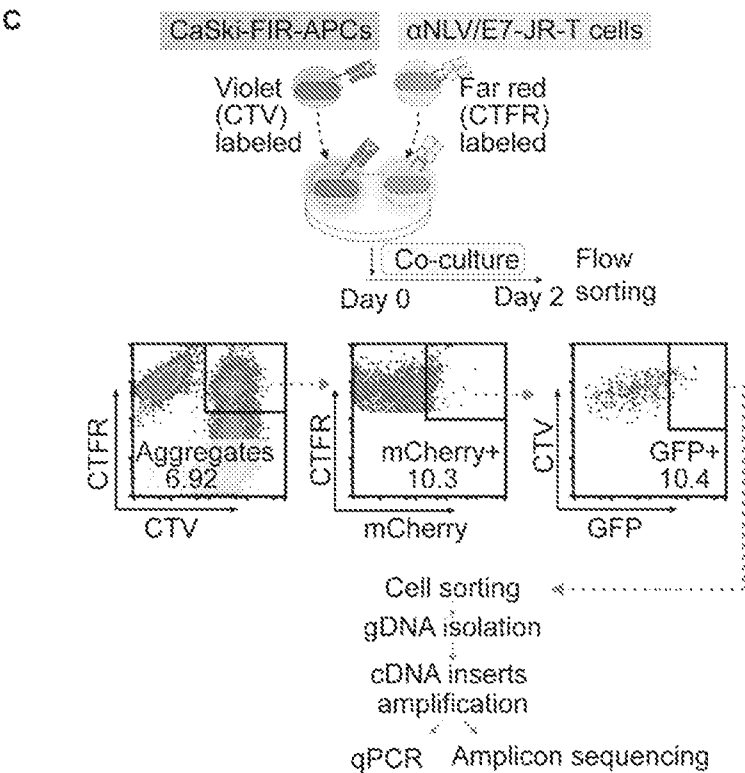


FIG. 6C

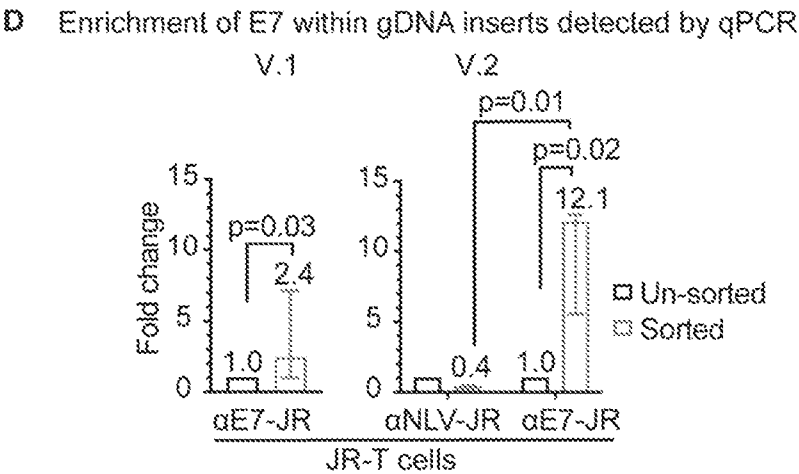


FIG. 6D

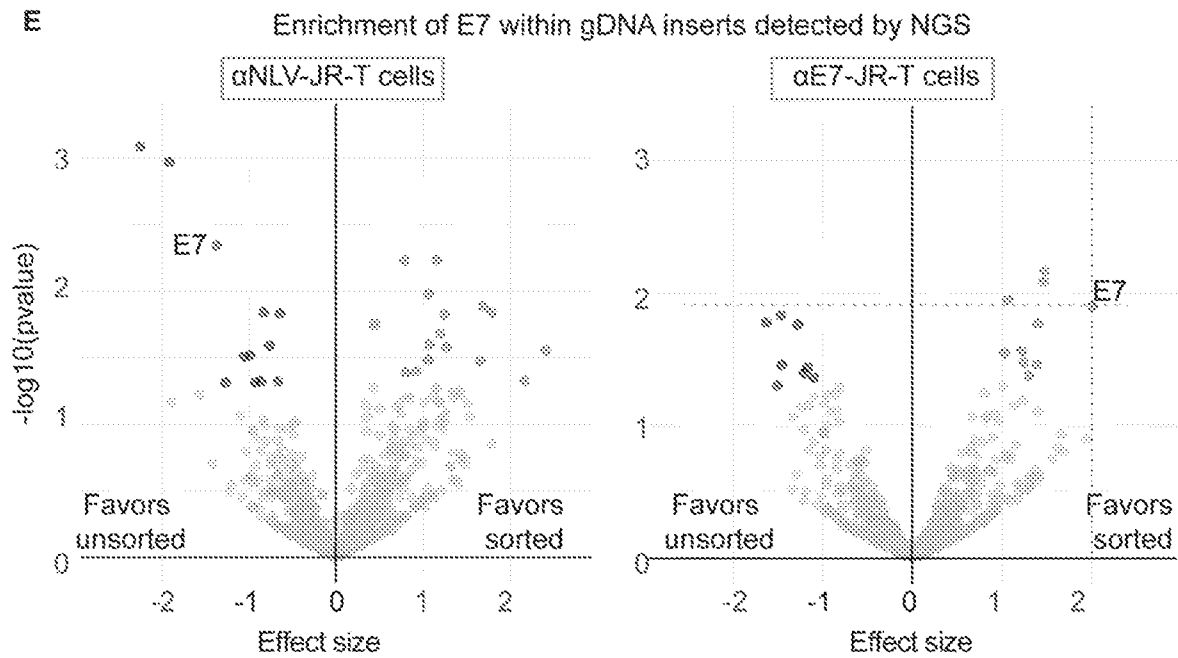


FIG. 6E

F

Abundance of E7 detected by NGS

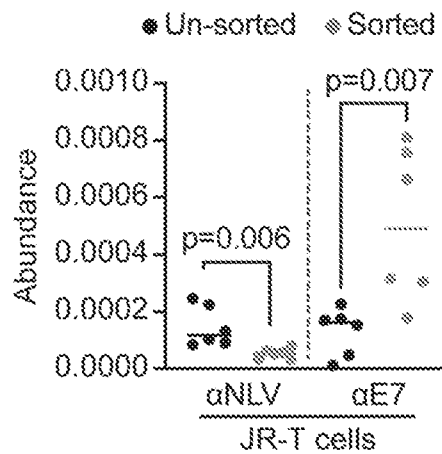


FIG. 6F

7/7

SEQ ID NO:1
MLGIWTLPLPLVLTSVARLSSKSVNAQVTDINSKGLELRKTVTTVETQNLLEGLHHDGQFCHKPCPPGERKARDCTVNGD
EPDCVPCQEGKEYTDKAHFSSKRRRLCDEGHGLEVEINCTRTQNTKCRCKPNFFCNSTVCEHCDPCTKCEHGIKE
CTLTSNTKCKEEGSEFALPVGLIVGVTALGLLIIGVNCVIMTQVKKKPLCLQREAKVPHLPAGKARGTQGPQQHLLI
TAPSSSSSLESSASALDRRAPTRNQAPGVEASGAGEARASTGSSDSSPGGHGTQVNVTCIVNVCSSSHSSQCSQAS
STMGDTSSPSESPKDEQVPFSKEECAFRSQLETPETLLGSTEEKPLPLGVPDAGMKPS

SEQ ID NO:2
MLGIWTLPLPLVLTSVARLSSKSVNAQVTDINSKGLELRKTVTTVETQNLLEGLHHDGQFCHKPCPPGERKARDCTVNGD
EPDCVPCQEGKEYTDKAHFSSKRRRLCDEGHGLEVEINCTRTQNTKCRCKPNFFCNSTVCEHCDPCTKCEHGIKE
CTLTSNTKCKEEGS

SEQ ID NO:3
FALPVGLIVGVTALGLLIIGVVN

SEQ ID NO:4
CVIMTQVKKKPLCLQREAKVPHLPAGKARGTQGPQQHLLITAPSSSSSLESSASALDRRAPTRNQAPGVEASGAG
EARASTGSSDSSPGGHGTQVNVTCIVNVCSSSHSSQCSQASSTMGDTSSPSESPKDEQVPFSKEECAFRSQLETPETL
LGSTEEKPLPLGVPDAGMKPS

SEQ ID NO:5
STSLYKKVGMGLGIWTLPLPLVLTSVARLSSKSVNAQVTDINSKGLELRKTVTTVETQNLLEGLHHDGQFCHKPCPPGERK
ARDCTVNGDEPDCVPCQEGKEYTDKAHFSSKRRRLCDEGHGLEVEINCTRTQNTKCRCKPNFFCNSTVCEHCDPC
TKCEHGIKECTLTSNTKCKEEGSEFALPVGLIVGVTALGLLIIGVNCVIMTQVKKKPLCLQREAKVPHLPAGKARGTQ
GPEQQHLLITAPSSSSSLESSASALDRRAPTRNQAPGVEASGAGEARASTGSSDSSPGGHGTQVNVTCIVNVCSSSH
SSQCSQASSTMGDTSSPSESPKDEQVPFSKEECAFRSQLETPETLLGSTEEKPLPLGVPDAGMKPSDICKPIPNPLLGL
DST

FIG. 7

A

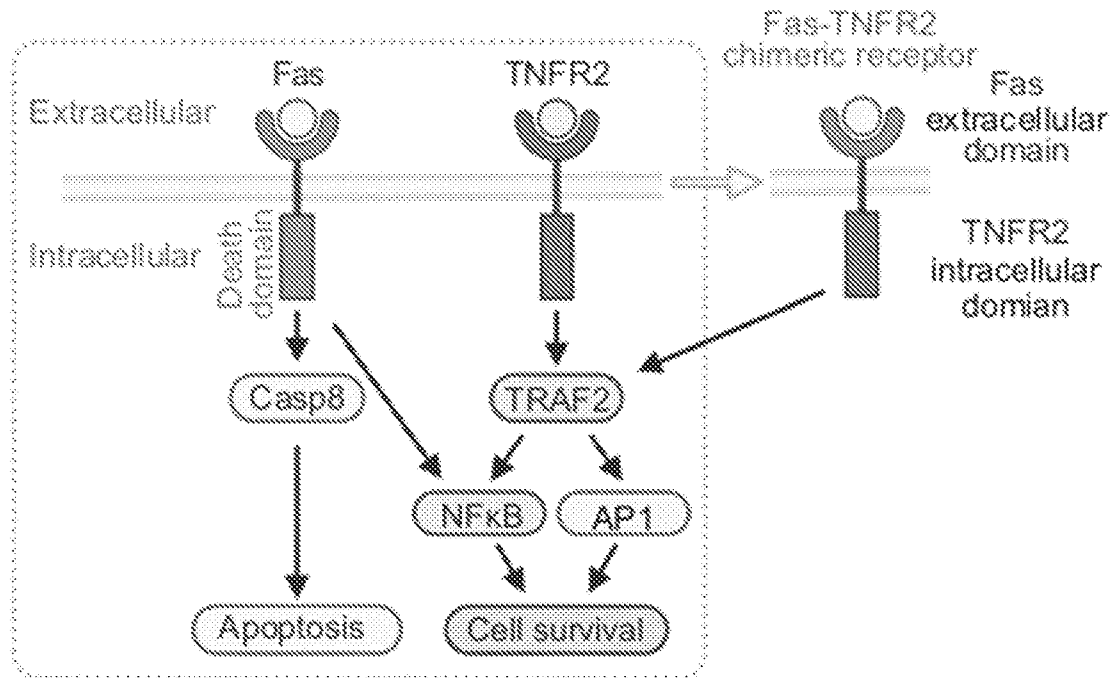


FIG. 8A

B

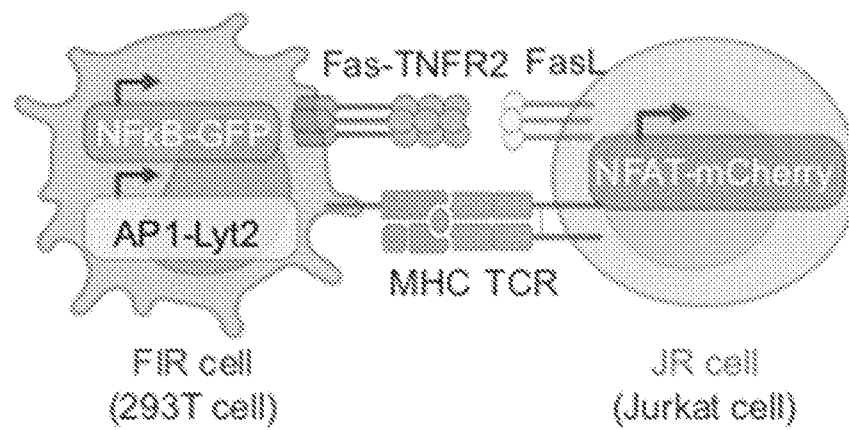


FIG. 8B

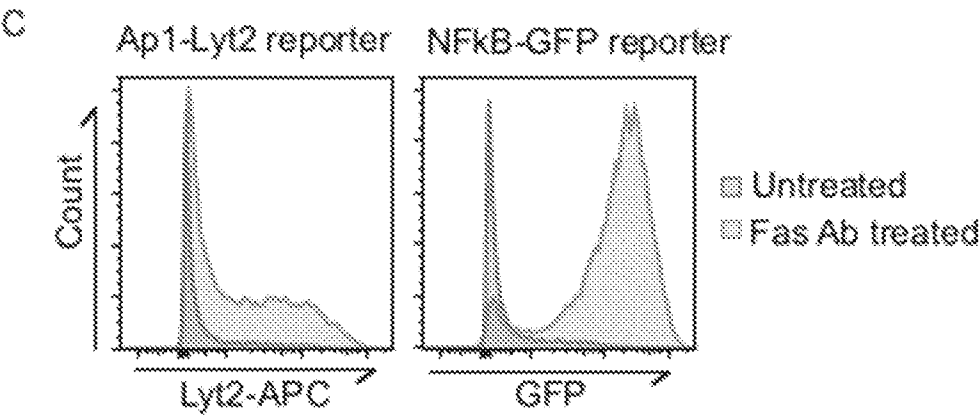


FIG. 8C

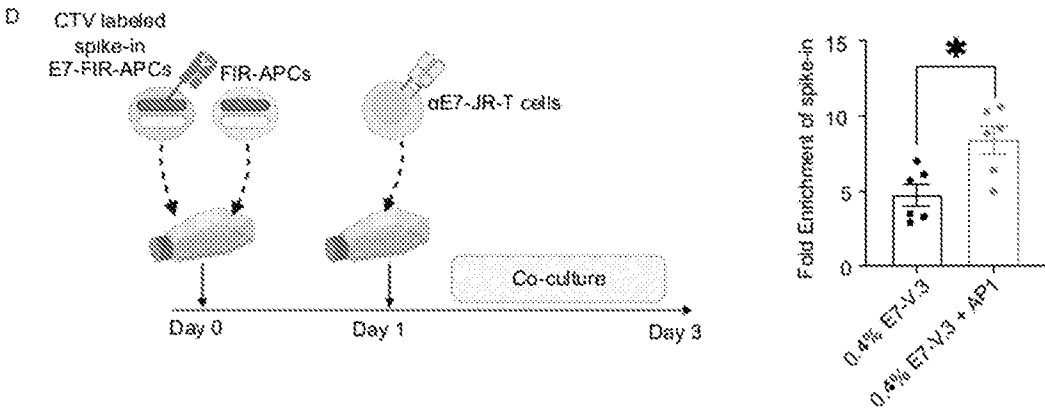


FIG. 8D

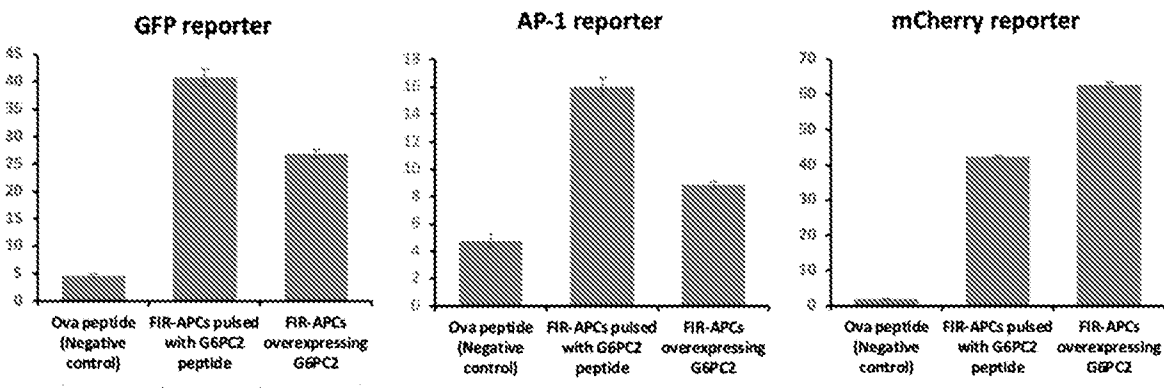


FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/035894

A. CLASSIFICATION OF SUBJECT MATTERIPC: **C07K 14/705** (2024.01); **C12N 15/62** (2024.01); **C12Q 1/6897** (2024.01); **G01N 33/533** (2024.01); **C12N 15/63** (2024.01); **C12N 5/10** (2024.01); **C07K 14/74** (2024.01)CPC: **C07K 14/705**; **C12N 15/62**; **C12N 15/1086**; **C12N 5/0634**; **C12Q 1/6897**; **C07K 2319/03**; **C07K 2319/60**; **C07K 14/70578**; **C07K 14/70539**; **C12N 15/63**; **C12N 5/10**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2022/0348936 A1 (MCMASTER UNIVERSITY) 03 November 2022 (03.11.2022) entire document	1-3, 12, 13
Y	entire document	4, 5, 9, 11
Y	US 2021/0292389 A1 (SANGAMO THERAPEUTICS FRANCE) 23 September 2021 (23.09.2021) entire document	4, 5, 11
Y	US 2020/0009190 A1 (FRED HUTCHINSON CANCER RESEARCH CENTER) 09 January 2020 (09.01.2020) entire document	9
A	US 2022/0010023 A1 (CITY OF HOPE et al.) 13 January 2022 (13.01.2022) entire document	1-33
A	US 7,754,208 B2 (LEDBETTER et al.) 13 July 2010 (13.07.2010) entire document	1-33



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“D” document cited by the applicant in the international application

“E” earlier application or patent but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

18 September 2024 (18.09.2024)

Date of mailing of the international search report

05 November 2024 (05.11.2024)

Name and mailing address of the ISA/US

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,648,219 A (MACKAY et al.) 15 July 1997 (15.07.1997) entire document	1-33
A	US 2018/0073013 A1 (MAX-DELBRUCK-CENTRUM FUR MOLEKULARE MEDIZIN IN DER HELMHOLTZ-GEMEINSCHAFT et al.) 15 March 2018 (15.03.2018) entire document	1-33
A	NEEFIES et al. Towards a systems understanding of MHC class I and MHC class II antigen presentation, Nature Reviews, Vol. 11, Pgs. 823-836. entire document	1-33

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/035894

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/035894

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: **43, 56, 59-74**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I: claims 1-33 are drawn to chimeric protein molecules.

Group II: claims 34-42, 44-55, 57, and 58 are drawn to systems.

The inventions listed in Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I, chimeric protein molecules, are not present in Group II; the special technical features of Group II, systems, are not present in Group I.

Additionally, even if Groups I and II were considered to share the technical features of a chimeric protein molecule, these shared technical features do not represent a contribution over the prior art.

US 2022/0010023 A1 to City of Hope et al. (hereinafter, "Hope") teaches a chimeric protein molecule (T cells expressing a chimeric antigen receptor; Para. [0004]).

The inventions listed in Groups I and II therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/035894

Box No. III **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-33**

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.