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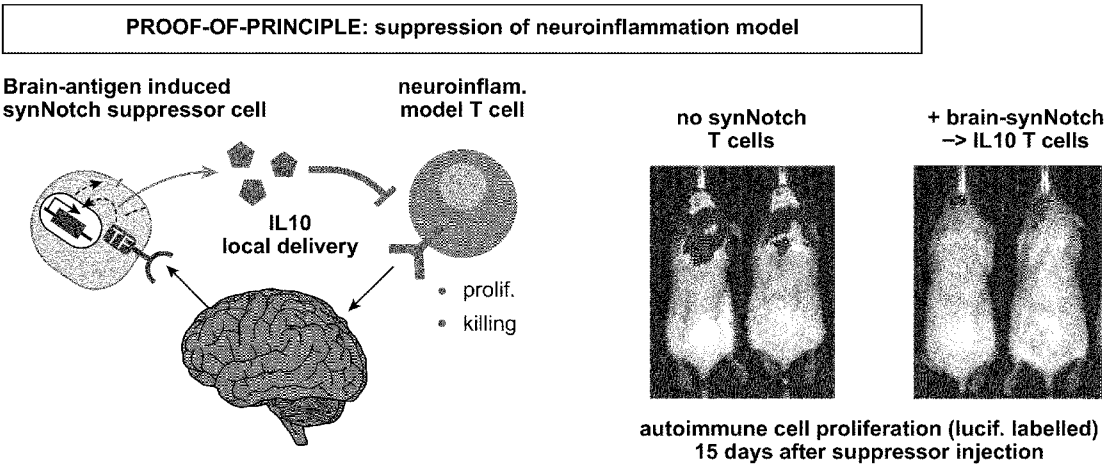


FIG. 6

(57) Abstract: Provided herein is a cell therapy comprising: (i) a cytotoxic T cell comprising an engineered immune receptor that recognizes an antigen on a target cell; and (ii) an immunosuppressive cell comprising a molecular circuit comprising (a) a binding-triggered transcriptional switch (BTTS) that recognizes an antigen on a non-target cell and (b) an anti-inflammatory protein, wherein: binding of the engineered immune receptor to the antigen on the target cell in the absence of the immunosuppressive cell activates the cytotoxic T cell and binding of the BTTS to the antigen on the surface of a non-target cell activates expression of the anti-inflammatory protein by the immunosuppressive cell and protects the non-target cell from the cytotoxic T cell. Methods of treatment are also provided.

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TWO CELL NOT GATE COMPRISING SYNTHETIC IMMUNO-SUPPRESSIVE CELLS

CROSS-REFERENCING

This application claims the benefit of U.S. provisional application serial no. 63/453,700,
5 filed on March 21, 2023, which application is incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant no. DK116264 awarded
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INCORPORATION BY REFERENCE OF SEQUENCE LISTING

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A Sequence Listing is provided herewith as a Sequence Listing XML,
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15 contents of the Sequence Listing XML are incorporated herein by reference in their entirety.

INTRODUCTION

Despite recent clinical success in using engineered T cells to treat hematologic cancers, a
major barrier in expanding their use to solid tumors is the challenge of specific tumor
20 recognition. While it is possible to engineer chimeric antigen receptors (CARs) directed toward
tumor associated antigens, many of those antigens, especially in the case of solid tumors, are
also expressed, often at lower levels, in other normal tissues, leading to cases of toxic cross-
reactivity. While toxicity can in some cases be ameliorated by reducing CAR T dosage, the
small therapeutic window caused by poor discrimination leads to a tradeoff between efficacy
25 and toxicity. The difficulty of finding absolutely tumor unique surface antigens that can be
distinctly recognized by CARs has led some to question the capability of such engineered T
cells to ultimately achieve success in safely treating solid tumors.

Current approaches for engineering CAR T cells, however, focus only on recognition of
a single target antigen. If one considers that solid tumors express an array of antigens, it is
30 possible that improved specificity could be achieved through recognition of combinatorial
antigen signatures. Such considerations, however, have only recently become actionable with

advances in synthetic biology approaches to engineering T cell therapies. Engineered cells are unique among therapeutic modalities in that they can in principle be engineered with multi-antigen recognition circuits. For example, recent advances have shown that it is possible to engineer CAR T cells that recognize target cells with combinatorial Boolean logic: one can engineer T cells with multi-receptor circuits that function as AND gates (requiring two antigens to be present), NOT gates (requiring one antigen but not the other to be present), and OR gates (requiring the presence of either of two antigens).

This disclosure provides a new “NOT” gate that relies on Boolean logic to improve therapeutic efficacy.

SUMMARY

Provided herein is a cell therapy comprising: (i) a cytotoxic immune cell comprising an engineered immune receptor that recognizes an antigen on a target cell; and (ii) an immunosuppressive cell comprising a molecular circuit comprising (a) a binding-triggered transcriptional switch (BTTS) that recognizes an antigen on a non-target cell and (b) a nucleic acid encoding an anti-inflammatory protein, wherein: binding of the engineered immune receptor to the antigen on the target cell in the absence of the immunosuppressive cell activates the cytotoxic immune cell and binding of the BTTS to the antigen on the surface of a non-target cell activates expression of the anti-inflammatory protein by the immunosuppressive cell and protects the non-target cell from the cytotoxic immune cell. Methods of treatment are also provided.

A method of treating a subject is also provided. In these embodiments, the method may comprise administering to the subject the cell therapy, where the cells may be administered together or separately. In these embodiments, the engineered immune receptor may recognize an antigen that is expressed on cancerous cells; and the BTTS recognizes an antigen that is not on the cancerous cells (e.g., an antigen that is expressed in a non-target tissue). For example, the antigen recognized by the BTTS is tissue and/or organ specific.

The present therapy is believed to reduce “on-target/off-tumor” killing by the cytotoxic immune cells.

Examples of such circuits and their use are described in further detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig 1. shows synNotch induced production of suppressive cytokine TGFb.

Figs. 2A and 2B show that suppressor cells that produce combination of TGFb (suppressive cytokine) and CD25 (IL2 sink) are very effective at suppressing CAR T killing in vitro.

Figs. 3A and 3B show that suppressor cells that produce combination of IL10 (suppressive cytokine) and CD25 (IL2 sink) are very effective at suppressing CAR T killing in vitro.

Figs. 4A and 4B shows that suppressor cells that produce combination of TGFb (suppressive cytokine) and CD25 (IL2 sink) are very effective at suppressing CAR T killing of tumors in vivo.

Fig. 5. shows that engineered T cells overexpressing CD25 increases consumption of IL2 and cell proliferation.

Fig. 6. shows that synNotch→IL10 synthetic suppressor cells can block autoimmune cell proliferation in brain and CNS in mouse neuroinflammation model.

Figs. 7A-7D show that synNotch circuits in CD4+ T cells can reconstitute Treg-like functions to drive local immune suppression.

Fig. 7A: For many inflammatory disorders, one therapeutic strategy would be to locally suppress immune responses without systemic immune suppression. In this example, engineered synthetic suppressor cells - human CD4+ T cells that contain a tissue-targeted synthetic Notch (synNotch) receptor - locally induce Treg-like immune suppressive functions. In this case, the cell produces production of inhibitory cytokines and pro-inflammatory cytokine sinks (CD25 and IL-10/TGFbeta1).

Fig. 7B SynNotch induced suppressive payloads are produced at high levels comparable to activated FoxP3+ regulatory T cells.

Fig. 7C Human CD4+ T cells engineered to express CD25 leads to increase consumption of IL2 (measured by ELISA) and increased expansion (measured by flow cytometry) in vitro compared to an untransduced T cell control.

Fig. 7D Synthetic suppressor cells with synNotch circuits that produce a combination of TGFβ1 and CD25 are more potent at suppression of CAR T cell expansion in vitro compared to each individual payload.

Figs. 8A-8C show that synthetic suppressor cells locally block inflammation without systemic immune suppression in vivo.

Fig. 8A: Synthetic suppressor cells can act block off-target CAR T cell toxicity without systemic immune suppression. Two tumor model (K562 cells) with one dual antigen tumor (Her2+ CD19+) and one single antigen tumor (Her2+) is used to test antigen-specific suppression of CAR T cells by synthetic suppressor cells (T cells injected i.v.). Human anti-Her2 CAR T cells can kill both tumors, while human synthetic suppressor cells expressing an anti-CD19 SynNotch will only induce SynNotch in the dual antigen tumor.

Fig. 8B Synthetic suppressors with anti-CD19 SynNotch circuits producing both CD25 and TGFβ1 are effective at suppressing anti-Her2 CAR T cell killing of the dual antigen tumor, but circuits producing either individual payloads was not sufficient for suppression.

Fig. 8C Flow analysis of isolated tumors shows reduced expansion of CAR T cells in dual antigen tumor.

Fig. 9 shows data obtained from replicates with different T cell donors.

Fig. 10 shows the present system is an improvement over other CAR NOT gates in vivo.

Fig. 11 shows some of the general principles of how tumor recognition can be enhanced using a two cell NOT gate.

Fig. 12 shows that suppressor T cells can protect bystander cells under immune attack.

Fig. 13 shows that suppressor T cells are not self-inactivating when active because synNotch bypasses native TCR signaling requirements.

Fig. 14 shows suppressor T cells can be programmed to induce non-native suppression
5 programs.

DEFINITIONS

As used herein, the terms "treatment," "treating," "treat" and the like, refer to obtaining a desired pharmacologic and/or physiologic effect and/or a response related to the treatment. The
10 effect can be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or can be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment," as used herein, covers any treatment of a disease in a mammal, particularly in a human, and includes: (a) preventing the disease from occurring in a subject which can be predisposed to the disease but has not yet been diagnosed as
15 having it; (b) inhibiting the disease, i.e., arresting its development; and (c) relieving the disease, i.e., causing regression of the disease.

A "therapeutically effective amount" or "efficacious amount" refers to the amount of an agent (including biologic agents, such as cells), or combined amounts of two agents, that, when administered to a mammal or other subject for treating a disease, is sufficient to effect such
20 treatment for the disease. The "therapeutically effective amount" will vary depending on the agent(s), the disease and its severity and the age, weight, etc., of the subject to be treated.

The terms "individual," "subject," "host," and "patient," used interchangeably herein, refer to a mammal, including, but not limited to, murines (e.g., rats, mice), non-human primates, humans, canines, felines, ungulates (e.g., equines, bovines, ovines, porcines, caprines),
25 lagomorphs, etc. In some cases, the individual is a human. In some cases, the individual is a non-human primate. In some cases, the individual is a rodent, e.g., a rat or a mouse. In some cases, the individual is a lagomorph, e.g., a rabbit.

As used herein, the term "binding-triggered transcriptional switch" or "BTTS" refers to any polypeptide or complex of the same that is capable of transducing a specific binding event
30 on the outside of the cell (e.g., binding of an extracellular domain of the BTTS) to activation of a recombinant promoter within the nucleus of the cell. Many BTTSs work by releasing a

transcription factor that activates the promoter. In these embodiments, the BTTS is made up of one or more polypeptides that undergo proteolytic cleavage upon binding to the antigen to release a gene expression regulator that activates the recombinant promoter. For example, a BTTS may comprise (i) an extracellular domain comprising the antigen binding region of an antigen-specific antibody; (ii) a proteolytically cleavable sequence comprising one or more proteolytic cleavage sites; and (iii) an intracellular domain, wherein binding of the antigen binding region to the antigen induces cleavage of the sequence at the one or more proteolytic cleavage sites, thereby releasing the intracellular domain and wherein the intracellular domain activates transcription of an expression cassette. A BTTS can be based on synNotch, A2, MESA, or force receptor, for example, although others are known or could be constructed. In some embodiments, a SNIPR (Zhu et al 2022 Cell. 185: 1431-1443 and WO2021061856) may be used.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. In some embodiments, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains, which enables the sFv to form the desired structure for antigen binding. For a review of sFv, see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "nanobody" (Nb), as used herein, refers to the smallest antigen binding fragment or single variable domain (V_{HH}) derived from naturally occurring heavy chain antibody and is known to the person skilled in the art. They are derived from heavy chain only antibodies, seen in camelids (Hamers-Casterman et al. (1993) *Nature* 363:446; Desmyter et al. (2015) *Curr. Opin. Struct. Biol.* 32:1). In the family of "camelids" immunoglobulins devoid of light polypeptide chains are found. "Camelids" comprise old world camelids (*Camelus bactrianus* and *Camelus dromedarius*) and new world camelids (for example, *Llama paccos*, *Llama glama*, *Llama guanicoe* and *Llama vicugna*). A single variable domain heavy chain antibody is referred to herein as a nanobody or a V_{HH} antibody.

The terms "synthetic", "chimeric" and "engineered" as used herein generally refer to artificially derived polypeptides or polypeptide encoding nucleic acids that are not naturally occurring. Synthetic polypeptides and/or nucleic acids may be assembled de novo from basic subunits including, e.g., single amino acids, single nucleotides, etc., or may be derived from pre-

existing polypeptides or polynucleotides, whether naturally or artificially derived, e.g., as through recombinant methods. Chimeric and engineered polypeptides or polypeptide encoding nucleic acids will generally be constructed by the combination, joining or fusing of two or more different polypeptides or polypeptide encoding nucleic acids or polypeptide domains or polypeptide domain encoding nucleic acids. Chimeric and engineered polypeptides or polypeptide encoding nucleic acids include where two or more polypeptide or nucleic acid “parts” that are joined are derived from different proteins (or nucleic acids that encode different proteins) as well as where the joined parts include different regions of the same protein (or nucleic acid encoding a protein) but the parts are joined in a way that does not occur naturally.

The term “recombinant”, as used herein describes a nucleic acid molecule, e.g., a polynucleotide of genomic, cDNA, viral, semisynthetic, and/or synthetic origin, which, by virtue of its origin or manipulation, is not associated with all or a portion of the polynucleotide sequences with which it is associated in nature. The term recombinant as used with respect to a protein or polypeptide means a polypeptide produced by expression from a recombinant polynucleotide. The term recombinant as used with respect to a host cell or a virus means a host cell or virus into which a recombinant polynucleotide has been introduced. Recombinant is also used herein to refer to, with reference to material (e.g., a cell, a nucleic acid, a protein, or a vector) that the material has been modified by the introduction of a heterologous material (e.g., a cell, a nucleic acid, a protein, or a vector).

The term “operably linked” refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For instance, a promoter is operably linked to a coding sequence if the promoter affects its transcription or expression. Operably linked nucleic acid sequences may but need not necessarily be adjacent. For example, in some instances a coding sequence operably linked to a promoter may be adjacent to the promoter. In some instances, a coding sequence operably linked to a promoter may be separated by one or more intervening sequences, including coding and non-coding sequences. Also, in some instances, more than two sequences may be operably linked including but not limited to e.g., where two or more coding sequences are operably linked to a single promoter.

The terms “polynucleotide” and “nucleic acid,” used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides.

Thus, this term includes, but is not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases.

5 The terms “polypeptide,” “peptide,” and “protein”, used interchangeably herein, refer to a polymeric form of amino acids of any length, which can include genetically coded and non-genetically coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones. The term includes fusion proteins, including, but not limited to, fusion proteins with a heterologous amino acid sequence, fusions
10 with heterologous and homologous leader sequences, with or without N-terminal methionine residues; immunologically tagged proteins; and the like.

A “vector” or “expression vector” is a replicon, such as plasmid, phage, virus, or cosmid, to which another DNA segment, i.e. an “insert”, may be attached so as to bring about the replication of the attached segment in a cell.

15 The term “heterologous”, as used herein, means a nucleotide or polypeptide sequence that is not found in the native (e.g., naturally-occurring) nucleic acid or protein, respectively. Heterologous nucleic acids or polypeptide may be derived from a different species as the organism or cell within which the nucleic acid or polypeptide is present or is expressed. Accordingly, a heterologous nucleic acids or polypeptide is generally of unlike evolutionary
20 origin as compared to the cell or organism in which it resides.

The term “cancer-associated” refers to an antigen that is expressed in cancerous cells but not significantly non-cancerous cells of the same type. Some cancer-associated antigens are expressed on cancer cells and in normal tissues. For example, MSLN is considered a cancer-associated antigen since it is aberrantly expressed various cancer cells (e.g., lung cancers
25 (adenocarcinoma and squamous carcinoma), ovary, peritoneum, endometrium, pancreas, stomach and colon, etc.) but it is also expressed on normal mesothelial cells in the pleura, pericardium, and peritoneum and in epithelial cells on the surface of the ovary, tunica vaginalis, rete testis, and fallopian tubes in trace amounts.

The term “activates expression of”, in the context of activating the expression of a
30 nucleic acid or protein, refers to activating the expression of the protein encoded by a nucleic

acid. As would be understood, “activates expression of” includes transcription of the coding sequence to produce mRNA and translation of the mRNA to produce protein.

Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular
5 embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

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

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 also be used in the practice or testing of the present invention, the preferred methods and
20 materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for
25 example, reference to “a cell” includes a plurality of such cells and reference to “the cell” includes reference to one or more cells and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements,
30 or use of a “negative” limitation.

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

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DETAILED DESCRIPTION

The present disclosure provides a two cell “NOT” gate therapeutic that dampens the ability of engineered cytotoxic immune cells to kill off-target cells. This principle is illustrated in Fig. 11.

The cell therapy may comprise: (i) a cytotoxic immune cell (e.g., a CD8⁺ T cell or an NK cell) comprising an engineered immune receptor (e.g., engineered T cell receptor (TCR) or chimeric antigen receptor (CAR)) that recognizes an antigen on a target cell and (ii) a immunosuppressive cell (e.g., a CD4⁺ T cell) comprising a molecular circuit comprising: (a) a binding-triggered transcriptional switch (BTTS) that recognizes an antigen on a non-target cell and one or any combination of: (b) a nucleic acid encoding a pro-inflammatory cytokine sink (e.g., CD25, at least the extracellular domain of IL-1R, IL-12R/CD25, IL-18R, TNFR1, TNFR2, IFNGR, GM-CSFR, a domain thereof that binds to its cognate ligand, or an antibody that is tethered to the cell and binds to a pro-inflammatory cytokine), (c) a nucleic acid encoding an anti-inflammatory cytokine (e.g., IL-1ra, IL-4, IL-10, IL-11, IL-13, IL-35, and TGF- β , or a variant thereof), (d) a nucleic acid encoding an immune inhibitory receptor, or ligand thereof

(e.g., PD1, CTLA4, BTLA, CD160, KIRLG-1, 2B4, Lag-3, Tim-3, or TIGIT, etc.); and (e) a nucleic acid encoding an ectonucleotidase (CD39 or CD73). In these embodiments, binding of the engineered immune receptor to the antigen on the target cell in the absence of the immunosuppressive cell activates the cytotoxic immune cell; and binding of the BTTS to the antigen on the surface of a non-target cell activates expression of one or any combination of (b)-(e) by the immunosuppressive cell and protects the non-target cell from the cytotoxic immune cell.

In any embodiment, the immunosuppressive immune cell may be a T cell, a B cell, a macrophage, or a neutrophil. For example, in some embodiments, the immunosuppressive immune cell may be a CD4⁺ T cell.

In any embodiment, the engineered immune receptor may recognize a cancer antigen and the BTTS may recognize tissue and/or organ specific antigen that is at a different site to the antigen. For example, if the cancer antigen is expressed in brain tumor and in the liver, then the BTTS may recognize a liver-specific antigen.

In this circuit, binding of the BTTS to an antigen on a non-target cell activates expression of one or any combination of (b)-(e) by the immunosuppressive cell. Specifically, binding of the BTTS to a marker on the surface of a target cell may activate expression of (a), (b), (c), (d), (e), (a) and (b), (a) and (c), (a) and (d), (a) and (e), (b) and (c), (b) and (d), (b) and (e), (c) and (d), (c) and (e) or (d) and (e), etc., by the immunosuppressive cell. For example, binding of the BTTS to a marker on the surface of a target cell activates expression of the anti-inflammatory cytokine of (b) and/or the pro-inflammatory cytokine sink of (c) by the engineered immune cell. In any embodiment, the circuit may comprise components (a) and (b), (a) and (c) or (a), (b) and (c). In some embodiments, binding of the BTTS to the antigen on the surface of a non-target cell activates expression of TGFβ1, CD25 and, optionally, IL-10.

Binding-triggered transcriptional switches (BTTSs)

The BTTS is a cleavable fusion protein that contains: (a) an extracellular binding domain comprising a protein binding domain (e.g., scFv or nanobody) that binds to a cell surface marker on a cell, (b) an optional force sensing region, (c) a transmembrane domain, (d) one or more force-dependent cleavage sites that are cleaved, e.g., when the force sensing region is activated, and (e) an intracellular domain comprising a transcriptional activator, where binding of the binding domain to the marker on the surface of the other cell induces proteolytic cleavage of the

one or more force-dependent cleavage sites to release the transcriptional activator.

In this switch, the fusion protein is cleaved to release the intracellular domain when the extracellular domain of the fusion protein engages with a marker on another cell. As such, in many cases, the fusion protein may contain a force sensing region (which is typically in the extracellular domain) and one or more force-dependent cleavage sites that are cleaved, e.g.,
5 when the force sensing region is activated. The position of the force-dependent cleavage sites may vary and, in some embodiments the fusion protein may contain at least two cleavage sites. In some cases, one of the cleavage sites may be extracellular and the other may be in the transmembrane domain or within 10 amino acids of the transmembrane domain in the intracellular domain. In any embodiment, the force sensing region and/or the one or more force-
10 dependent cleavage sites may be from a Delta/Serrate/Lag2 (DSL) superfamily protein, as reviewed by Pintar et al (Biology Direct 2007 2: 1-13). For example, the force sensing region and/or the one or more force-dependent cleavage sites may be from Notch (see Morsut Cell. 2016 164: 780-91), von Willebrand Factor (vWF), amyloid-beta, CD16, CD44, Delta, a cadherin, an ephrin-type receptor or ephrin ligand, a protocadherin, a filamin, a synthetic E
15 cadherin, interleukin-1 receptor type 2 (IL1R2), major prion protein (PrP), a neuregulin or an adhesion-GPCR. Several other examples of this type of protein are known and listed in Pintar, supra. Many members of this family appear to share a similar architecture of a region that unfolds and opens up a protease cleavage site (e.g., EGF-like repeats; see Cordle et al Nat. Struct. Mol. Biol. 2008 15: 849–857), a trans-membrane segment, and a relatively short (~100–
20 150 amino acids) intracellular domain. These sequences permit the binding-triggered release of a transcriptional activator from the membrane in their natural environment and can be readily adapted herein.

In some cases, the one or more ligand-inducible proteolytic cleavage sites are selected from S1, S2, and S3 proteolytic cleavage sites. In some cases, the S1 proteolytic cleavage site is
25 a furin-like protease cleavage site comprising the amino acid sequence Arg-X-(Arg/Lys)-Arg, where X is any amino acid. In some cases, the S2 proteolytic cleavage site is an ADAM-17-type protease cleavage site comprising an Ala-Val dipeptide sequence. In some cases, the S3 proteolytic cleavage site is a γ -secretase cleavage site comprising a Gly-Val dipeptide sequence. The S3 proteolytic cleavage site is in the transmembrane domain. In many cases, the shear force
30 generated by binding of the extracellular domain of this fusion protein to another cells unfolds

the force sensing region (which, in the case of Notch contains EGF-like repeats whereas in other proteins is made up of other sequences such as the A2 domain in vWF (see, e.g., J Thromb Haemost. 2009 7:2096-105, Lippok Biophys J. 2016 110: 545-54, Lynch Blood. 2014 123: 2585-92, Crawley, Blood. 2011 118:3212-21 and Xy J Biol Chem. 2013 288:6317-24) or modified A2 domain that has, e.g., the R1597W, E1638K and I1628T substitutions. The architecture of such proteins is described in, e.g., Morsut et al, Cell. 2016 164: 780-91, WO2016138034 and WO2019099689, among other places).

In some cases, the fusion protein includes an S1 ligand-inducible proteolytic cleavage site. An S1 ligand-inducible proteolytic cleavage site can be located between the HD-N segment and the HD-C segment. In some cases, the S1 ligand-inducible proteolytic cleavage site is a furin-like protease cleavage site. A furin-like protease cleavage site can have the canonical sequence Arg-X-(Arg/Lys)-Arg, where X is any amino acid; the protease cleaves immediately C-terminal to the canonical sequence. For example, in some cases, an amino acid sequence comprising an S1 ligand-inducible proteolytic cleavage site can have the amino acid sequence GRRRRELDPM (SEQ ID NO:1), where cleavage occurs between the "RE" sequence. As another example, an amino acid sequence comprising an S1 ligand-inducible proteolytic cleavage site can have the amino acid sequence RQRRELDPM (SEQ ID NO:2), where cleavage occurs between the "RE" sequence.

In some cases, the fusion protein polypeptide includes an S2 ligand-inducible proteolytic cleavage site. An S2 ligand-inducible proteolytic cleavage site can be located within the HD-C segment. In some cases, the S2 ligand-inducible proteolytic cleavage site is an ADAM-17-type protease cleavage site. An ADAM-17-type protease cleavage site can comprise an Ala-Val dipeptide sequence, where the enzyme cleaves between the Ala and the Val. For example, in some cases, amino acid sequence comprising an S2 ligand-inducible proteolytic cleavage site can have the amino acid sequence KIEAVKSE (SEQ ID NO:3), where cleavage occurs between the "AV" sequence. As another example, an amino acid sequence comprising an S2 ligand-inducible proteolytic cleavage site can have the amino acid sequence KIEAVQSE (SEQ ID NO:4), where cleavage occurs between the "AV" sequence.

In some cases, the fusion protein includes an S3 ligand-inducible proteolytic cleavage site. An S3 ligand-inducible proteolytic cleavage site can be located within the TM domain. In some cases, the S3 ligand-inducible proteolytic cleavage site is a gamma-secretase (γ -secretase)

cleavage site. A γ -secretase cleavage site can comprise a Gly-Val dipeptide sequence, where the enzyme cleaves between the Gly and the Val. For example, in some cases, an S3 ligand-inducible proteolytic cleavage site has the amino acid sequence VGCGVLLS (SEQ ID NO:5), where cleavage occurs between the “GV” sequence. In some cases, an S3 ligand-inducible proteolytic cleavage site comprises the amino acid sequence GCGVLLS (SEQ ID NO:6).

In some cases, the fusion protein polypeptide lacks an S1 ligand-inducible proteolytic cleavage site. In some cases, the BTTS lacks an S2 ligand-inducible proteolytic cleavage site. In some cases, the BTTS lacks an S3 ligand-inducible proteolytic cleavage site. In some cases, the BTTS lacks both an S1 ligand-inducible proteolytic cleavage site and an S2 ligand-inducible proteolytic cleavage site. In some cases, the BTTS includes an S3 ligand-inducible proteolytic cleavage site; and lacks both an S1 ligand-inducible proteolytic cleavage site and an S2 ligand-inducible proteolytic cleavage site.

In some embodiments, the fusion protein may have an vWF A2 sequence or a variation thereof, an ADAMTS13 cleavage site (which may be described by the consensus sequence HEXXHXXGXXHD; SEQ ID NO:7; Crawley, Blood. 2011 118:3212-21), and an S3 or γ -secretase cleavage site, although many other arrangements exist. In some embodiments, the switch may contain components that are borrowed from Notch. In other embodiments, the switch may not contain components that are from Notch.

For simplicity, BTTSs, including but not limited to chimeric notch receptor polypeptides, are primarily single polypeptide chains. However, BTTSs, including chimeric notch receptor polypeptides, may be divided or split across two or more separate polypeptide chains where the joining of the two or more polypeptide chains to form a functional BTTS, e.g., a chimeric notch receptor polypeptide, may be constitutive or conditionally controlled. For example, constitutive joining of two portions of a split BTTS may be achieved by inserting a constitutive heterodimerization domain between the first and second portions of the split polypeptide such that upon heterodimerization the split portions are functionally joined.

Useful BTTSs that may be employed in the subject methods include, but are not limited to, modular extracellular sensor architecture (MESA) polypeptides. A MESA polypeptide comprises: a) a ligand binding domain; b) a transmembrane domain; c) a protease cleavage site; and d) a functional domain. The functional domain can be a transcription regulator (e.g., a transcription activator, a transcription repressor). In some cases, a MESA receptor comprises

two polypeptide chains. In some cases, a MESA receptor comprises a single polypeptide chain. Non-limiting examples of MESA polypeptides are described in, e.g., U.S. Patent Publication No. 2014/0234851; the disclosure of which is incorporated herein by reference in its entirety.

Useful BTTSs that may be employed in the subject methods include, but are not limited to, polypeptides employed in the TANGO assay. The subject TANGO assay employs a TANGO polypeptide that is a heterodimer in which a first polypeptide comprises a tobacco etch virus (Tev) protease and a second polypeptide comprises a Tev proteolytic cleavage site (PCS) fused to a transcription factor. When the two polypeptides are in proximity to one another, which proximity is mediated by a native protein-protein interaction, Tev cleaves the PCS to release the transcription factor. Non-limiting examples of TANGO polypeptides are described in, e.g., Barnea et al. (Proc Natl Acad Sci USA. 2008 Jan. 8; 105(1):64-9); the disclosure of which is incorporated herein by reference in its entirety.

Useful BTTSs that may be employed in the subject methods include, but are not limited to von Willebrand Factor (vWF) cleavage domain-based BTTSs, such as but not limited to e.g., those containing a unmodified or modified vWF A2 domain. A subject vWF cleavage domain-based BTTS will generally include: an extracellular domain comprising a first member of a binding pair; a von Willebrand Factor (vWF) cleavage domain comprising a proteolytic cleavage site; a cleavable transmembrane domain and an intracellular domain. Non-limiting examples of vWF cleavage domains and vWF cleavage domain-based BTTSs are described in Langridge & Struhl (Cell (2017) 171(6):1383-1396); the disclosure of which is incorporated herein by reference in its entirety.

Useful BTTSs that may be employed in the subject methods include, but are not limited to chimeric Notch receptor polypeptides, such as but not limited to e.g., synNotch polypeptides, non-limiting examples of which are described in PCT Pub. No. WO 2016/138034, U.S. Patent No. 9,670,281, U.S. Patent No.9,834,608, Roybal et al. Cell (2016) 167(2):419-432, Roybal et al. Cell (2016) 164(4):770-9, and Morsut et al. Cell (2016) 164(4):780-91; the disclosures of which are incorporated herein by reference in their entirety. The "SNIPR" switch is another example of a BTTS (Zhu et al 2022 Cell. 185: 1431-1443 and WO2021061856), although others exist and/or can be readily designed.

Expression of the BTTS in the cell may be constitutive or inducible, e.g., by binding of another BTTS to an antigen on another cell in the patient.

Examples of transcriptional activators that can be part of the fusion protein are numerous and include artificial transcription factors (ATFs) such as, e.g., Zinc-finger-based artificial transcription factors (including e.g., those described in Sera T. Adv Drug Deliv Rev. 2009 61(7-8):513-26; Collins et al. Curr Opin Biotechnol. 2003 14(4):371-8; Onori et al. BMC Mol Biol. 2013 14:3. In some cases, the transcriptional activator may contain a GAL4 DNA binding domain, which binds to the Gal4 responsive UAS, which has been well characterized in the art. Examples of suitable transcriptional activators include GAL4-VP16 and GAL4-VP64, although many others could be used. As would be appreciated, the identity of the transcription activators may vary. In some embodiments, the transcription factor may have a DNA binding domain that binds to a corresponding promoter sequence and an activation domain. In many embodiments, the DNA binding domain transcription factor may be independently selected from Gal4-, LexA-, Tet-, Lac-, dCas9-, zinc-finger- and TALE-based transcription factors. TALE- and CRISPR/dCas9-based transcription factors are described in Lebar (Methods Mol Biol. 2018 1772: 191-203), among others. The binding sites for such domains are well known or can be designed at will. The transcription factors can have any suitable activation domain, e.g., VP16, VP64, Ela, Sp1, VP16, CTF, GAL4 among many others.

Extracellular binding domains

The extracellular binding domain of the BTTS may bind to a tissue- or organ-specific cell-surface marker, a disease-specific cell-surface marker, or an off-target cell-surface marker, depending on how the cell is being used. For example, if one wanted to dampen the effects of the cytotoxic cells in the brain and/or spinal cord then the BTTS may have an extracellular domain that binds to a brain and/or CNS-specific cell-surface marker (e.g., MOG, CDH10, BCAN, CSPG5, PTPRZ1 or NRCAM) which are both preferentially expressed in the brain). Likewise if one wanted to dampen the effects of the cytotoxic cells in the pancreas, then the BTTS may have an extracellular domain that binds to a pancreatic cell surface marker (e.g., GP2, CD133, ion transport regulator 2 (FXRD2), tetraspanin 7 (TSPAN7), transmembrane protein 27 (TMEM27), discoidin domain receptor tyrosine kinase 1 (DDR1) and delta/notch-like EGF repeat containing (DNER), dispatched homologue 2 (DISP2), seizure related 6 homologue like (SEZ6L2), low density lipoprotein receptor-related protein 11 (LRP11), HEPACAM family member 2 (HEPACAM2), TSPAN7 and TMEM27, etc.) Tissue-specific cell-surface markers are available for the eye, retina, heart, skeletal muscle, smooth muscle,

adrenal gland, parathyroid gland, thyroid gland, pituitary gland, lung, bone marrow, lymphoid tissue, liver, gallbladder, testis, epididymis, prostate, seminal vesicle, ductus deferens, adipose tissue, brain, salivary gland, esophagus, tongue, stomach, intestine, pancreas, kidney, urinary bladder, breast, vagina, cervix, endometrium, fallopian tube, ovary, placenta, skin, blood, etc. In another example, the extracellular binding domain may bind to a tissue or organ-specific cell surface marker in a transplanted organ (e.g., pancreas, liver, lung, or kidneys, etc.), thereby protecting it from attack from killer T cells. In any embodiment, the BTTS may bind to CD19, for example.

If one wanted to dampen "off-target" immune responses (which may occur when cell therapies, e.g., CAR T therapies, attack sites that are off target) the extracellular binding domain may bind to a marker in the off-target sites. As would be apparent, this marker may vary depending on the therapy being used. However, many off-target markers are listed as "NOT" antigens in Dannenfelser (Cell Syst. 2020 11: 215-228) WO 2017/193059, WO 2020/097395 and PCT/US2021/045796).

Anti-inflammatory cytokines

If the protein induced by the BTTS binding to its antigen is an anti-inflammatory cytokine, the cytokine will be secreted from the cell. In these embodiments, the circuit may comprise a nucleic acid containing a promoter that is activated by the released transcriptional activator, and a coding sequence encoding an anti-inflammatory cytokine. In this disclosure, the term "anti-inflammatory cytokine" is intended to encompass natural molecules that have anti-inflammatory activity (e.g., IL-1ra, IL-4, IL-10, IL-11, IL-13, IL-35 and TGF- β), as well as non-natural or "engineered" cytokines that have anti-inflammatory activity. As would be appreciated, cytokines are secreted from the cell and their coding sequence will encode a secretion signal. IL10 variants are described in Saxton et al (Science 2021 371: 6535); TGF β mimics are described in Johnston et al (Science Immun. 2020 5: 50); IL35 variants are described in Collison et al (Science 2021 371: 6535); and CD25-biased IL2 variants are described in Khoryati et al (Science Imm. 2020 5: 50), which publications are incorporated by reference for disclosure of the sequences.

Pro-inflammatory cytokine sinks

The term "pro-inflammatory cytokine sink" is intended to refer to a protein that specifically binds to a pro-inflammatory cytokine (e.g., IL-2, CCL-21, IL-12, IL-7, IL-15 or IL-

21, etc.) and prevents it from binding with its cognate receptor on another immune cell. In some embodiments, the cytokine sink comprises at least the extracellular domain of a receptor for a pro-inflammatory cytokine, e.g., at least the extracellular domain of IL-1R, IL-2R/CD25, IL-12R, IL-18R, TNFR1, TNFR2, IFNGR, GM-CSFR, etc., or a part thereof that binds to its cognate ligand. For example, the cytokine sink may have the extracellular domain of IL-1R (which binds to IL-1), IL-2R or CD25 (which binds to IL-2), IL-12R, IL-18R (which binds to IL-18), TNFR1 and TNFR2 (which binds to TNF- α), IFNGR (which binds to IFN γ) and GM-CSFR (which binds to GM-CSF), or a subunit thereof that binds to its ligand. This domain may be tethered to the cell via a transmembrane domain or it may be secreted. If the domain is tethered to the cell then, in some embodiments, a truncated or mutated form of the receptor may be used so that the receptor is incapable of signaling. In other embodiments, the full-length receptor may be used. In these embodiments, the cell may not have the internal machinery to transduce a signal from that receptor to the nucleus. In one embodiment, sink may contain the extracellular domain of CD25 (which is the receptor for IL-2), although others could be used too. In any embodiment, the cell may express CD25, which not only acts as a cytokine sink but it also causes the engineered CD4⁺ T cell to proliferate when it bind to its ligand. Stimulation of CD25 should survival/persistence of the T cells in the host.

In alternative embodiments, an antibody (e.g., a scFv) that binds to the pro-inflammatory cytokine may be used. In these embodiments, the antibody may be tethered to the cell, e.g., via a transmembrane domain, or secreted.

In these embodiments, the circuit may comprise a nucleic acid containing a promoter that is activated by the released transcriptional activator, and a coding sequence encoding a pro-inflammatory cytokine sink.

Ectonucleotidase

Ectonucleotidases are nucleotide metabolizing enzymes that are expressed on the plasma membrane and have externally oriented active sites. These enzymes metabolize nucleotides to nucleosides. Extracellular adenosine generated by the ectonucleotidases CD39 and CD73 is a newly recognized “immune checkpoint mediator” that is believed to interfere with anti-tumor immune responses. Expressing an ectonucleotidase such as CD39 or CD73 on a cell should dampen the immune response around that cell. In these embodiments, the circuit may comprise

a nucleic acid containing a promoter that is activated by the released transcriptional activator, and a coding sequence encoding a ectonucleotidase .

Combinations

As noted above, in some embodiments expression of two or more of (a)-(e) may be induced by binding of the BTTS to the cell surface marker. In these embodiments, the different proteins may be on different constructs with the same promoter or their expression may be coordinated by an IRES. Other ways for co-expressing two proteins are known. The two or more of (a)-(e) may be on the same vector or different vectors.

Circuits

As noted above, binding of BTTS to the non-target cell activates expression of one or more other proteins. In these embodiments, binding of the binding domain of the BTTS to the antigen on the surface of another cell induces proteolytic cleavage of the one or more force-dependent cleavage sites to release the transcriptional activator. The released transcriptional activator then binds to a promoter that drives the expression of the one or more other proteins, thereby inducing expression of the one or more other proteins. The general principles of a circuit are described in WO 2016/138034, U.S. Patent No. 9,670,281, U.S. Patent No.9,834,608, Roybal et al. Cell (2016) 167(2):419-432, Roybal et al. Cell (2016) 164(4):770-9, and Morsut et al. Cell (2016) 164(4):780-91, among others.

Immune receptors

As noted above, the present circuits make use of an engineered immune receptor that recognizes an antigen on a target cell, where binding of the engineered immune receptor to the antigen on the target cell (in the absence of the immunosuppressive cell) activates the cytotoxic immune cell and induces the cytotoxic immune cell to kill the target cell. CARs and TCRs are examples of such immune receptors, although others are known.

The terms “chimeric antigen receptor” and “CAR”, used interchangeably herein, refer to artificial multi-module molecules capable of triggering or inhibiting the activation of an immune cell which generally but not exclusively comprise an extracellular domain (e.g., a ligand/antigen binding domain), a transmembrane domain and one or more intracellular signaling domains. The term CAR is not limited specifically to CAR molecules but also includes CAR variants. CAR variants include split CARs wherein the extracellular portion (e.g., the ligand binding portion) and the intracellular portion (e.g., the intracellular signaling portion) of a CAR are

present on two separate molecules. CAR variants also include ON-switch CARs which are conditionally activatable CARs, e.g., comprising a split CAR wherein conditional heterodimerization of the two portions of the split CAR is pharmacologically controlled (e.g., as described in PCT publication no. WO 2014/127261 A1 and US Patent Application No.

5 2015/0368342 A1, the disclosures of which are incorporated herein by reference in their entirety). CAR variants also include bispecific CARs, which include a secondary CAR binding domain that can either amplify or inhibit the activity of a primary CAR. CAR variants also include inhibitory chimeric antigen receptors (iCARs) which may, e.g., be used as a component of a bispecific CAR system, where binding of a secondary CAR binding domain results in
10 inhibition of primary CAR activation. CAR molecules and derivatives thereof (i.e., CAR variants) are described, e.g., in PCT Application No. US2014/016527; Fedorov et al. Sci Transl Med (2013) ;5(215):215ra172; Glienke et al. Front Pharmacol (2015) 6:21; Kakarla & Gottschalk 52 Cancer J (2014) 20(2):151-5; Riddell et al. Cancer J (2014) 20(2):141-4; Pegram et al. Cancer J (2014) 20(2):127-33; Cheadle et al. Immunol Rev (2014) 257(1):91-106; Barrett
15 et al. Annu Rev Med (2014) 65:333-47; Sadelain et al. Cancer Discov (2013) 3(4):388-98; Cartellieri et al., J Biomed Biotechnol (2010) 956304; the disclosures of which are incorporated herein by reference in their entirety. Useful CARs also include the anti-CD19—4-1BB—CD3 ζ CAR expressed by lentivirus loaded CTL019 (Tisagenlecleucel-T) CAR-T cells as commercialized by Novartis (Basel, Switzerland).

20 The terms “T cell receptor” and “TCR” are used interchangeably and will generally refer to a molecule found on the surface of T cells, or T lymphocytes, that is responsible for recognizing fragments of antigen as peptides bound to major histocompatibility complex (MHC) molecules. The TCR complex is a disulfide-linked membrane-anchored heterodimeric protein normally consisting of the highly variable alpha (α) and beta (β) chains expressed as part of a
25 complex with CD3 chain molecules. Many native TCRs exist in heterodimeric $\alpha\beta$ or $\gamma\delta$ forms. The complete endogenous TCR complex in heterodimeric $\alpha\beta$ form includes eight chains, namely an alpha chain (referred to herein as TCR α or TCR alpha), beta chain (referred to herein as TCR β or TCR beta), delta chain, gamma chain, two epsilon chains and two zeta chains. In some instance, a TCR is generally referred to by reference to only the TCR α and TCR β chains,
30 however, as the assembled TCR complex may associate with endogenous delta, gamma, epsilon and/or zeta chains an ordinary skilled artisan will readily understand that reference to a TCR as

present in a cell membrane may include reference to the fully or partially assembled TCR complex as appropriate.

Recombinant or engineered individual TCR chains and TCR complexes have been developed. References to the use of a TCR in a therapeutic context may refer to individual recombinant TCR chains. As such, engineered TCRs may include individual modified TCR α or modified TCR β chains as well as single chain TCRs that include modified and/or unmodified TCR α and TCR β chains that are joined into a single polypeptide by way of a linking polypeptide.

As noted above, the immune receptor may be bind to a cancer-associated antigen. In some embodiments, the antigen may be associated with hematological cancers (e.g., CD19, CD20, CD22, CD25, CD30 or CD33) or a solid tumor. Examples of cancer-associated antigens that are in solid tumors are listed in the table below.

Mesothelin	Trop2 (TACSTD2)	KREMEN2
FAP	MUC1	GRIN2D
EGFRvIII	ROR1	CELSR3
IL13RA2	Claudin18.2	KISS1R
EPHA2	GPC3	CDH6
PSMA (FOLH1)	CA9	TREM1
HER2	EPCAM	MC1R
EGFR	FOLR1	LIFR
PSCA	L1CAM	PRR7
ALPPL2	CD133 (PROM1)	PTPRN
GD2 (B4GALNT1)	CEA (CEACAM5)	CACNG7
BCAN	MUC16	TRPM1
MOG	PDL1 (CD274)	
CSPG5	ROR2	
CD70	VEGFR2 (KDR)	
MET	CD44v6	
AXL	NKG2D (KLRK1)	
MCAM	MLANA (MART1)	
DLL3, DLL4	NY-ESO	
nectin4	TYRP-1	
nectin2	AFP	
nectin3	MAGE family	

nectin1	WT-1	
ALK		

Methods of treatment

A method of treatment is described below. In general terms, this method may comprise administering a cell therapy described above to the subject. In some embodiments, primary
5 immune cells may be purified from an individual, constructs encoding the above proteins may be introduced into the cells *ex vivo*, and the recombinant cells may be expanded and administered to the subject, e.g., by injection. In other embodiments, pre-made allogeneic cells (which may have abrogated MHC class I molecules) may be used instead.

In some embodiments, the subject may have cancer. In these embodiments, the subject
10 may be receiving a course of cytotoxic immune cells (e.g., CAR T or NK cells) that kill cancer cells in an antigen-specific manner. In these embodiments, the BTTS can be targeted to off-target sites (i.e., normal tissue that is not cancerous), thereby providing a way to protect those sites. In these embodiments, the BTTS may be an extracellular binding domain that binds to cells that are not part of the cancer. Exemplary cell surface markers for off-target sites may be
15 listed as "NOT" antigens in Dannenfelser (Cell Syst. 2020 11: 215-228) WO 2017/193059, WO 2020/097395 and PCT/US2021/045796), as described above.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art
20 with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless
25 indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s);

nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); and the like.

In the following examples, synthetic suppressor cells have been shown to locally block CD8⁺ T cell-mediated killing of cancer cells highly effectively, in vitro and in vivo. Suppression of an autoinflammatory EAE (mouse MS model for CNS inflammation) has also been shown in vivo.

Example 1

synNotch induced production of suppressive cytokine TGFb

Engineered immune cells can produce immuno-suppressive payloads in response to a specific antigen. Human CD4⁺ T cells can selectively induce immune inhibitory cytokine TGFβ1 in response to CD19 antigen using SynNotch (measured by flow cytometry). The results shown in Fig. 1 show that expression of the suppressive cytokine TGFb can be induced by synNotch binding to CD19 on another cell.

Example 2

Suppressor cells that produce combination of TGFb (suppressive cytokine) and CD25 (IL2 sink) are very effective at suppressing CAR T killing in vitro.

T cells inducibly producing a combination of inhibitory cytokine TGFβ1 and pro-inflammatory cytokine sink CD25 using synNotch show strong suppression of CAR T cell proliferation and killing in vitro. In vitro immune suppression was assayed by co-culturing three cells: (1) human CD4⁺ T cells with anti-CD19 SynNotch inducing production of CD25, TGFβ1, or both payloads, (2) K562 target cells expressing both a synNotch antigen, CD19, and a CAR antigen, Her2, and (3) human CD8⁺ T cells expressing an anti-Her2 4-1BB CAR. Cell counts were tracked over time using flow cytometry. Human CD4⁺ T cells with synNotch inducing production of both payloads is most effective at suppressing CAR T cell activity, proliferation of the CAR T cells and killing of the K562 target cells. These experiments and results are illustrated in Figs. 2A and 2B.

Example 3

Suppressor cells that produce combination of IL10 (suppressive cytokine) and CD25 (IL2 sink) are very effective at suppressing CAR T killing in vitro.

T cells inducibly producing a combination of inhibitory cytokine IL10 and pro-inflammatory cytokine sink CD25 using synNotch show strong suppression of CAR T cell proliferation and killing in vitro. In vitro immune suppression was assayed by co-culturing three cells: (1) human CD4+ T cells with anti-CD19 SynNotch inducing production of CD25, IL10, or both payloads, (2) K562 target cells expressing both a synNotch antigen, CD19, and a CAR antigen, Her2, and (3) human CD8+ T cells expressing an anti-Her2 4-1BB CAR. Cell counts were tracked over time using flow cytometry. Human CD4+ T cells with synNotch inducing production of both payloads is most effective at suppressing CAR T cell activity, proliferation of the CAR T cells and killing of the K562 target cells. These experiments and results are illustrated in Figs. 3A and 3B.

Example 4

Suppressor cells that produce combination of TGFb (suppressive cytokine) and CD25 (IL2 sink) are very effective at suppressing CAR T killing of tumors in vivo

Synthetic immune-suppressive cells can locally suppress immune response. K562 tumors, Her2+ and Her2+ CD19+, were subcutaneously injected in the flanks of N.S.G. mice. These mice were treated with either no T cells, anti-Her2 CAR T cells only, or anti-Her2 CAR T cells and synthetic suppressor cells (human CD4+ T cells with an anti-CD19 synNotch inducibly producing TGFβ1 and CD25) by i.v. injection after 7 days. Tumor volume was monitored by caliper measurement. Synthetic suppressor cells show strong local suppression of CAR T cell killing in the CD19+ tumor without suppressing CAR T cell killing of the CD19- tumor. These experiments and results are illustrated in Figs. 4A and 4B.

Example 5

Engineered T cells overexpressing CD25 increases consumption of IL2 and cell proliferation.

Human CD4+ T cells constitutively expressing CD25 show increased consumption of IL2 (measured by ELISA) and increased proliferation (measured by flow cytometry) in vitro

when exogenous IL2 is added to the media. These experiments and results are illustrated in Fig. 5.

Example 6

5 **synNotch→IL10 synthetic suppressor cells can block autoimmune cell proliferation in brain and CNS in mouse neuroinflammation model.**

IL10 expression can be activated by mouse brain specific antigen (CDH10). These experiments and results are illustrated in Fig. 6.

10

Example 7

SynNotch circuits in CD4+ T cells can reconstitute Treg-like functions to drive immune suppression.

Human CD4+ T cells with anti-CD19 synNotch circuits inducing different immune
15 suppressive payloads (CD25, IL10, or TGFb) were mixed with CD19+ or CD19- K562 target cells. The level of CD25 on the engineered CD4+ T cells was measured by antibody staining after 72 hours of incubation with target cells. The level of TGFb or IL10 cytokine secretion was measured by ELISA of the supernatant after 72 hours. This data is shown in Fig. 7B.

Human CD4+ T cells were engineered to constitutively overexpress CD25. T cells were
20 grown in media that contained added IL2. Cell counts were measured by flow cytometry of in vitro cell culture and IL2 levels were measured by ELISA of supernatant. This data is shown in Fig. 7C.

Human CD8+ T cells were engineered with an anti-Her2 CAR (killer T cells). Human
CD4+ T cells (suppressor T cells) were engineered with anti-CD19 synNotch circuits that either
25 induces one or two payloads. These T cells were mixed with K562 target cells that are engineered to express both Her2 and CD19. Cell counts were measured by flow cytometry. This data is shown in Fig. 7D.

Together, this data shows that:

BTTS circuits in CD4⁺ T cells induce antigen-specific production of immune
30 suppressive payloads;

suppressor T cells inducing immune suppressive payloads block CAR T cell expansion and killing in vitro; and

suppressor T cells producing the combination of an inhibitory cytokine (TGFb or IL10) with CD25 leads to significantly stronger suppression of CAR T cell expansion and killing in vitro.

Example 8

Synthetic suppressor cells act locally to block inflammation without systemic immune suppression in vivo

Two K562 tumors (Her2+ or Her2+ CD19+) were injected subcutaneously into two flanks of NSG mice. A week after tumor injection, anti-Her2 CAR T cells only or anti-Her2 CAR T cells and anti-CD19 synthetic suppressor T cells inducing different suppressive payloads were injected i.v. Tumor volume was measured by calipers. This data is shown in Figs. 8A-8C.

Together, this data shows that:

present two cell system provides a robust two-cell NOT gate for CAR T cells in vivo; and

the present two cell NOT gate is capable of blocking T cells from killing locally (dual antigen tumor) without systemic suppression (single antigen tumor) in vivo.

In the experiments shown in Fig. 9, the experimental setup is as for Fig. 8. For iCAR NOT gate (Fig. 10), anti-Her2 CAR T cells were engineered to co-express a PD-1 based anti-CD19 iCAR (PMID: 24337479).

Fig. 9 shows replicates data using different T cell donors. The data is consistent between replicates.

Fig. 10 shows a comparison over an iCAR not gate. The two cell NOT gate has improved performance over the iCAR NOT gate.

Together, the data show in Figs. 9 and 10 demonstrates that:

two-cell NOT gate circuits are highly reproducible in vivo (Fig. 9); and

the dynamic range of two-cell NOT gate is better than other strategies in vivo: complete killing of single antigen tumor; complete protection of dual antigen was observed (Fig. 10).

Example 9

Suppressor T cells can protect bystander cells under immune attack

The data shown in Fig. 12 shows that suppressor T cells protect bystander target cells that are in the neighborhood of cells with synNotch priming ligand. In this experiment target
5 cells that have CD19 (synNotch priming ligand) were mixed with cells that do not, in vitro. The data shows that suppressor T cells can protect both priming cells and bystander cells from CAR T cell killing. This data shows that suppressor T cells can overcome heterogenous priming antigen expression.

Example 10

Suppressor T cells are not self-inactivating

Because the synNotch receptor is orthogonal to native TCR signaling, suppressor T cells do not self-inactivate when synNotch is active. Suppressor cells can continue to produce suppressive payloads like TGFb that inhibit TCR signaling during suppression. See Fig. 13.

15 This data shows that suppressor T cell induce suppressive responses that are not dependent on TCR signaling.

Example 11

Suppressor T cells can be programmed to induce non-native suppression programs

20 Suppressor T cells can produce a diverse set of suppressive signals and combinations, including non-native combinations like PD-L1 + CD25 which show synergistic suppression of CAR T cells. The data shown in Fig 14 shows that synthetic suppressor T cells can induce custom programs that are not in endogenous suppressor cells such as Tregs.

25 While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit
30 and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

CLAIMS

What is claimed is:

1. A cell therapy comprising:

(i) a cytotoxic immune cell comprising an engineered immune receptor that recognizes an antigen on a target cell; and

(ii) an immunosuppressive immune cell comprising a molecular circuit comprising:

(a) a binding-triggered transcriptional switch (BTTS) that recognizes an antigen on a non-target cell; and

one or any combination of:

(b) a nucleic acid encoding a pro-inflammatory cytokine sink,

(c) a nucleic acid encoding an anti-inflammatory cytokine;

(d) a nucleic acid encoding an immune inhibitory receptor, or ligand thereof; and

(e) a nucleic acid encoding an ectonucleotidase;

wherein:

binding of the engineered immune receptor to the antigen on the target cell in the absence of the immunosuppressive immune cell activates the cytotoxic immune cell; and

binding of the BTTS to the antigen on the surface of a non-target cell activates expression of the protein(s) encoded by one or any combination of (b)-(e) by the immunosuppressive immune cell and protects the non-target cell from the cytotoxic immune cell.

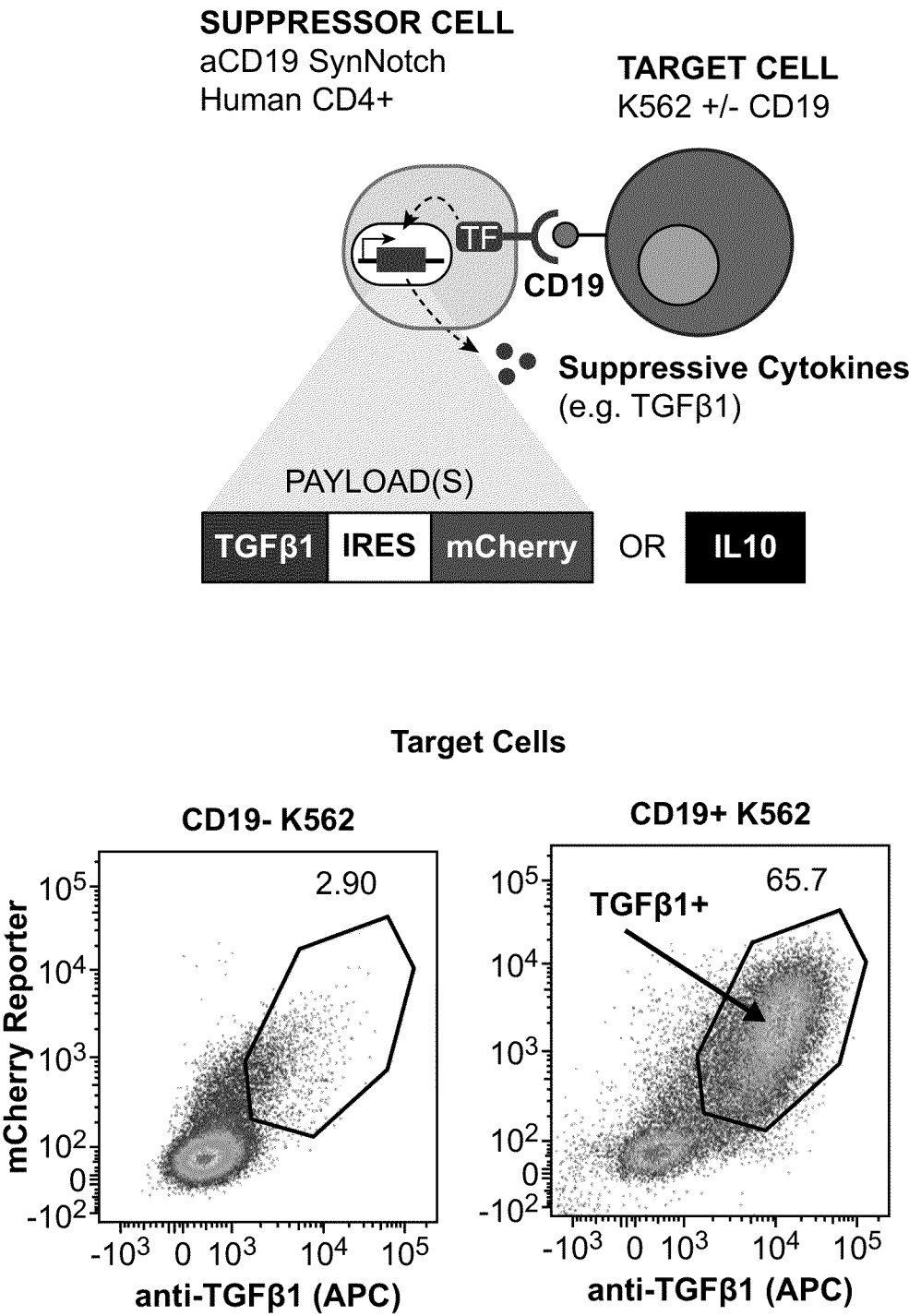
2. The cell therapy of claim 1, wherein the engineered immune receptor is an engineered T cell receptor (TCR) or chimeric antigen receptor (CAR).

3. The cell therapy of claim 1 or 2, wherein the cytotoxic immune cell is a CD8⁺ T cell or NK cell.

4. The cell therapy of any prior claim, wherein the immunosuppressive immune cell is a T cell, a B cell, a macrophage, or a neutrophil.

5. The cell therapy of any prior claim, wherein the immunosuppressive immune cell is a CD4⁺ T cell.
6. The cell therapy of any prior claim, wherein the engineered immune receptor recognizes a cancer antigen.
7. The cell therapy of any prior claim, wherein the antigen on the non-target cell is tissue and/or organ specific.
8. The cell therapy of any prior claim, wherein the anti-inflammatory cytokine is IL-1ra, IL-4, IL-10, IL-11, IL-13, IL-35, and TGF- β , or a variant thereof.
9. The cell therapy of any prior claim, wherein the cytokine sink comprises at least the extracellular domain of a receptor that binds to a pro-inflammatory cytokine.
10. The cell therapy of any prior claim, wherein the cytokine sink is CD25.
11. The cell therapy of any prior claim, wherein the cytokine sink comprises at least the extracellular domain of IL-1R, IL-12R/CD25, IL-18R, TNFR1, TNFR2, IFNGR, GM-CSFR or a subunit thereof that binds to its cognate ligand.
12. The cell therapy of any prior claim, wherein the cytokine sink is an antibody that is tethered to the cell and binds to a pro-inflammatory cytokine.
13. The cell therapy of any prior claim, wherein the immune inhibitory receptor is PD1, CTLA4, BTLA, CD160, KRLG-1, 2B4, Lag-3, Tim-3, or TIGIT.
14. The cell therapy of any prior claim, wherein the ectonucleotidase is CD39 or CD73.
15. The cell therapy of any prior claim, wherein the circuit comprises components (a) and (b).

16. The cell therapy of any prior claim, wherein the circuit comprises components (a) and (c).
17. The cell therapy of any prior claim, wherein the circuit comprises components (a), (b) and (c).
18. The cell therapy of any prior claim, wherein binding of the BTTS to the antigen on the surface of a non-target cell activates expression of TGF β 1, CD25 and, optionally, IL-10.
19. The cell therapy of any prior claim, wherein the BTTS comprises:
- i. an extracellular binding domain that recognizes an antigen on a non-target cell,
 - ii. a force sensing region,
 - iii. a transmembrane domain,
 - iv. one or more force-dependent cleavage sites that are cleaved when the force sensing region is activated, and
 - v. an intracellular domain comprising a transcriptional activator, where binding of the extracellular binding domain to the antigen on the non-target cell induces proteolytic cleavage of the one or more force-dependent cleavage sites to release the transcriptional activator, and
wherein the released transcriptional activator induces expression of the expression of one or any combination of (b)-(e) in the cell.
20. A method of treating a subject, comprising:
administering to the subject a cell therapy of any of claims 1-19.
21. The method of claim 20, wherein the subject has cancer,
the engineered immune receptor recognizes an antigen on cancerous cells; and
the BTTS recognizes an antigen that is not on the cancerous cells.
22. The method of claim 20 or 21, wherein the antigen recognized by the BTTS is tissue and/or organ specific.



Assay: 100K cells each; 24h

FIG. 1

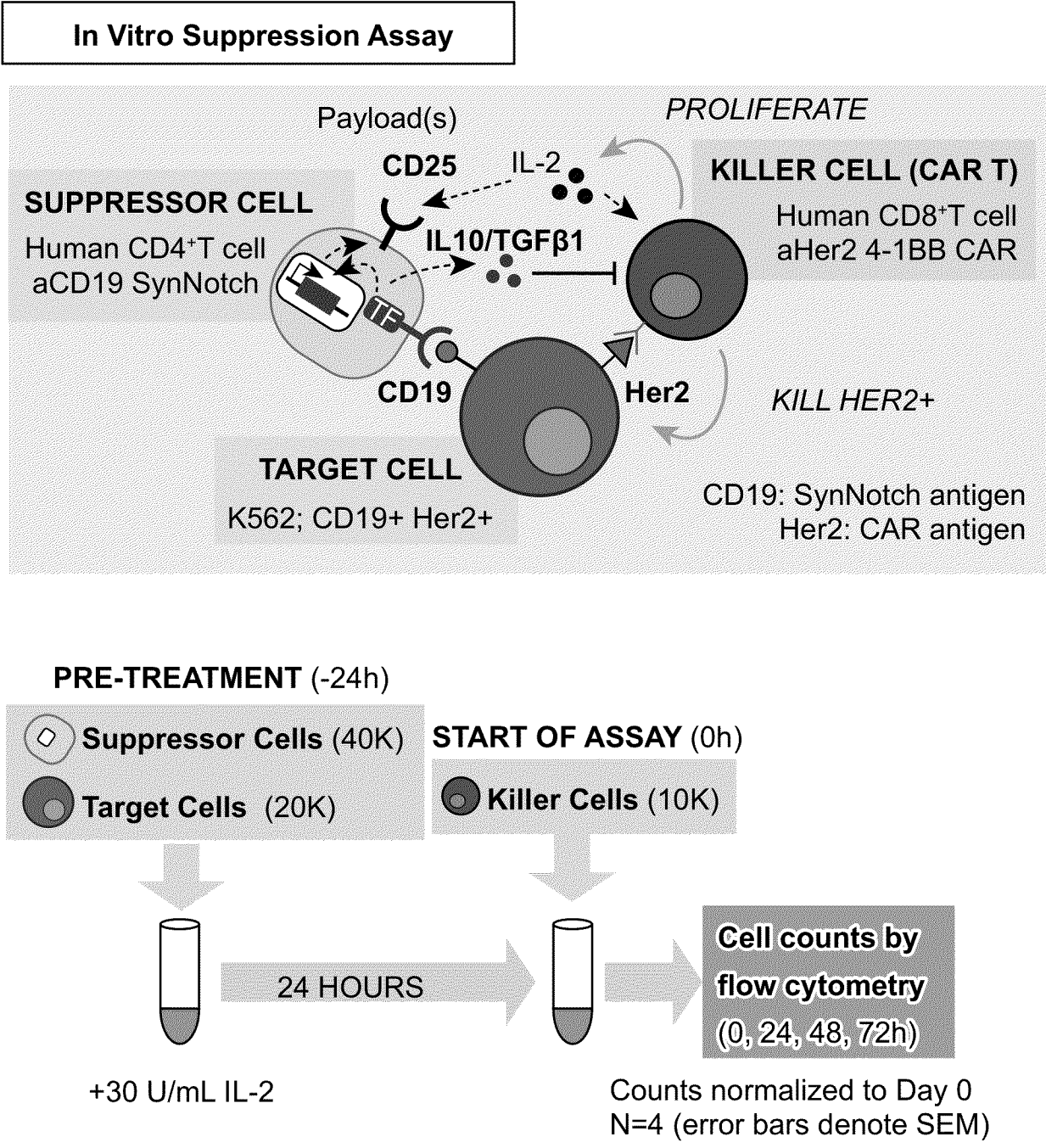


FIG. 2A

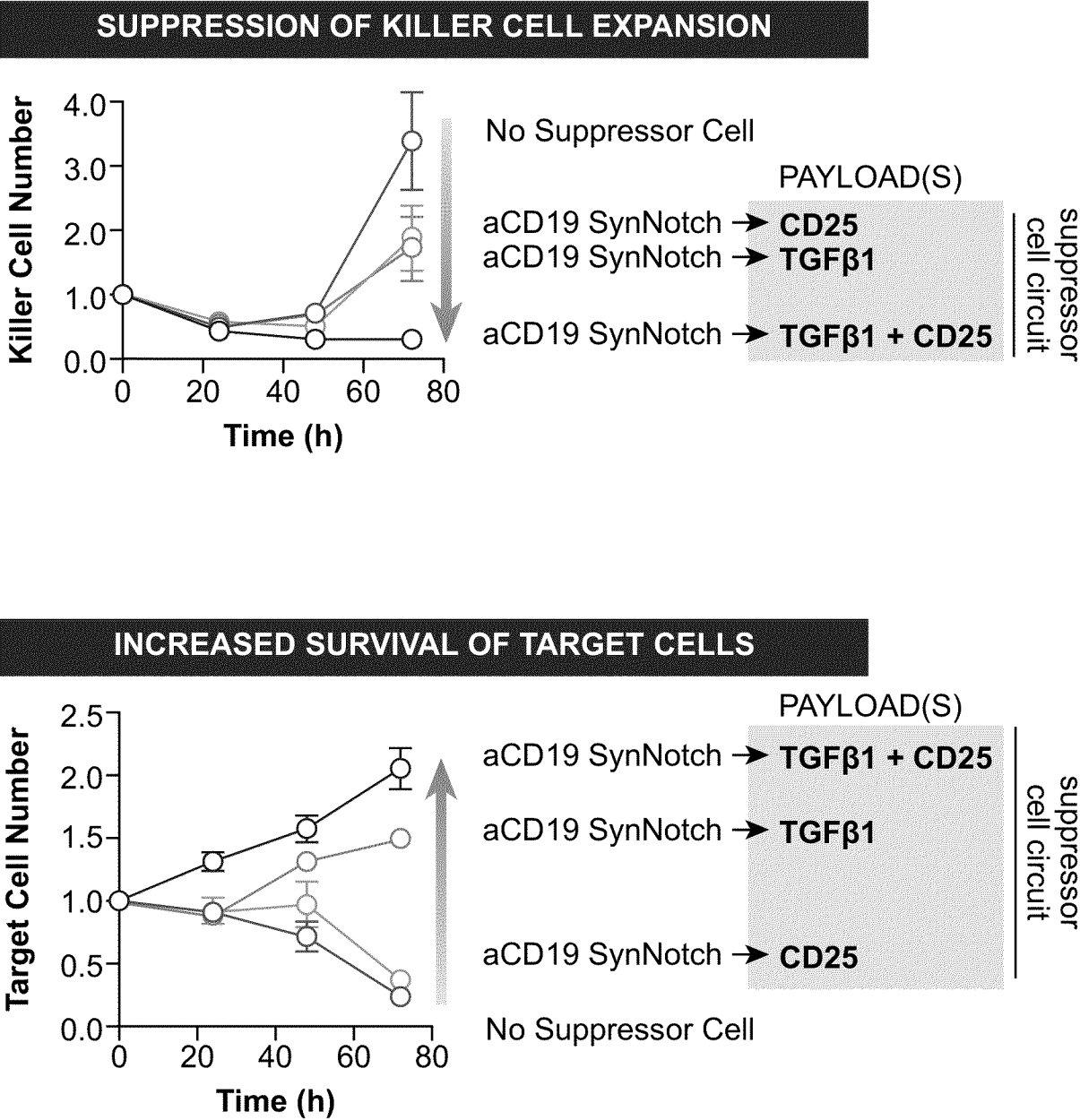


FIG. 2B

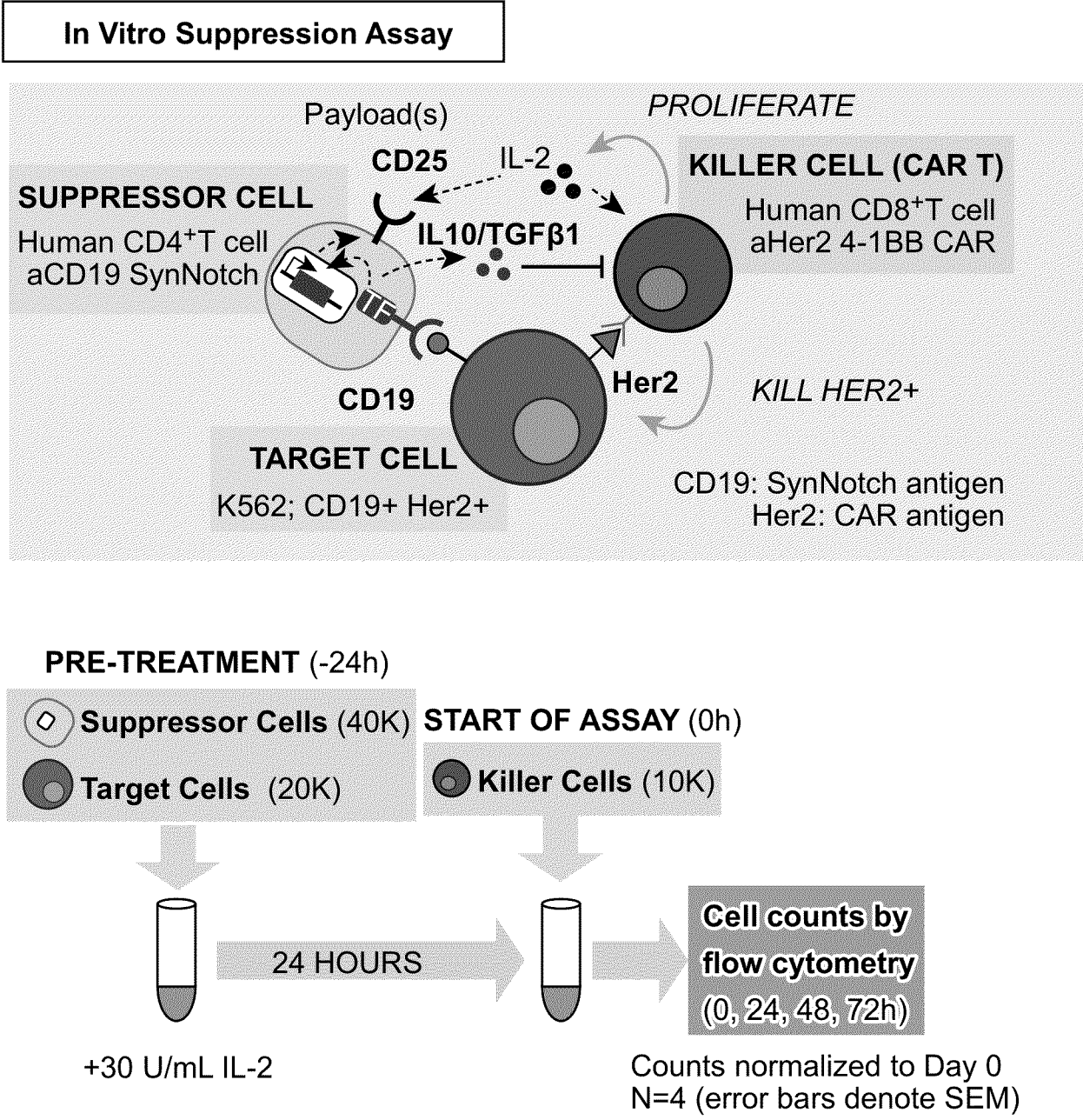


FIG. 3A

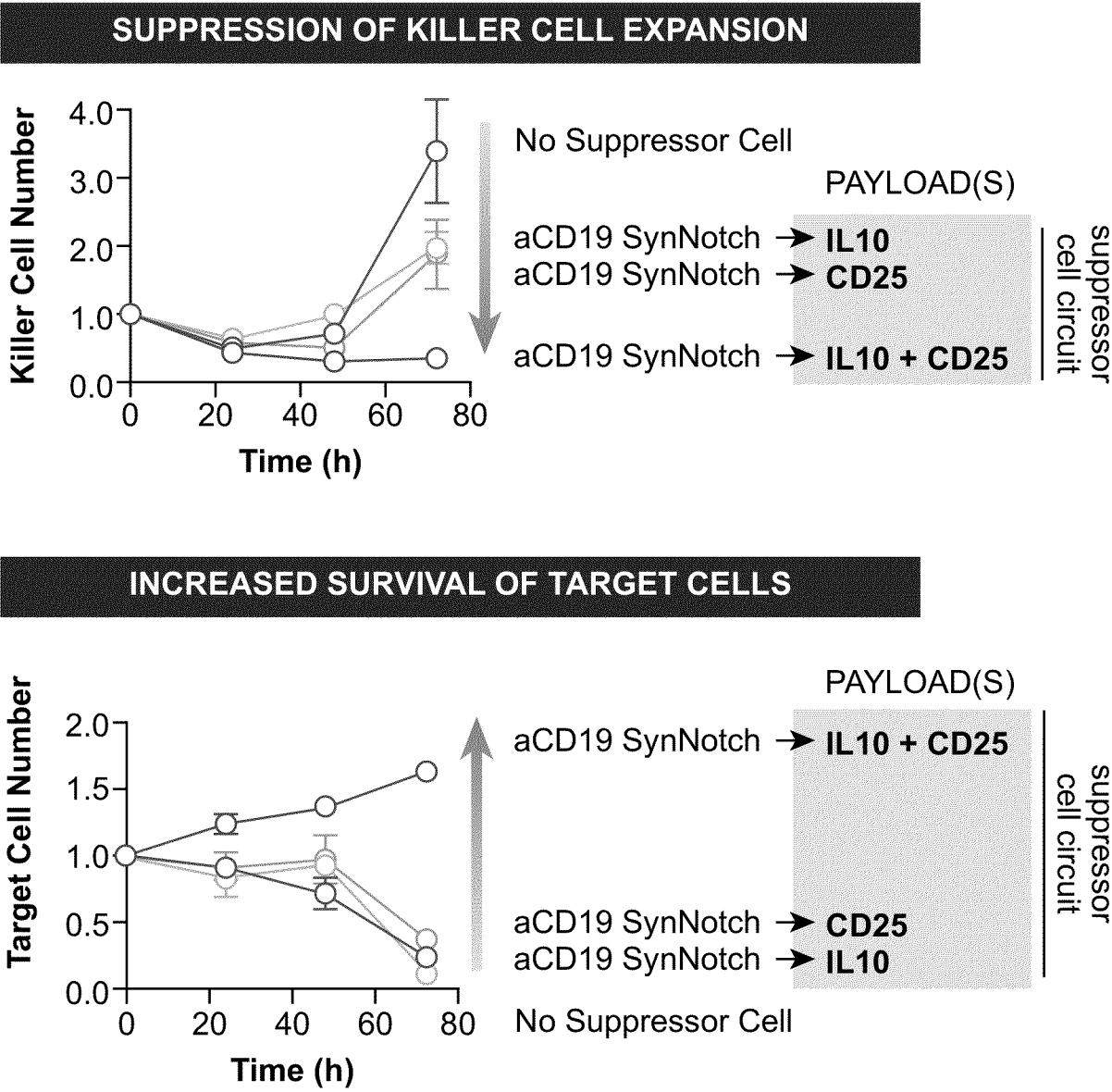


FIG. 3B

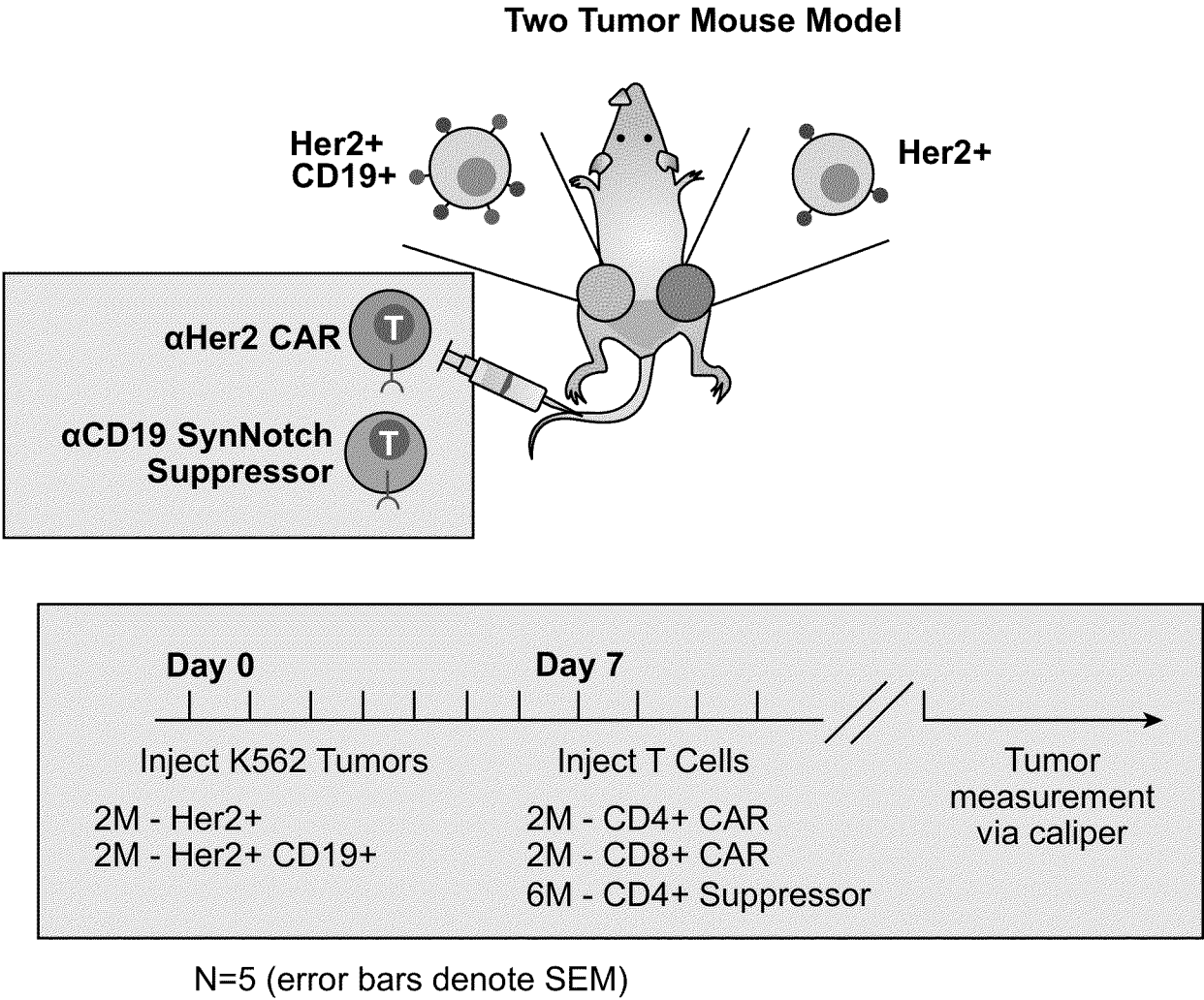


FIG. 4A

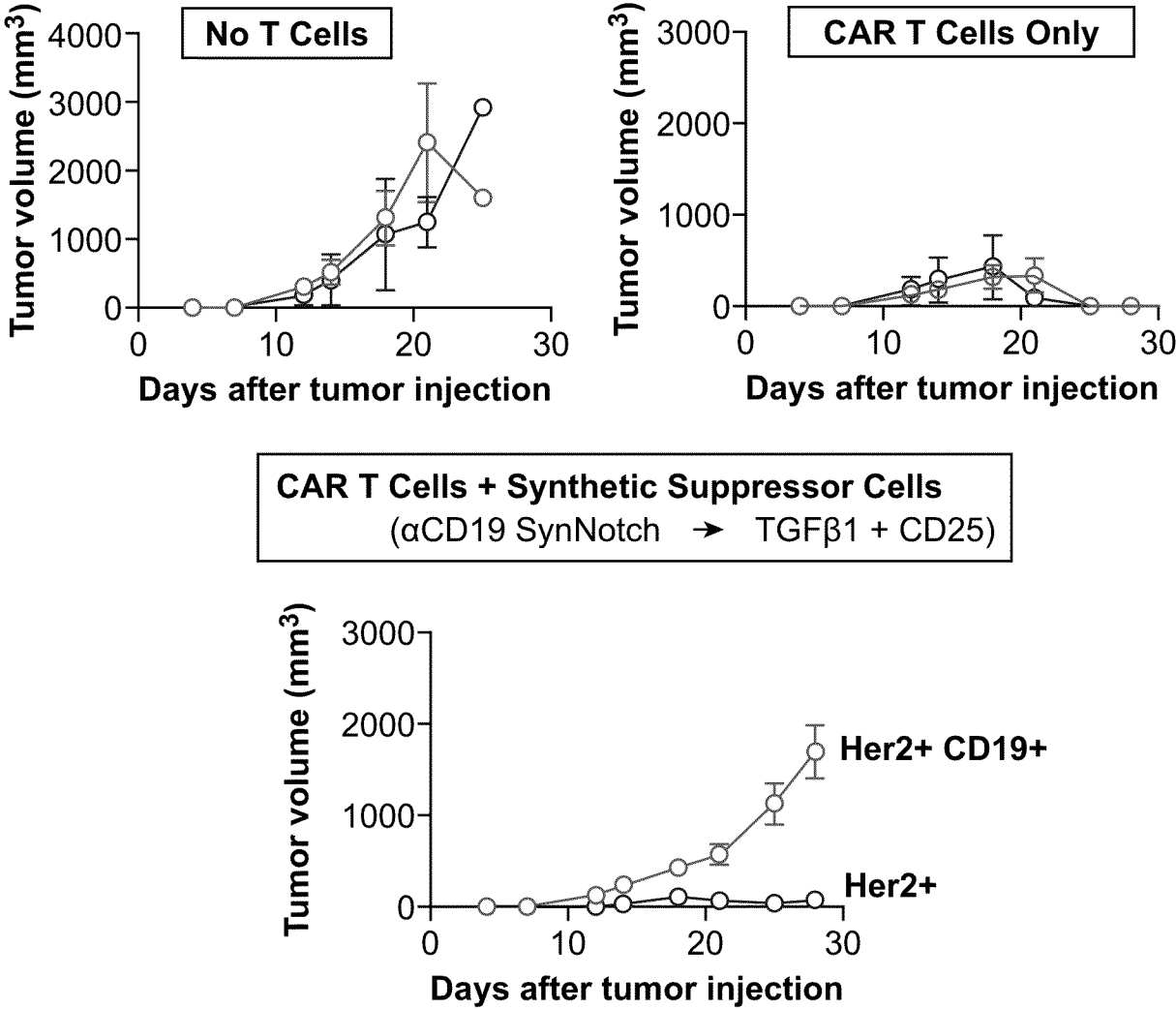


FIG. 4B

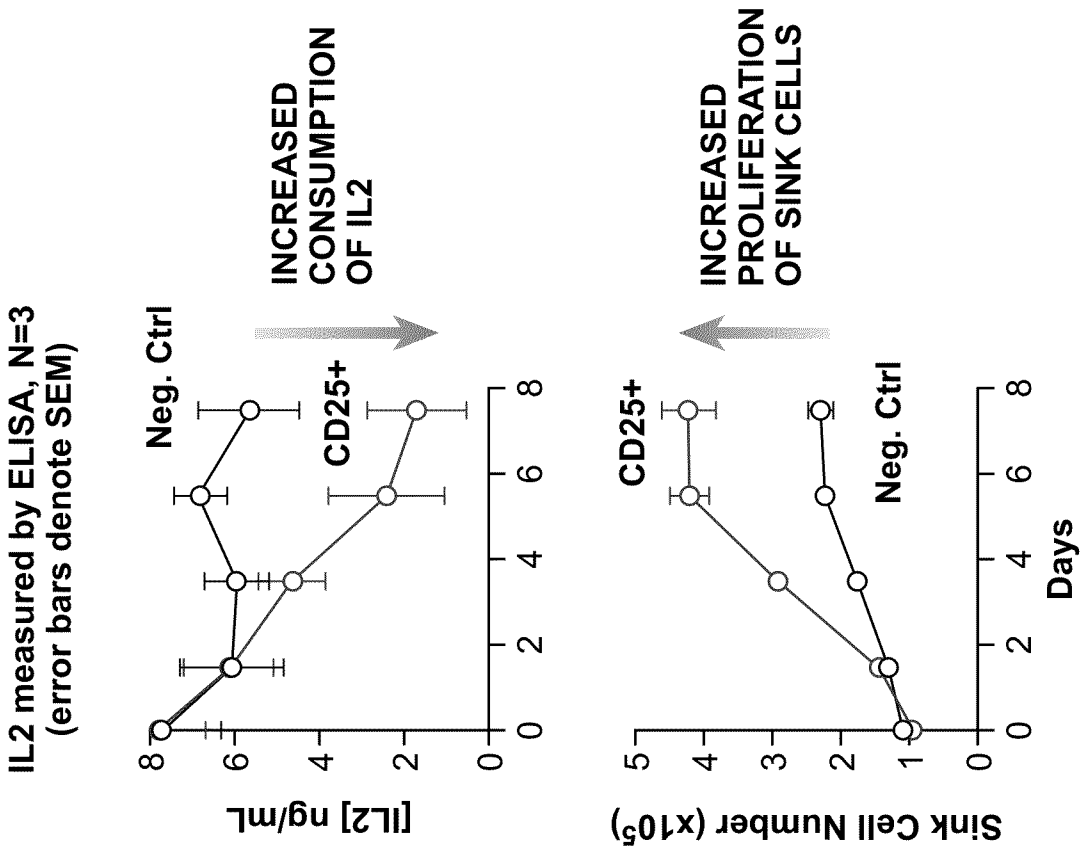
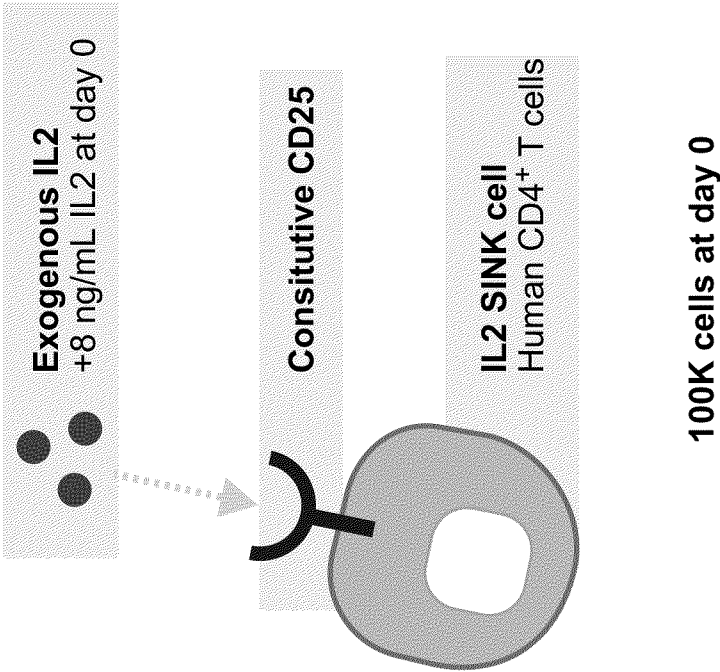


FIG. 5



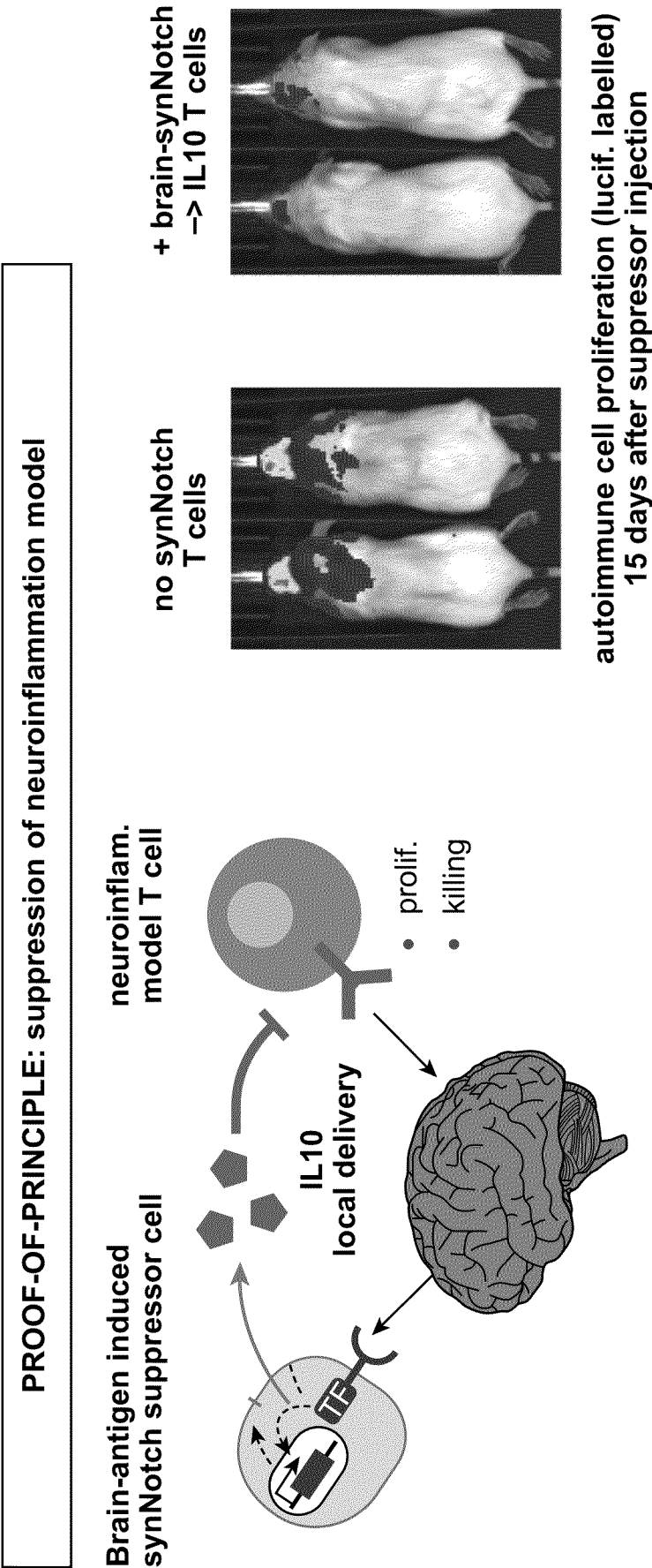


FIG. 6

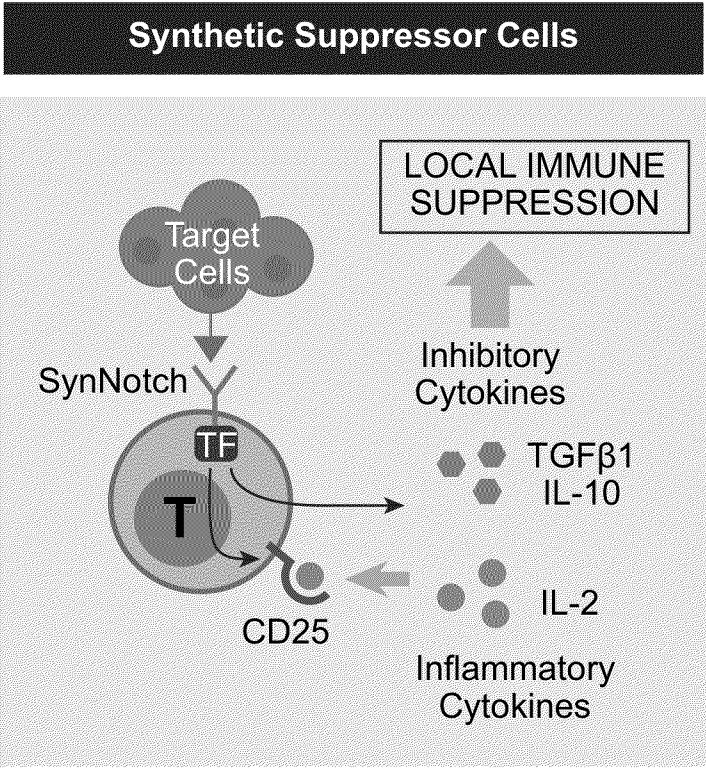


FIG. 7A

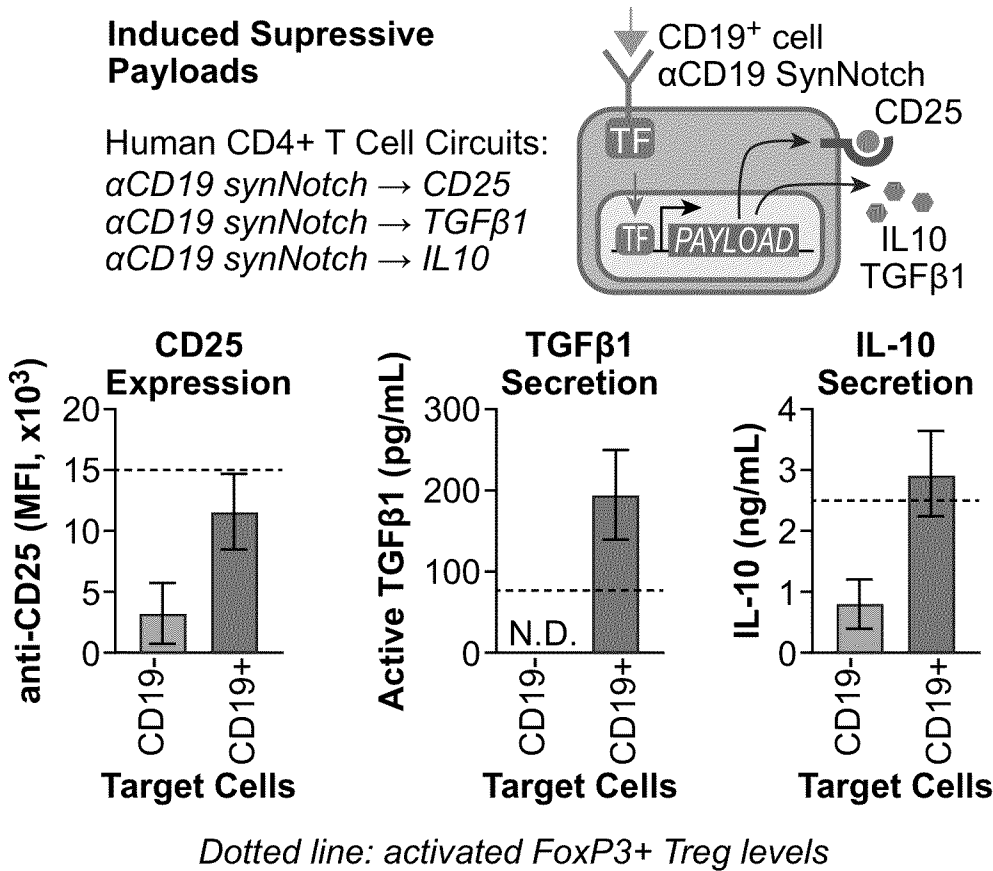


FIG. 7B

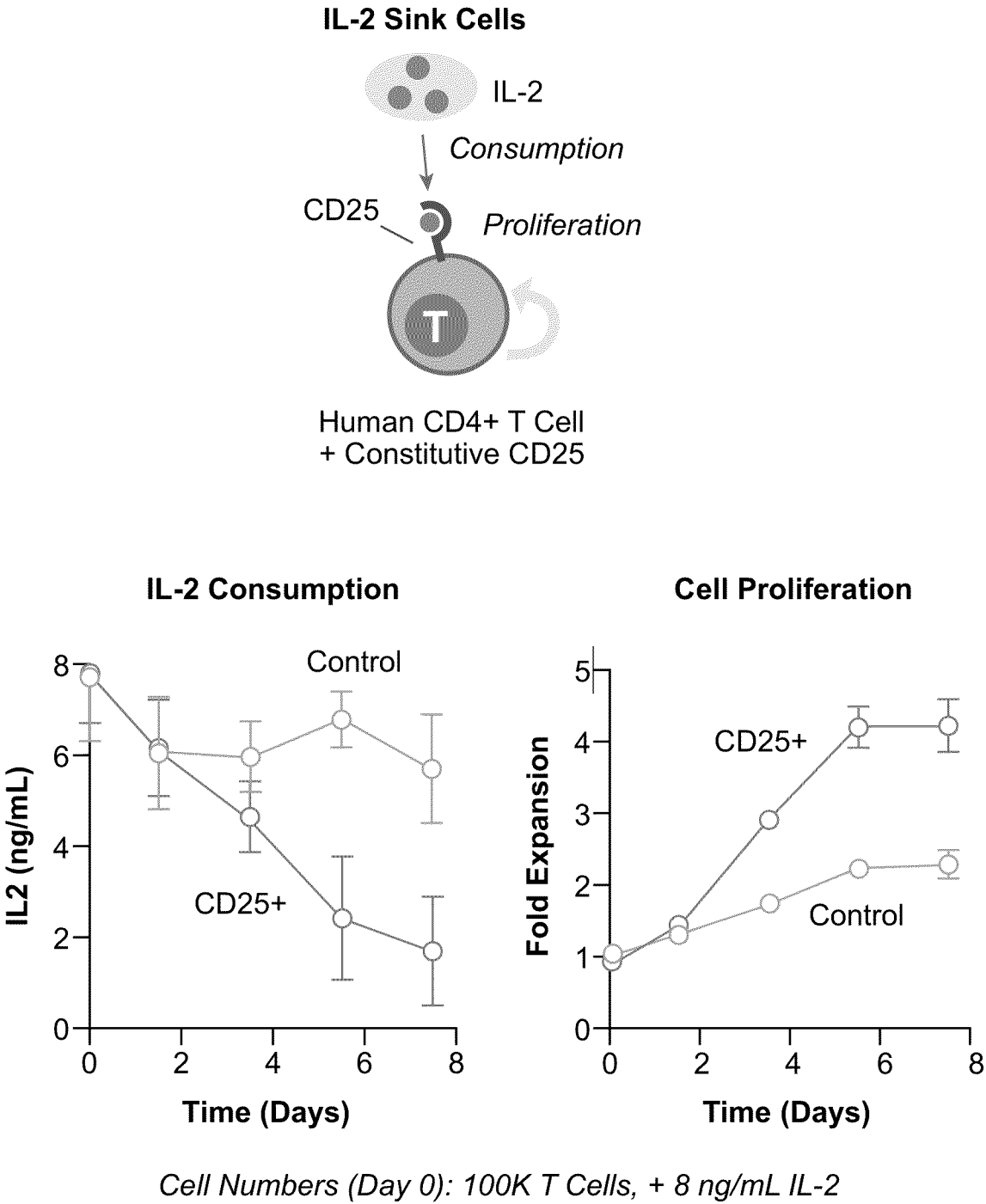
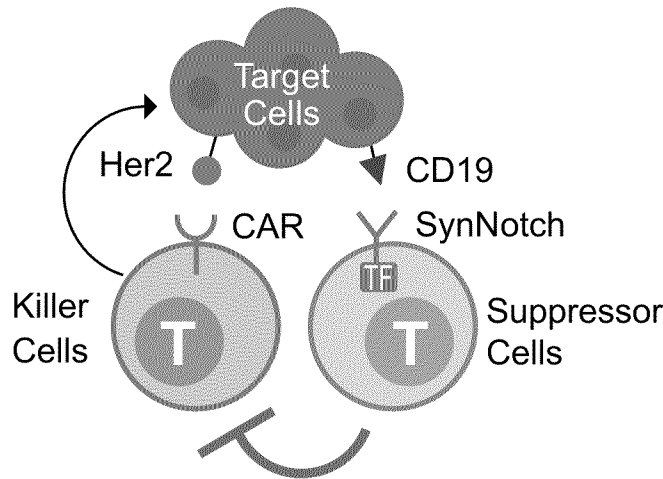


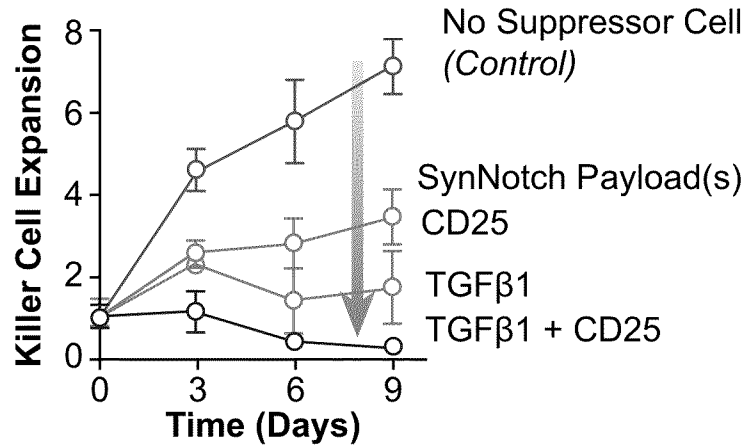
FIG. 7C

Suppression of CAR T Cell Killing *in vitro*



Cell type numbers on Day 0:
40K Target (K562; Her2+, CD19+),
20K Suppressor (CD4+), 10K Killer (CD8+)

Killer (CAR T) Cell Expansion



Target Cell Expansion

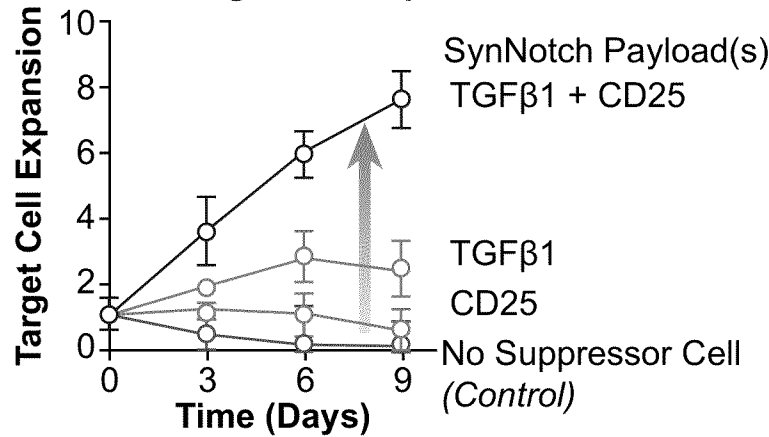


FIG. 7D

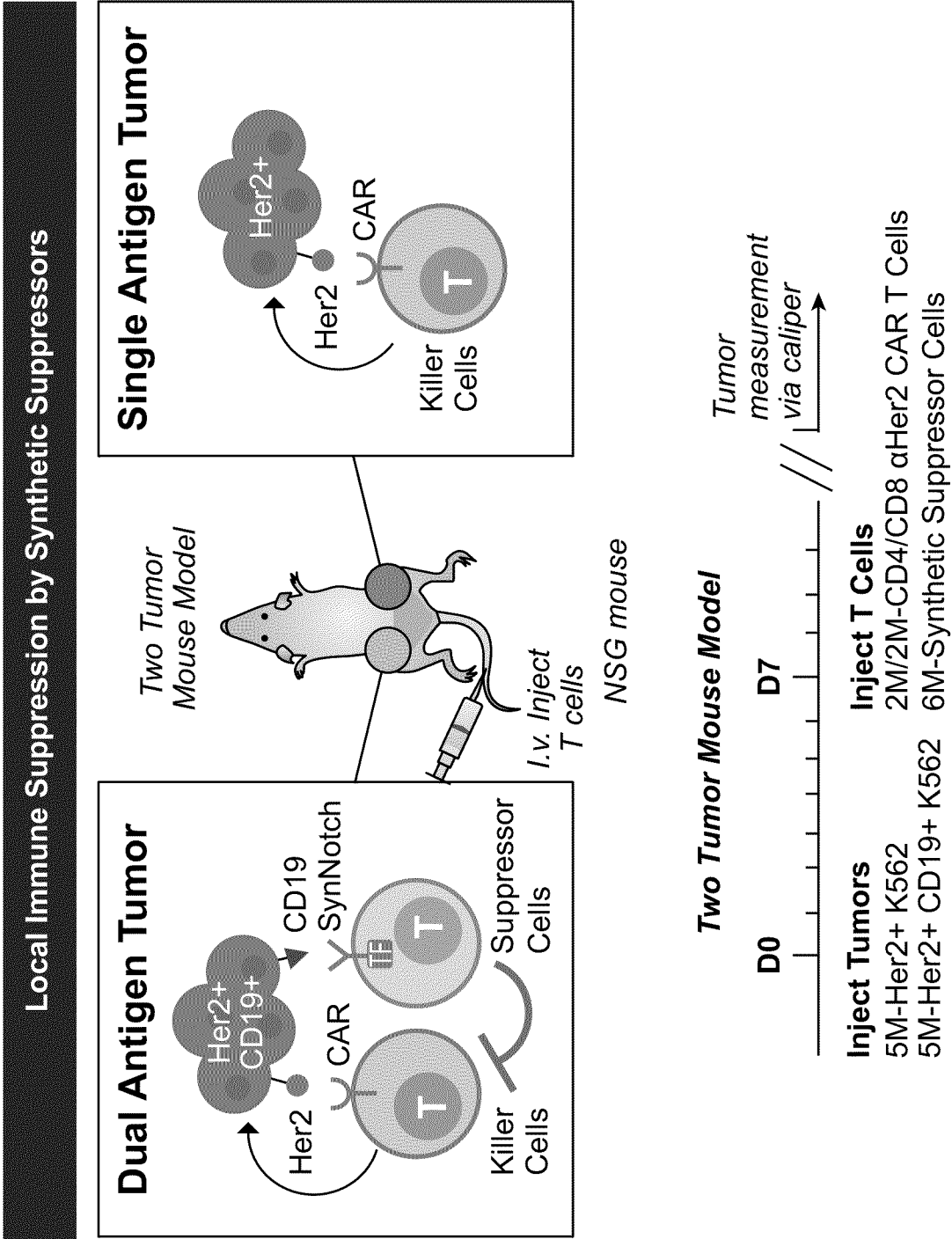


FIG. 8A

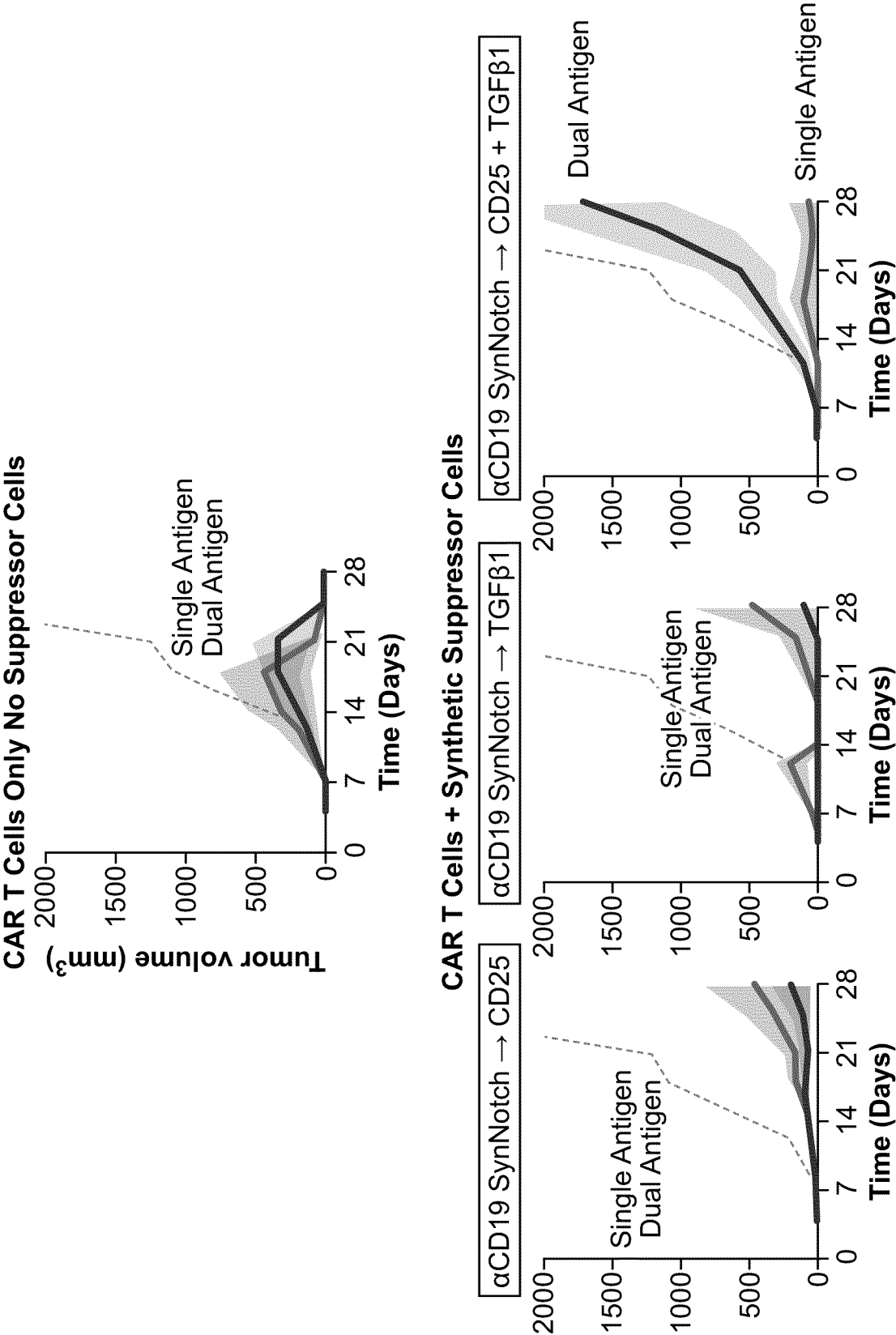


FIG. 8B

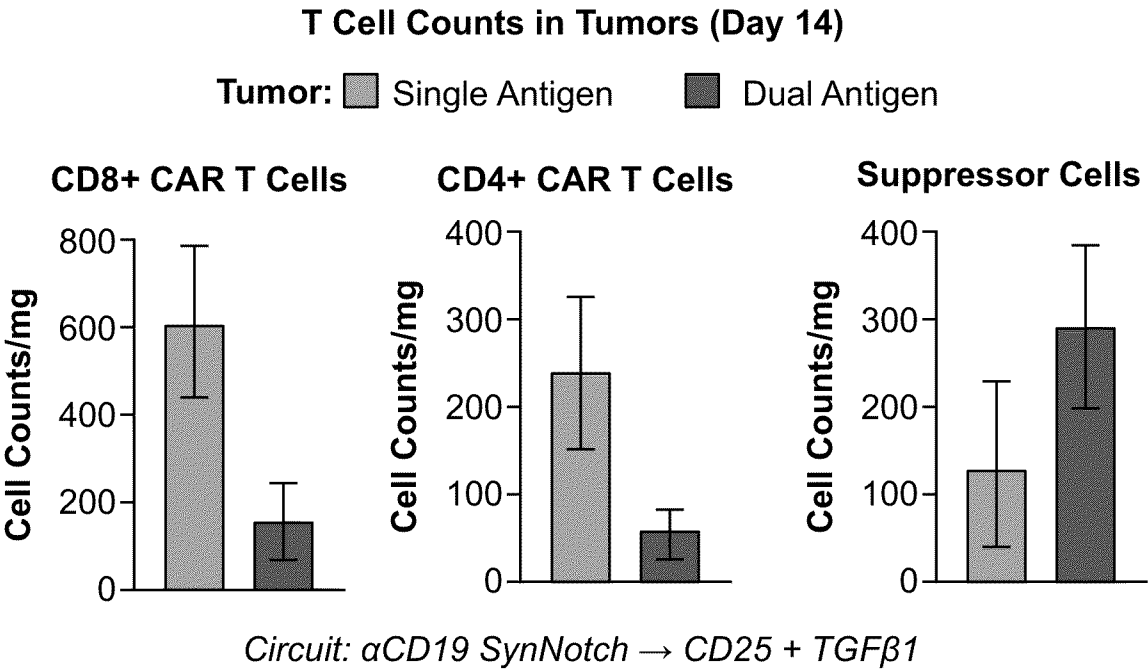


FIG. 8C

Local Suppression in Two Tumor Model (Different Human T Cell Donors)

Circuit: $\alpha CD19 \text{ SynNotch} \rightarrow CD25 + TGF\beta 1$

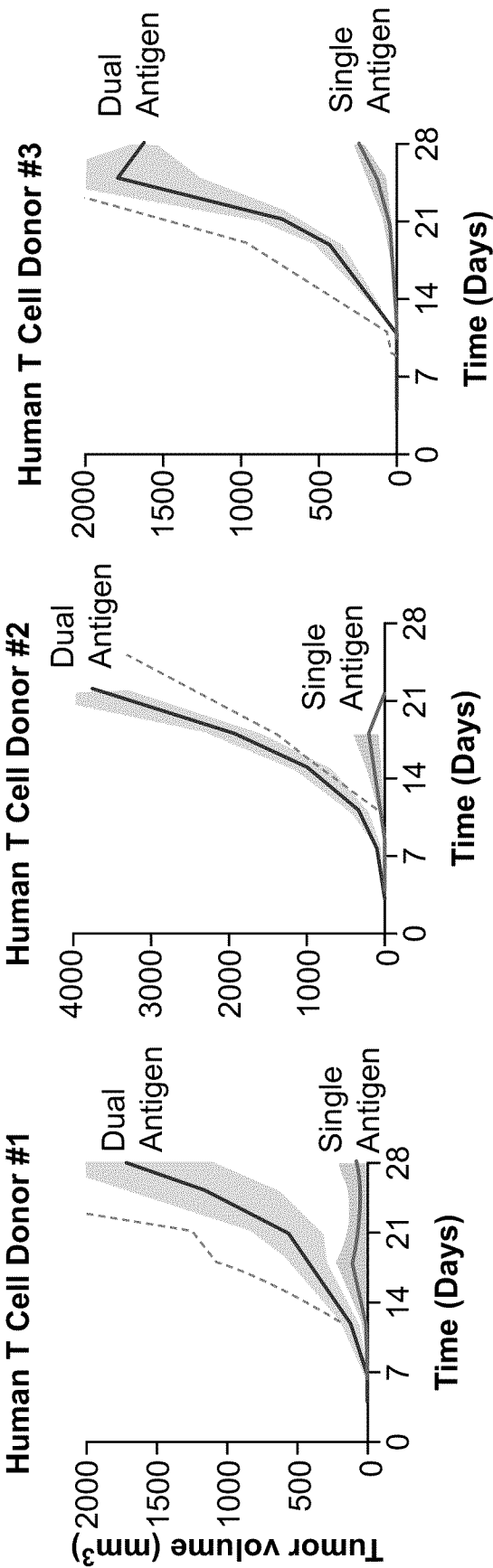


FIG. 9

Improvement over other CAR NOT gates in vivo.

*dynamic range of two cell NOT gate is much better
(complete killing of single antigen tumor; complete protection of dual antigen)*

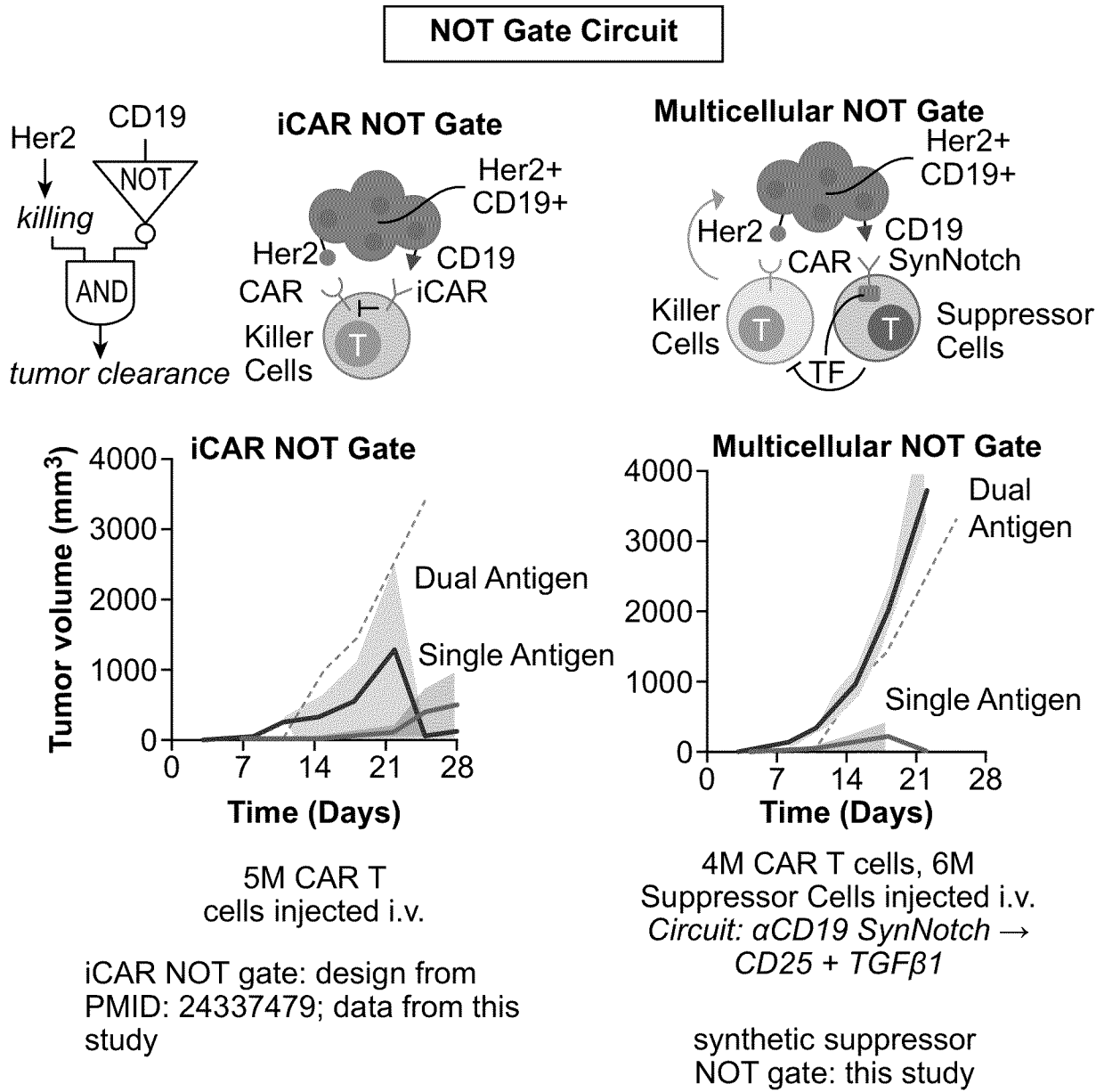


FIG. 10

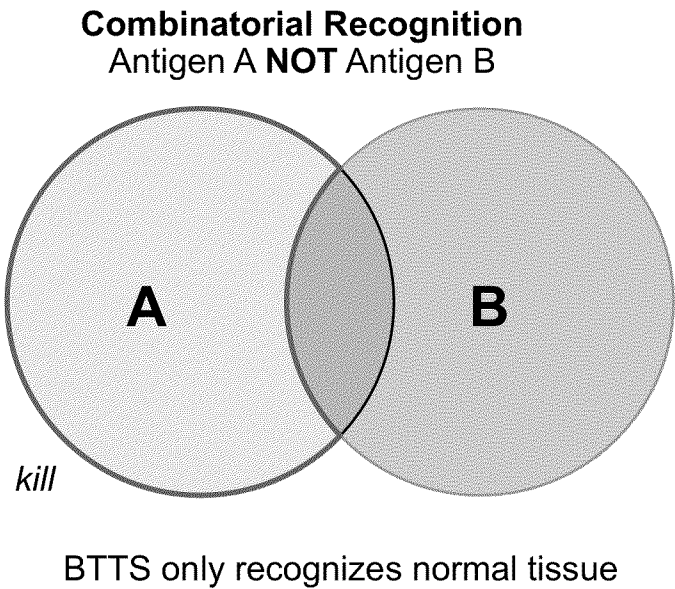
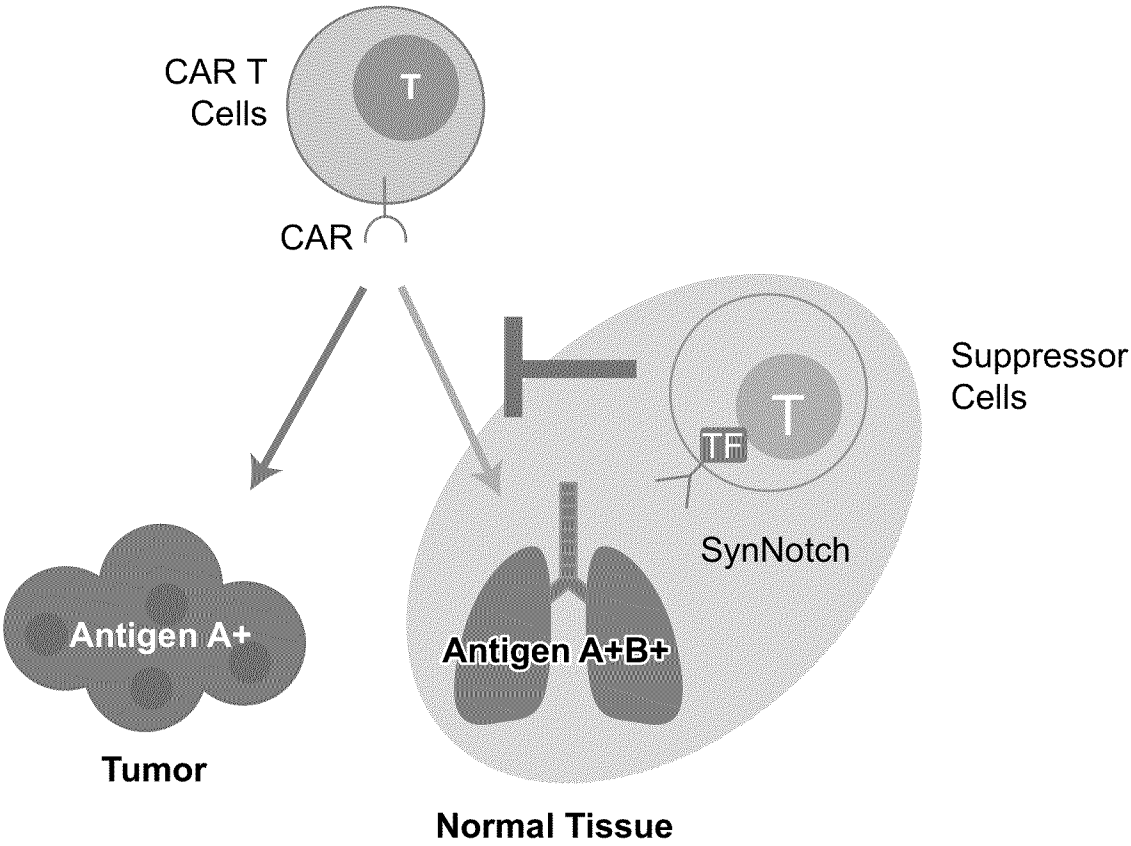


FIG. 11

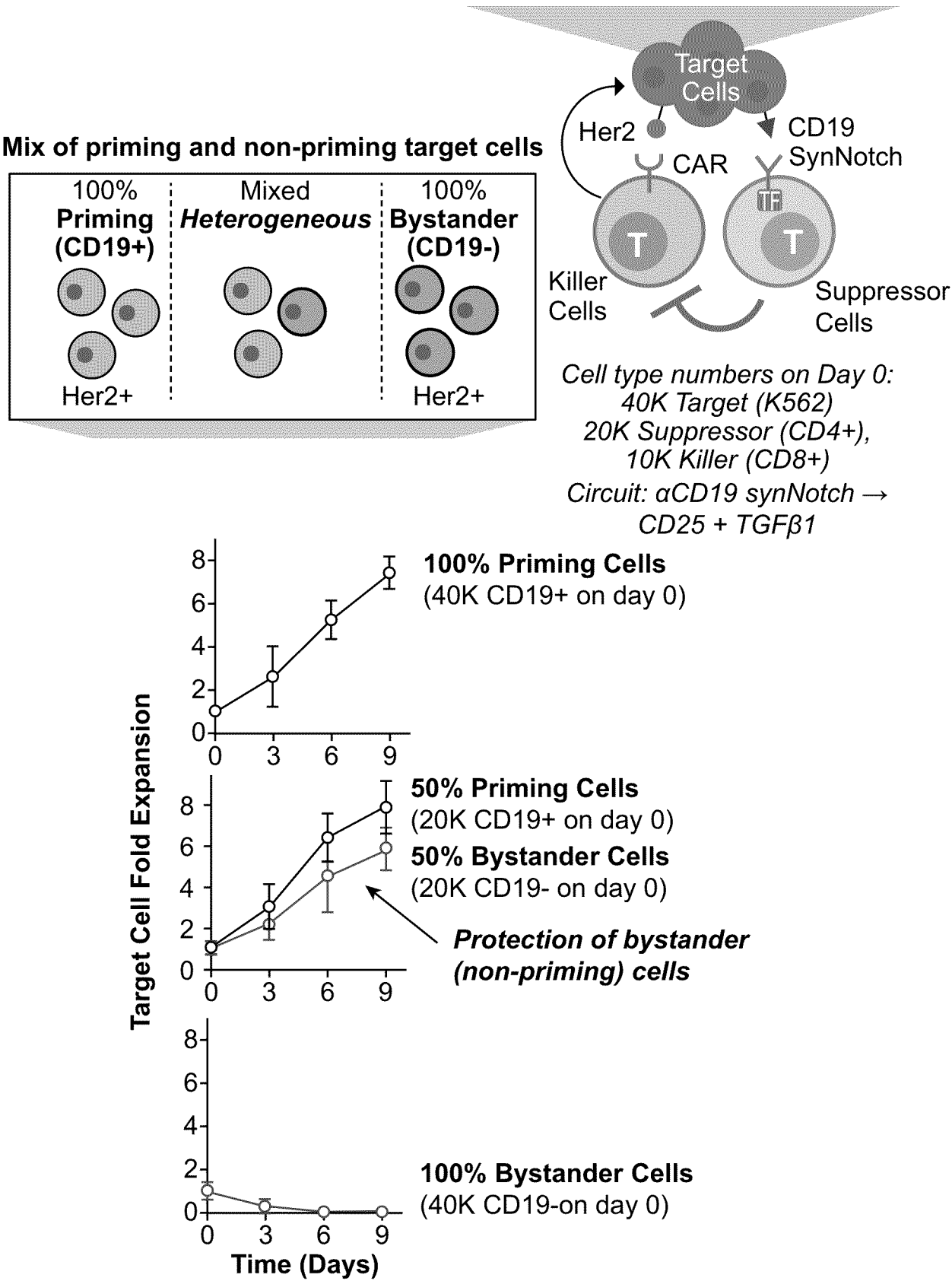
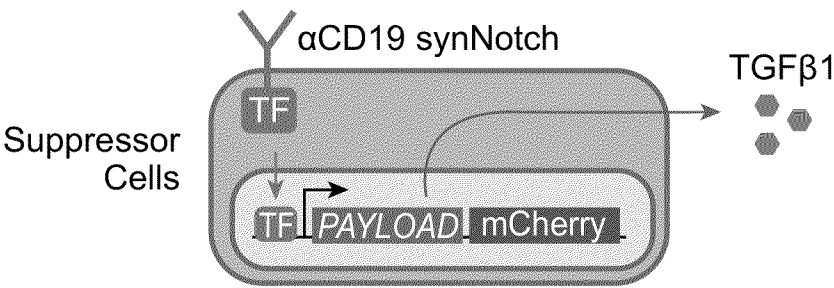


FIG. 12



Suppression Assay from (Fig. S1B)
Circuit: α CD19 synNotch $\rightarrow$ TGF β 1 (mCherry Reporter)

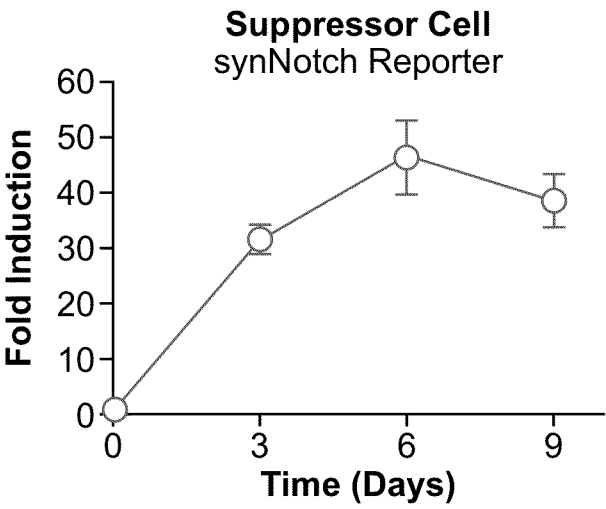


FIG. 13

FIG. 14



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/020272

A. CLASSIFICATION OF SUBJECT MATTER

IPC: **A61K 35/17** (2024.01); **A61K 48/00** (2024.01); **A61P 35/00** (2024.01); **C07K 14/705** (2024.01); **C12N 5/0783** (2024.01)
CPC: **A61K 39/4611**; **A61K 48/00**; **A61K 39/4621**; **A61K 39/4631**; **A61K 39/464402**; **A61P 35/00**; **C12N 5/0636**; **A61K 35/17**; **C07K 14/705**; **A61K 39/46433**; **C12N 2510/00**; **C07K 2319/00**

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.
Y	US 2022/0127373 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al.) 28 April 2022 (28.04.2022) entire document	1-3
Y	US 2018/0355011 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 13 December 2018 (13.12.2018) entire document	1-3
A	WO 2022/118310 A1 (LEPTON PHARMACEUTICALS LTD.) 09 June 2022 (09.06.2022) entire document	1-3



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

14 May 2024 (14.05.2024)

Date of mailing of the international search report

24 May 2024 (24.05.2024)

Name and mailing address of the ISA/US

**Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450**

Facsimile No. **571-273-8300**

Authorized officer

**MATOS
TAINA**

Telephone No. **571-272-4300**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/020272

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.: **4-22**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/020272

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: