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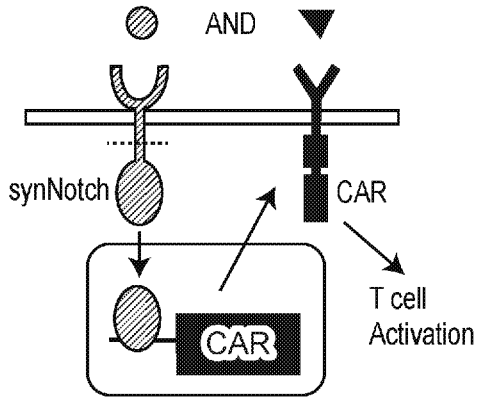
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(54) Title: METHODS OF TREATING EGFRVIII EXPRESSING GLIOBLASTOMAS

FIG. 1A



(57) Abstract: Methods are provided for treating a subject for an EGFRvIII expressing glioblastoma. The methods of the present disclosure involve administering to the subject a molecular circuit that is primed by EGFRvIII to induce one or more encoded therapeutics specific for one or more antigens expressed by the glioblastoma. Nucleic acids containing sequences encoding all or portions of such circuits are also provided, as well as cells, expression cassettes and vectors that contain such nucleic acids. Also provided are kits for practicing the described methods.

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METHODS OF TREATING EGFRvIII EXPRESSING GLIOBLASTOMAS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Patent Application Serial Nos. 62/654,012 filed April 6, 2018 and 62/722,681 filed August 24, 2018; the disclosures of which applications are herein incorporated by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under grant nos. R01 CA196277, P50 GM081879 and R35 NS105068 awarded by the National Institutes of Health. The government has certain rights in the invention.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING PROVIDED AS A TEXT FILE

[0003] A Sequence Listing is provided herewith as a text file, "UCSF-564WO_SeqList_ST25.txt" created on March 29, 2019 and having a size of 156 KB. The contents of the text file are incorporated by reference herein in their entirety.

INTRODUCTION

[0004] Among neuroepithelial tumors, the most frequent (50-60%) is glioblastoma. Glioblastoma multiforme (GBM) is highly anaplastic and develops from a diffuse astrocytoma or *de novo*. GBM is often found in the cerebral hemispheres and its peak incidence occurs at an age of 45–70 years. The median survival of patients with GBM is typically less than 2 years. GBM tumors commonly appear as a heterogeneous mixture containing cells of various phenotypes and polymorphisms. Heterogeneity in GBM tumors at the cellular level undoubtedly contributes to the aggressive pathology of the disease and may play a role in tumor recurrences following treatment (see e.g., Soeda et al., Scientific Reports (2015) 5:7979). Targeted therapies for GBM have been deployed, including chimeric antigen receptor (CAR) T cell therapies directed to the neo-antigen epidermal growth factor receptor variant III (EGFRvIII) (see e.g., Johnson et al., Sci Transl Med.(2015) 7(275):275ra22). However, in some instances, these

therapies have resulted in antigen loss and resistance to treatment (see e.g., O'Rourke et al., Sci Transl Med. (2017) 9(399)).

SUMMARY

[0005] Methods are provided for treating a subject for an EGFRvIII expressing glioblastoma. The methods of the present disclosure involve administering to the subject a molecular circuit that is primed by EGFRvIII to induce one or more encoded therapeutics specific for one or more antigens expressed by the glioblastoma. Nucleic acids containing sequences encoding all or portions of such circuits are also provided, as well as cells, expression cassettes and vectors that contain such nucleic acids. Also provided are kits for practicing the described methods.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] **FIG. 1A-1D** depict examples of prime/kill circuits, with or without diffusible components and employing antigen recognition of EGFRvIII priming antigen expressed on cancerous cells.

[0007] **FIG. 2** depicts a schematic representation of a mixed tumor cell line model employed in the examples.

[0008] **FIG. 3** depicts a schematic representation of a synNotch CAR T cell circuit that engages a priming antigen, EGFRvIII, to then expresses a CAR bi-specific for EphA2 and IL-13RA2.

[0009] **FIG. 4** demonstrates that the 'prime and kill' circuit was effective at driving the full eradication of heterogeneous populations of tumor cells having differing amounts of priming antigen-expressing tumor cells.

[0010] **FIG. 5** depicts delayed killing kinetics in heterogeneous cell populations with lower ratios of priming antigen.

[0011] **FIG. 6A-6C** show the effective *in vivo* treatment of glioblastomas, in a representative preclinical mouse model, using an EGFRviii prime/kill circuit as described herein.

[0012] **FIG. 7** depicts a schematic representation of a heterogeneous GBM tumor treatment model as described in the examples.

[0013] **FIG. 8** depicts a timeline relevant to the heterogeneous GBM tumor treatment model.

[0014] **FIG. 9** depicts that mice treated with synCART showed a reduction in tumor burden as compared to controls.

[0015] **FIG. 10** depicts that mice treated with synCART showed increased survival as compared to controls.

[0016] **FIG. 11** demonstrates that expression of killing CAR in the synCART system is localized to the microenvironment bearing the priming antigen.

[0017] **FIG. 12** depicts a schematic representation of a dual tumor model was employed to assess the ability of prime-and-kill T cells to ignore off-target/bystander cells.

[0018] **FIG. 13** depicts a timeline relevant to the dual tumor model.

[0019] **FIG. 14** demonstrates that synCART treated mice showed a reduction in brain tumor burden but bystander flank cells were unaffected.

[0020] **FIG. 15A-15D** demonstrates killing of GBM target cells using a 2-receptor IF/THEN circuit dependent on the presence of EGFRvIII priming cells for CAR activity and target cell killing.

[0021] **FIG. 16** depicts cells that contain IF/THEN circuits with and without OR gate functionality at the relevant binding triggered transcriptional switch, the antigen-specific therapeutic, or both.

DEFINITIONS

[0022] As used herein, the term "heterogeneous", when used in reference to cancer, generally refers to a cancer displaying some level of intracancer or intratumor heterogeneity, e.g., at the molecular, cellular, tissue or organ level. A heterogeneous cancer is composed of at least two different cell types, where different cell types may be defined in variety of ways. For example, different cell types may differ genomically (e.g., through the presence of a mutation in one cell type that is absent in another), transcriptionally (e.g., through expression of a gene in one cell type that is not expressed in another, through enhanced or reduced expression of a gene in one cell type as compared to another, etc.), or proteomically (e.g., through expression of a protein in one cell type that is not expressed in another, through enhanced or reduced expression of a protein in one cell type as compared to another, etc.). In some instances, cancer heterogeneity may be identified based on the presence of two or more phenotypically different cells present in a cancer, including e.g., where such phenotypically different cells are identified through clinical testing (e.g., histology, immunohistochemistry, in situ hybridization, cytometry, transcriptomics, mutational analysis, whole genome sequencing, proteomics, etc.).

[0023] As such, a heterogeneous cancer, as defined herein, will generally include at least one cancerous cell type and at least one other cell type, where the one other cell type may be a second cancerous cell type or a non-cancerous cell type. For example, a heterogeneous cancer may include a first cancerous cell type and a second cancerous cell type. Alternatively, a heterogeneous cancer may include a cancerous cell type and a non-cancerous cell type. Although a heterogeneous cancer will include at least two different cell types, such cancers are not so limited and may include e.g., more than two different cell types, three or more different cell types, four or more different cell types, five or more different cell types, etc., where at least one cell type is cancerous and the additional cell types may each be cancerous or non-cancerous.

[0024] As summarized above, heterogeneity of a cancer may be defined by differing gene or protein expression by different subpopulations of cells of the cancer. For example, in some instances, a first subpopulation of cells may express a first gene product from a first gene that is not expressed by a second subpopulation of cells, where such a second cell population may or may not express a second gene product from a second gene that defines the second population. Put another way, subpopulations of cells within a heterogeneous cancer may, in some instances, each be defined by the presence or absence (or relative levels) of one or more expressed gene products, where useful expressed gene products for defining cell types may include but are not limited to biomarkers, antigens, wild-type proteins, mutated proteins, wild-type transcripts, mutated transcripts, etc.

[0025] Cancer heterogeneity, in some instances, may include or exclude heterogeneity at the subject level, i.e., intrapatient heterogeneity. As used herein, the term "intrapatient heterogeneity" generally refers to heterogeneity observed between multiple cancers, e.g., multiple tumors, present in a single subject. For example, a primary tumor and a metastasis with a subject may be heterogeneous, e.g., differentially expressing a particular gene product, such as a biomarker, an antigen or a mutated protein. Multiple heterogeneous cancers may arise in a subject through various mechanisms including but not limited to mutation, clonal expansion, metastasis, selection, and combinations thereof. For example, two different intrapatient heterogeneous cancers arising by metastasis of a primary tumor may be heterogeneous with respect to the tissues in which they reside. Alternatively, two different intrapatient heterogeneous cancers derived from the same primary tumor may arise due to mutation and clonal expansion, where one cancer is a subclone of the other. Various other mechanism by

which different intrapatient heterogeneous cancers may arise are possible and fall within the scope of the term as used herein.

[0026] Cancer heterogeneity, in some instances as used herein, may exclude heterogeneity at the population level, i.e., interpatient heterogeneity. As used herein, the term "interpatient heterogeneity" generally refers to differences observed between two cancers or two tumors present in separate subjects or patients.

[0027] 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 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 having it; (b) inhibiting the disease, i.e., arresting its development; and (c) relieving the disease, i.e., causing regression of the disease.

[0028] 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 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.

[0029] 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), 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.

[0030] The term "refractory", used herein, refers to a disease or condition that does not respond to treatment. With regard to cancer, "refractory cancer", as used herein, refers to cancer that does not respond to treatment. A refractory cancer may be resistant at the beginning of treatment or it may become resistant during treatment. Refractory cancer may also called resistant cancer.

[0031] The term "histology" and "histological" as used herein generally refers to microscopic analysis of the cellular anatomy and/or morphology of cells obtained from a multicellular organism including but not limited to plants and animals.

[0032] The term "cytology" and "cytological" as used herein generally refers to a subclass of histology that includes the microscopic analysis of individual cells, dissociated cells, loose cells, clusters of cells, etc. Cells of a cytological sample may be cells in or obtained from one or more bodily fluids or cells obtained from a tissue that have been dissociated into a liquid cellular sample.

[0033] 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 hetero-dimerization 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. 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 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 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—CD3ζ

CAR expressed by lentivirus loaded CTL019 (Tisagenlecleucel-T) CAR-T cells as commercialized by Novartis (Basel, Switzerland).

[0034] 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 complex with CD3 chain molecules. Many native TCRs exist in heterodimeric αβ or γδ forms. The complete endogenous TCR complex in heterodimeric αβ form includes eight chains, namely an alpha chain (referred to herein as TCRα or TCR alpha), beta chain (referred to herein as TCRβ 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 TCRα and TCRβ chains, 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.

[0035] 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 TCRα or modified TCRβ chains as well as single chain TCRs that include modified and/or unmodified TCRα and TCRβ chains that are joined into a single polypeptide by way of a linking polypeptide.

[0036] As used herein, by "chimeric bispecific binding member" is meant a chimeric polypeptide having dual specificity to two different binding partners (e.g., two different antigens). Non-limiting examples of chimeric bispecific binding members include bispecific antibodies, bispecific conjugated monoclonal antibodies $(mab)_2$, bispecific antibody fragments (e.g., $F(ab)_2$, bispecific scFv, bispecific diabodies, single chain bispecific diabodies, etc.), bispecific T cell engagers (BiTE), bispecific conjugated single domain antibodies, micabodies and mutants thereof, and the like. Non-limiting examples of chimeric bispecific binding members also include those chimeric bispecific agents described in Kontermann. *MAbs.* (2012) 4(2): 182–197; Stamova et al. *Antibodies* 2012, 1(2), 172-198; Farhadfar et al. *Leuk Res.* (2016) 49:13-21; Benjamin et al. *Ther Adv Hematol.* (2016) 7(3):142-56; Kiefer et al. *Immunol Rev.*

(2016) 270(1):178-92; Fan et al. *J Hematol Oncol.* (2015) 8:130; May et al. *Am J Health Syst Pharm.* (2016) 73(1):e6-e13; the disclosures of which are incorporated herein by reference in their entirety.

[0037] A "biological sample" encompasses a variety of sample types obtained from an individual or a population of individuals and can be used in various ways, including e.g., the isolation of cells or biological molecules, diagnostic assays, etc. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by mixing or pooling of individual samples, treatment with reagents, solubilization, or enrichment for certain components, such as cells, polynucleotides, polypeptides, etc. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples. The term "biological sample" includes urine, saliva, cerebrospinal fluid, interstitial fluid, ocular fluid, synovial fluid, blood fractions such as plasma and serum, and the like. The term "biological sample" also includes solid tissue samples, tissue culture samples (e.g., biopsy samples), and cellular samples. Accordingly, biological samples may be cellular samples or acellular samples.

[0038] The terms "antibodies" and "immunoglobulin" include antibodies or immunoglobulins of any isotype, fragments of antibodies which retain specific binding to antigen, including, but not limited to, Fab, Fv, scFv, and Fd fragments, chimeric antibodies, humanized antibodies, single-chain antibodies, nanobodies, single-domain antibodies, and fusion proteins comprising an antigen-binding portion of an antibody and a non-antibody protein.

[0039] "Antibody fragments" comprise a portion of an intact antibody, for example, the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies (Zapata et al., Protein Eng. 8(10): 1057-1062 (1995)); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, a designation reflecting the ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen combining sites and is still capable of cross-linking antigen.

[0040] "Single-chain Fv" or "sFv" 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, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[0041] 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.

[0042] As used herein, the term "affinity" refers to the equilibrium constant for the reversible binding of two agents and is expressed as a dissociation constant (Kd). Affinity can be at least 1-fold greater, at least 2-fold greater, at least 3-fold greater, at least 4-fold greater, at least 5-fold greater, at least 6-fold greater, at least 7-fold greater, at least 8-fold greater, at least 9-fold greater, at least 10-fold greater, at least 20-fold greater, at least 30-fold greater, at least 40-fold greater, at least 50-fold greater, at least 60-fold greater, at least 70-fold greater, at least 80-fold greater, at least 90-fold greater, at least 100-fold greater, or at least 1000-fold greater, or more, than the affinity of an antibody for unrelated amino acid sequences. Affinity of an antibody to a target protein can be, for example, from about 100 nanomolar (nM) to about 0.1 nM, from about 100 nM to about 1 picomolar (pM), or from about 100 nM to about 1 femtomolar (fM) or more. As used herein, the term “avidity” refers to the resistance of a complex of two or more agents to dissociation after dilution. The terms “immunoreactive” and “preferentially binds” are used interchangeably herein with respect to antibodies and/or antigen-binding fragments.

[0043] The term “binding” refers to a direct association between two molecules, due to, for example, covalent, electrostatic, hydrophobic, and ionic and/or hydrogen-bond interactions, including interactions such as salt bridges and water bridges. Non-specific binding would refer

to binding with an affinity of less than about 10^{-7} M, e.g., binding with an affinity of 10^{-6} M, 10^{-5} M, 10^{-4} M, etc.

[0044] A "orthogonal" or "orthogonalized" member or members of a binding pair are modified from their original or wild-type forms such that the orthogonal pair specifically bind one another but do not specifically or substantially bind the non-modified or wild-type components of the pair. Any binding partner/specific binding pair may be orthogonalized, including but not limited to e.g., those binding partner/specific binding pairs described herein.

[0045] The terms "domain" and "motif", used interchangeably herein, refer to both structured domains having one or more particular functions and unstructured segments of a polypeptide that, although unstructured, retain one or more particular functions. For example, a structured domain may encompass but is not limited to a continuous or discontinuous plurality of amino acids, or portions thereof, in a folded polypeptide that comprise a three-dimensional structure which contributes to a particular function of the polypeptide. In other instances, a domain may include an unstructured segment of a polypeptide comprising a plurality of two or more amino acids, or portions thereof, that maintains a particular function of the polypeptide unfolded or disordered. Also encompassed within this definition are domains that may be disordered or unstructured but become structured or ordered upon association with a target or binding partner. Non-limiting examples of intrinsically unstructured domains and domains of intrinsically unstructured proteins are described, e.g., in Dyson & Wright. *Nature Reviews Molecular Cell Biology* 6:197-208.

[0046] 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.

[0047] 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).

[0048] 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.

[0049] 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.

[0050] 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 with heterologous and homologous leader sequences, with or without N-terminal methionine residues; immunologically tagged proteins; and the like.

[0051] 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.

[0052] 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 origin as compared to the cell or organism in which it resides.

[0053] 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 embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0054] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the 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 included in the invention.

[0055] 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 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.

[0056] 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 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, or use of a "negative" limitation.

[0057] 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.

[0058] 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

[0059] As summarized above, the present disclosure provides methods of treating a subject for an EGFRvIII expressing glioblastoma. The methods of the present disclosure involve administering to the subject a molecular circuit that is primed by EGFRvIII to induce one or more encoded therapeutics specific for one or more antigens expressed by the glioblastoma. The circuit may be administered in the form of cells encoding the molecular circuit, vector(s) that

deliver nucleic acids encoding the circuit to cells of the subject, or the like. Accordingly, nucleic acids containing sequences encoding all or portions of such circuits are also provided, as well as cells, expression cassettes and vectors that contain such nucleic acids. Also provided are kits for practicing the described methods.

[0060] The subject circuits may integrate the expression of EGFRvIII on a glioblastoma multiforme (GBM) cell and at least a second antigen expressed on a second cell of the GBM to produce a desired outcome with respect to the second cell. The integration of two antigens expressed by different cells of a heterogeneous cell population to result in a desired targeting event may be referred to herein as "*trans*-targeting".

[0061] For example, an employed circuit may integrate EGFRvIII as a "priming antigen" expressed by a first GBM cell, referred to as a "priming cell", and a second antigen (e.g., a "targeting antigen" or "targeted antigen" or "killing antigen") expressed by a second cell, e.g., a nearby cell, of the GBM, referred to as a "targeted cell", to target the second cell type in *trans*. A therapeutic cell modified with such a circuit is primed by the presence of the EGFRvIII antigen on the first cell to then target the targeted cell.

[0062] For comparison, in this context *cis*-targeting refers to integrating of two antigens to target a single cell which expresses both a priming antigen and a targeting antigen to produce a desired outcome with respect to the single cell. Thus, in *cis*-targeting, the targeted cell expresses both the priming antigen and the targeting antigen such that the two antigens are expressed in *cis* with respect to the cell. In *trans*-targeting, the targeted cell expresses only the targeting antigen and not the priming antigen such that the two antigens are expressed in *trans* with respect to the two cells. As such, *trans* targeting may be employed to target a cell that does not express a priming antigen, e.g., does not express EGFRvIII. In some instances, a circuit of the present disclosure may employ both *trans*-targeting and *cis*-targeting, i.e., *cis*- and *trans*-targeting may be combined in a single circuit. In some instances, a circuit of the present disclosure may employ only *trans*-targeting and may e.g., exclude *cis*-targeting.

[0063] The circuits of the present disclosure will generally employ at least one binding triggered transcriptional switch (BTTS) as described in more detail below. A therapeutic cell may be modified to express a BTTS responsive to an EGFRvIII priming antigen. The BTTS may be expressed in the plasma membrane of the cell. Binding of the BTTS to EGFRvIII may induce expression of a protein in the BTTS expressing cell. The induced protein may be a heterologous antigen-specific protein, such as a second BTTS or a heterologous antigen-specific therapeutic,

as described in more detail below. In the context of *cis*-targeting, binding of the BTTS to EGFRvIII expressed on a GBM priming cell induces expression of an antigen specific protein that is specific for a targeting antigen that is also expressed by the GBM priming cell (i.e., the GBM cell is both the priming cell and the targeted cell). In the context of *trans*-targeting, binding of the BTTS to EGFRvIII expressed on a GBM priming cell induces expression of an antigen specific protein that is specific for a targeting antigen that is expressed on a GBM cell that does not express the priming antigen (i.e., a GBM cell other than the priming cell).

[0064] In this manner, *trans*-targeting allows for targeting of cells by an antigen specific protein, such as an antigen-specific therapeutic, only in the presence of EGFRvIII-positive ("EGFRvIII(+)") priming cells. Correspondingly, *trans*-targeting allows for targeting of cells with an antigen specific protein, such as an antigen-specific therapeutic, in a heterogeneous cell population, such as a heterogeneous cancer, where the targeted cells do not express EGFRvIII, i.e., are EGFRvIII-negative ("EGFRvIII(-)") cells. Accordingly, such targeted EGFRvIII(-) GBM cells may be spatially associated with cells that do express EGFRvIII.

METHODS

[0065] As summarized above, the present disclosure provides methods of targeting EGFRvIII(-) cells in a heterogeneous EGFRvIII(+) GBM, including where such cells are targeted in *trans*. Such methods may include administering, to a subject in need thereof, a circuit encoding a BTTS responsive to EGFRvIII(+) that induces expression of an antigen-specific therapeutic, where the antigen-specific therapeutic may be responsive to one or more antigens other than EGFRvIII. Such circuits, when expressed on a therapeutic immune cell, may activate the immune cell to mediate the targeted killing of EGFRvIII(-) GBM cells in a EGFR(+) GBM tumor.

Methods of Treatment

[0066] As summarized above, the methods of the present disclosure find use in treating a subject for a GBM, including where a subject's GBM is heterogeneous for EGFRvIII. Such treatments may include obtaining a desired effect with respect to at least one EGFRvIII(-) cell type (or subpopulation thereof) of a heterogeneously positive EGFRvIII tumor. By the terms "heterogeneously positive EGFRvIII", "EGFRvIII(+) GBM" or "EGFRvIII(+) tumor", as used herein, is generally meant a GBM tumor containing at least some cells that express EGFRvIII. Such tumors may include cells that are EGFRvIII(-) or may evolve to contain cells that are EGFR(-) over the course of tumor progression.

[0067] In some instances, treatments may include obtaining a desired effect with respect to one cell type or more than one cell type (or subpopulation of cells) of the heterogeneous GBM, including two or more, three or more, four or more, five or more, etc., cell types or subpopulations of cells of the heterogeneous GBM. Desired effects of the treatments, as described in more detail below, will vary. For example, with respect to one or more targeted cell types, desired effects will vary and may include but are not limited to e.g., killing of the one or more targeted cell types, reducing the proliferation of the one or more targeted cell types, and the like.

[0068] The subject methods may include introducing into a subject in need thereof, cells that contain nucleic acid sequences encoding a circuit for *trans*-targeting of a cell of a heterogeneous GBM. The introduced cells may be immune cells, including e.g., myeloid cells or lymphoid cells.

[0069] In some instances, the instant methods may include contacting a cell with one or more nucleic acids encoding a circuit wherein such contacting is sufficient to introduce the nucleic acid(s) into the cell. Any convenient method of introducing nucleic acids into a cell may find use herein including but not limited viral transfection, electroporation, lipofection, bombardment, chemical transformation, use of a transducible carrier (e.g., a transducible carrier protein), and the like. Nucleic acids may be introduced into cells maintained or cultured *in vitro* or *ex vivo*. Nucleic acids may also be introduced into a cell in a living subject *in vivo*, e.g., through the use of one or more vectors (e.g., viral vectors) that deliver the nucleic acids into the cell without the need to isolate, culture or maintain the cells outside of the subject.

[0070] Introduced nucleic acids may be maintained within the cell or transiently present. As such, in some instance, an introduced nucleic acid may be maintained within the cell, e.g., integrated into the genome. Any convenient method of nucleic acid integration may find use in the subject methods, including but not limited to e.g., viral-based integration, transposon-based integration, homologous recombination-based integration, and the like. In some instance, an introduced nucleic acid may be transiently present, e.g., extrachromosomally present within the cell. Transiently present nucleic acids may persist, e.g., as part of any convenient transiently transfected vector.

[0071] An introduced nucleic acid encoding a circuit may be introduced in such a manner as to be operably linked to a regulatory sequence, such as a promoter, that drives the expression of one or more components of the circuit. The source of such regulatory sequences may vary and

may include e.g., where the regulatory sequence is introduced with the nucleic acid, e.g., as part of an expression construct or where the regulatory sequence is present in the cell prior to introducing the nucleic acid or introduced after the nucleic acid. As described in more detail herein, useful regulatory sequence can include e.g., endogenous promoters and heterologous promoters. For example, in some instances, a nucleic acid may be introduced as part of an expression construct containing a heterologous promoter operably linked to a nucleic acid sequence. In some instances, a nucleic acid may be introduced as part of an expression construct containing a copy of a promoter that is endogenous to the cell into which the nucleic acid is introduced. In some instances, a nucleic acid may be introduced without a regulatory sequence and, upon integration into the genome of the cell, the nucleic acid may be operably linked to an endogenous regulatory sequence already present in the cell. Depending on the confirmation and/or the regulatory sequence utilized, expression of each component of the circuit from the nucleic acid may be configured to be constitutive, inducible, tissue-specific, cell-type specific, etc., including combinations thereof.

[0072] Any convenient method of delivering the circuit encoding components may find use in the subject methods. In some instances, the subject circuit may be delivered by administering to the subject a cell expressing the circuit. In some instances, the subject circuit may be delivered by administering to the subject a nucleic acid comprising one or more nucleotide sequences encoding the circuit. Administering to a subject a nucleic acid encoding the circuit may include administering to the subject a cell containing the nucleic acid where the nucleic acid may or may not yet be expressed. In some instances, administering to a subject a nucleic acid encoding the circuit may include administering to the subject a vector designed to deliver the nucleic acid to a cell.

[0073] Accordingly, in the subject methods of treatment, nucleic acids encoding a circuit or components thereof may be administered *in vitro*, *ex vivo* or *in vivo*. In some instances, cells may be collected from a subject and transfected with nucleic acid and the transfected cells may be administered to the subject, with or without further manipulation including but not limited to e.g., *in vitro* expansion. In some instances, the nucleic acid, e.g., with or without a delivery vector, may be administered directly to the subject.

[0074] EGFRvIII(+) priming cells and targeted cells of a subject circuit will generally differ in at least the expression of EGFRvIII. In some instances, priming cells and targeted cells may differ in the expression of at least one surface expressed epitope, e.g., a surfaced expressed

protein, an antigen presented in the context of MHC, etc., other than EGFRvIII. In some instances, two different targeted cells may differ in the expression of at least one surface expressed epitope, e.g., a surfaced expressed protein, an antigen presented in the context of MHC, etc.

[0075] Differential expression between two cells or two cell types of a GBM will vary. For example, in some instances, a cell expresses one surface epitope not expressed by the other. In some instances, a cell expresses one surface epitope more highly than the surface epitope is expressed by the other cell. Where cells differ in the level, e.g., as compared to the presence/absence, of expression of a surface epitope the difference in level may vary but will generally be substantially different, e.g., sufficiently different to allow for practical targeting of one cell versus the other. Differences in expression between cells may range from less than one order of magnitude of expression to ten orders of magnitude of expression or more, including but not limited to e.g., 1 order of magnitude, 2 orders of magnitude, 3 orders of magnitude, 4 orders of magnitude, 5 orders of magnitude, 6 orders of magnitude, 7 orders of magnitude, 8 orders of magnitude, 9 orders of magnitude, 10 orders of magnitude, etc. In some instances, two cell types differing in level of expression of a particular epitope may be said to be "high" and "low" for the epitope, respectively, where high versus low expression may be differentiated using conventional methods known to the relevant artisan.

[0076] In some instances, the presence or absence of a particular epitope will be defined by the limit of detection of the method employed to detect the epitope, including e.g., where such limit of detection may or may not be based on an appropriate reference standard or positive or negative control. For example, where the epitope is present below the limit of detection the cell may be said to be "negative" for the epitope. Correspondingly, where the epitope is present below the level detected in a reference standard or appropriate control the cell may be said to be negative for the epitope. Where the epitope is present above the limit of detection the cell may be said to be "positive" for the epitope. Correspondingly, where the epitope is present above the level detected in a reference standard or appropriate control the cell may be said to be positive for the epitope.

[0077] As summarized above, EGFRvIII(+) priming cells and targeted cells in a heterogeneous GBM will generally be in sufficient proximity to allow for recognition of a targeted cell expressing a targeting antigen, but not the priming antigen, by a primed immune cell. Relative proximity between an EGFRvIII(+) priming cell and a targeted cell sufficient for *trans*-

targeting of the targeted cell will vary and, as described herein, may be modified as desired depending on how the subject circuit is designed (e.g., through the use of a more or less stable antigen-specific therapeutic, through the use of a diffusible payload, etc.). In some instances, the EGFRvIII(+) priming cell and the targeted cell may be adjacent. In some instances, the priming cell and the targeted cell may be non-adjacent. As such, the proximity, expressed in this context as the distance between, a priming cell and a targeted cell may range from about 1 cell diameter to 100 cell diameters or more, including but not limited to e.g., 1 to 100 cell diameters, 2 to 100 cell diameters, 5 to 100 cell diameters, 10 to 100 cell diameters, 1 to 50 cell diameters, 2 to 50 cell diameters, 5 to 50 cell diameters, 10 to 50 cell diameters, 1 to 25 cell diameters, 2 to 25 cell diameters, 5 to 25 cell diameters, 10 to 25 cell diameters, etc.

[0078] Heterogeneity of GBM tumors treated using the methods described herein will vary. For example, in some instances, the degree of heterogeneity in a heterogeneous GBM will vary. For example, with respect to each individual cell type present in a heterogeneous GBM, a subject cell type (e.g., an EGFRvIII priming cell, a first targeted cell type, a second targeted cell type, or another cell type) will represent less than 100% of the cells of the GBM including but not limited to e.g., less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, less than 50%, less than 45%, less than 40%, less than 35%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1% of the cells of the heterogeneous GBM.

[0079] In some instances, 75% or less of the cells of a heterogeneous GBM express EGFRvIII, including but not limited to e.g., 70% or less, 65% or less, 60% or less, 55% or less, 50% or less, 45% or less, 40% or less, 35% or less, 30% or less, 25% or less, or 20% or less. In some instances, methods of the present disclosure find use in treating a heterogeneous GBM in a subject where the percentage of cell of the GBM that express EGFRvIII ranges from 1% or more than 1% to 99% or less than 99%, including but not limited to e.g., from 1% to 99%, from 5% to 90%, from 10% to 85%, from 20% to 80%, from 25% to 75% and the like.

[0080] In some instances, a targeted cell (e.g., an EGFR(-) cell present in an EGFR(+) tumor) of a herein disclosed methods may represent less than 50% of the cells of the heterogeneous cancer or heterogeneous tumor, including but not limited to e.g., less than 45%, less than 40%, less than 35%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, less than

5%, less than 4%, less than 3%, less than 2%, or less than 1% of the cells of the heterogeneous cancer or a heterogeneous tumor.

[0081] In some instances, a particular cell type present in a heterogeneous EGFR(+) GBM (e.g., a EGFR(+) priming cell type, a targeted cell type or another cell type) may be a majority cell type of the heterogeneous cancer, including e.g., where the particular cell type represents 50% or greater, including e.g., 60% or greater, 70% or greater, 80% or greater, 90% or greater, 95% or greater, of the cells of the heterogeneous GBM. In some instances, a EGFR(+) priming cell of a herein disclosed method may represent 50% or greater of the cells of the heterogeneous GBM, including but not limited to e.g., 60% or greater, 70% or greater, 80% or greater, 90% or greater, 95% or greater, of the cells of the heterogeneous GBM. In some instances, a EGFRvIII(-) targeted cell of a herein disclosed method may represent 50% or greater of the cells of the heterogeneous GBM, including but not limited to e.g., 60% or greater, 70% or greater, 80% or greater, 90% or greater, 95% or greater, of the cells of the heterogeneous GBM.

[0082] The methods of the present disclosure may be employed to target and treat a variety of GBM tumors, including e.g., primary GBM, secondary GBM tumors, re-growing GBM tumors, recurrent GBM tumors, refractory GBM tumors and the like. For example, in some instances, the methods of the present disclosure may be employed as an initial treatment of a primary GBM identified in a subject. In some instances, the methods of the present disclosure may be employed as a non-primary (e.g., secondary or later) treatment, e.g., in a subject with a GBM that is refractory to at least one prior treatment, in a subject with a GBM that is re-growing following at least one prior treatment, in a subject with a mixed response to at least one prior treatment (e.g., a positive response in at least one tumor in the subject and a negative or neutral response in at least a second tumor in the subject, including e.g., a mixed response to a treatment for multiple GBM), and the like.

[0083] In some instances, the method of the present disclosure may be employed to target, treat or clear a subject for minimal residual disease (MRD) remaining after a prior GBM therapy. Targeting, treating and/or clearance of GBM MRD may be pursued using the instant methods whether or not the MRD is or has been determined to be refractory to the prior treatment. In some instances, a method of the present disclosure may be employed to target, treat and/or clear a subject of MRD following a determination that the MRD is refractory to a prior treatment or one or more available treatment options other than those employing the herein described circuits.

[0084] In some instances, the instant methods may be employed prophylactically for surveillance. For example, a subject in need thereof may be administered a treatment involving one or more of the herein described circuits when the subject does not have detectable disease but is at risk of developing a GBM or a recurrent GBM. In some instances, a prophylactic approach may be employed when a subject is at particularly high risk of developing a primary GBM that would be predicted to be a heterogeneous GBM. In some instances, a prophylactic approach may be employed when a subject has been previously treated for a GBM and is at risk of reoccurrence. Essentially any combination of EGFRvIII priming antigen and targeting antigen may be employed in prophylactic treatments, including those described herein.

[0085] In some instances, the herein described methods may be used to prophylactically surveil a subject for GBM cells expressing one or more mutations commonly present in GBM tumors, including mutations found in recurrent and/or refractory GBM or that occur in primary GBM. Mutations found in primary, recurrent and/or refractory GBM (and subtypes thereof) include but are not limited to e.g., IDH1 mutation, TP53 mutation, ALK mutation, RRM1 mutation, TUBB3 mutation, ATRX mutation, BRAF mutation, PTEN mutation, PDGFRA mutation, PTPN11 mutation, and SMARCA4 mutation. In some instances, methods may employ an antigen-specific therapeutic specific for one or more killing antigens, where the one or more killing antigens include one or more commonly mutated proteins, including surface expressed proteins.

[0086] In some instances, methods of the present disclosure may be employed to treat subjects that do not necessarily present with a heterogeneous GBM, including primary and non-primary GBMs, but are at an increased risk of developing such a heterogeneous GBM. For example, a subject having an apparently homogeneous EGFRvIII(+) GBM may be treated with a circuit to prophylactically surveil a subject for GBM cells expressing one or more mutations that occur in GBM. In some instances, a subject having an EGFRvIII(+) GBM, e.g., whether or not an apparently homogeneous EGFRvIII(+) GBM, may be treated with a circuit directed to a targeting antigen other than EGFRvIII as described herein in anticipation of the loss of EGFRvIII(+) in the GBM, i.e., EGFRvIII(+) antigen loss.

[0087] The methods of treating described herein may, in some instances, be performed in a subject that has previously undergone one or more conventional treatments. For example, in the case of oncology, the methods described herein may, in some instances, be performed following a conventional cancer therapy including but not limited to e.g., conventional chemotherapy, conventional radiation therapy, conventional immunotherapy, surgery, etc. In some instances,

the methods described herein may be used when a subject has not responded to or is refractory to a conventional therapy.

[0088] With respect to the GBM as a whole, desired effects of the described treatments may result in a reduction in the number of cells in the GBM, a reduction in the size of a GBM tumor, a reduction in the overall proliferation of the GBM, a reduction in the overall growth rate of a GBM tumor, etc. For example, an effective treatment is in some cases a treatment that, when administered in one or more doses to an individual in need thereof, reduces the number of cancer cells in the individual and/or reduces tumor mass in the individual, by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 75%, or more than 75%, compared to the number of cancer cells and/or tumor mass in the absence of the treatment. Reductions in the number of cancer cells or the size of the tumor mass may be defined with respect to the heterogeneous tumor as a whole or with respect to the targeted cells of the GBM.

[0089] In some embodiments, an effective treatment is a treatment that, when administered alone (e.g., in monotherapy) or in combination (e.g., in combination therapy) with one or more additional therapeutic agents, in one or more doses, is effective to reduce one or more of tumor growth rate, GBM cell number, and tumor mass, by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or more, compared to the tumor growth rate, GBM cell number, or tumor mass in the absence of the treatment. Reductions in the tumor growth rate, GBM cell number, or tumor mass may be defined with respect to the heterogeneous tumor as a whole or with respect to the targeted cells of the GBM.

[0090] In some instances, treatment may involve activation of an immune cell containing nucleic acid sequences encoding a circuit as described herein. Accordingly, the present disclosure correspondingly presents methods of activating an immune cell, e.g., where the immune cell expresses an EGFRvIII priming/targeting circuit as described herein and is contacted with a first cell of a GBM expressing a EGFRvIII priming antigen and a second cell of the GBM expressing a targeting antigen.

[0091] Immune cell activation, as a result of the methods described herein, may be measured in a variety of ways, including but not limited to e.g., measuring the expression level of one or more markers of immune cell activation. Useful markers of immune cell activation include but

are not limited to e.g., CD25, CD38, CD40L (CD154),CD69, CD71, CD95, HLA-DR, CD137 and the like. For example, in some instances, upon antigen binding by an immune cell receptor an immune cell may become activated and may express a marker of immune cell activation (e.g., CD69) at an elevated level (e.g., a level higher than a corresponding cell not bound to antigen). Levels of elevated expression of activated immune cells of the present disclosure will vary and may include an increase, such as a 1-fold or greater increase in marker expression as compared to un-activated control, including but not limited to e.g., a 1-fold increase, a 2-fold increase, a 3-fold increase, a 4-fold increase, etc.

[0092] In some instances, an immune cell modified to encode a circuit of the present disclosure, when bound to a targeted antigen, may have increased cytotoxic activity, e.g., as compared to an un-activated control cell. In some instances, activated immune cells encoding a subject circuit may show 10% or greater cell killing of antigen expressing target cells as compared to un-activated control cells. In some instances, the level of elevated cell killing of activated immune cells will vary and may range from 10% or greater, including but not limited to e.g., 20% or greater, 30% or greater, 40% or greater, 50% or greater, 60% or greater, 70% or greater, 80% or greater, 90% or greater, etc., as compared to an appropriate control.

[0093] In some instances, treatment may involve modulation, including induction, of the expression and/or secretion of a cytokine by an immune cell containing nucleic acid sequences encoding a circuit as described herein. Non-limiting examples of cytokines, the expression/secretion of which may be modulated, include but are not limited to e.g., Interleukins and related (e.g., IL-1-like, IL-1α, IL-1β, IL-1RA, IL-18, IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, IL-3, IL-5, GM-CSF, IL-6-like, IL-6, IL-11, G-CSF, IL-12, LIF, OSM, IL-10-like, IL-10, IL-20, IL-14, IL-16, IL-17, etc.), Interferons (e.g., IFN-α, IFN-β, IFN-γ, etc.), TNF family (e.g., CD154, LT-β, TNF-α, TNF-β, 4-1BBL, APRIL, CD70, CD153, CD178, GITRL, LIGHT, OX40L, TALL-1, TRAIL, TWEAK, TRANCE, etc.), TGF-β family (e.g., TGF-β1, TGF-β2, TGF-β3, etc.) and the like.

[0094] In some instances, activation of an immune cell through a circuit of the present disclosure may induce an increase in cytokine expression and/or secretion relative to that of a comparable cell where the circuit is not present or otherwise inactive. The amount of the increase may vary and may range from a 10% or greater increase, including but not limited to e.g., 10% or greater, 25% or greater, 50% or greater, 75% or greater, 100% or greater, 150% or

greater, 200% or greater, 250% or greater, 300% or greater, 350% or greater 400% or greater, etc.

Conventional Treatments and Combination Therapy

[0095] As will be readily understood, the methods of treating described herein may, in some instances, be combined with one or more conventional treatments. For example, in the case of oncology for GBM, the methods described herein may, in some instances, be combined with a conventional GBM therapy including but not limited to e.g., conventional chemotherapy, conventional radiation therapy, conventional immunotherapy, surgery, etc. Also as described above, in some instances, the methods of treating described herein may be employed following conventional therapy, e.g., to treat a heterogeneous GBM that is refractory to a conventional therapy, to treat a heterogeneous GBM that is recurrent after a conventional therapy, to treat a subject for MRD following conventional therapy, and the like.

[0096] In some instances, the methods described herein may be used before or after a conventional therapy. For example, the methods described herein may be used as an adjuvant therapy, e.g., after a subject has seen improvement from a conventional therapy, or may be used when a subject has not responded to a conventional therapy. In some instances, the methods described herein may be used prior to an additional therapy, e.g., to prepare a subject for an additional therapy, e.g., a conventional therapy as described herein.

[0097] Standard GBM therapies include surgery (e.g., surgical removal of cancerous tissue), radiation therapy, chemotherapeutic treatment, antibody treatment, biological response modifier treatment, and certain combinations of the foregoing.

[0098] Radiation therapy includes, but is not limited to, x-rays or gamma rays that are delivered from either an externally applied source such as a beam, or by implantation of small radioactive sources.

[0099] Antibodies suitable for use in, or under investigation for, GBM treatment include, but are not limited to, naked antibodies, e.g., trastuzumab (Herceptin) , bevacizumab (Avastin™), cetuximab (Erbitux™), panitumumab (Vectibix™), Ipilimumab (Yervoy™), rituximab (Rituxan), alemtuzumab (Lemtrada™), Oregovomab (OvaRex™), Lambrolizumab (pembrolizumab, MK-3475, Keytruda™), ranibizumab (Lucentis™) etc., and conjugated antibodies, e.g., conjugated antibodies of those listed above and the like.

[00100] Conventional cancer therapies also include targeted therapies for cancer including but not limited to e.g., Bevacizumab (Avastin) targeting VEGF ligand (approved for use in Glioblastoma) and the like.

[00101] Biological response modifiers suitable for use in connection with the methods of the present disclosure include, but are not limited to, (1) inhibitors of tyrosine kinase (RTK) activity; (2) inhibitors of serine/threonine kinase activity; (3) tumor-associated antigen antagonists, such as antibodies that bind specifically to a tumor antigen; (4) apoptosis receptor agonists; (5) interleukin-2; (6) interferon-α.; (7) interferon -γ; (8) colony-stimulating factors; (9) inhibitors of angiogenesis; and (10) antagonists of tumor necrosis factor.

[00102] Chemotherapeutic agents are non-peptidic (i.e., non-proteinaceous) compounds that reduce proliferation of cancer cells, and encompass cytotoxic agents and cytostatic agents. Non-limiting examples of chemotherapeutic agents include alkylating agents, nitrosoureas, antimetabolites, antitumor antibiotics, plant (vinca) alkaloids, and steroid hormones.

[00103] Agents that act to reduce cellular proliferation are known in the art and widely used. Such agents include alkylating agents, such as nitrogen mustards, nitrosoureas, ethylenimine derivatives, alkyl sulfonates, and triazenes, including, but not limited to, mechlorethamine, cyclophosphamide (Cytoxan™), melphalan (L-sarcolysin), carmustine (BCNU), lomustine (CCNU), semustine (methyl-CCNU), streptozocin, chlorozotocin, uracil mustard, chlormethine, ifosfamide, chlorambucil, pipobroman, triethylenemelamine, triethylenethiophosphoramine, busulfan, dacarbazine, and temozolomide.

[00104] Antimetabolite agents include folic acid analogs, pyrimidine analogs, purine analogs, and adenosine deaminase inhibitors, including, but not limited to, cytarabine (CYTOSAR-U), cytosine arabinoside, fluorouracil (5-FU), floxuridine (FudR), 6-thioguanine, 6-mercaptopurine (6-MP), pentostatin, 5-fluorouracil (5-FU), methotrexate, 10-propargyl-5,8-dideazafolate (PDDF, CB3717), 5,8-dideazatetrahydrofolic acid (DDATHF), leucovorin, fludarabine phosphate, pentostatine, and gemcitabine.

[00105] Suitable natural products and their derivatives, (e.g., vinca alkaloids, antitumor antibiotics, enzymes, lymphokines, and epipodophyllotoxins), include, but are not limited to, Ara-C, paclitaxel (Taxol®), docetaxel (Taxotere®), deoxycoformycin, mitomycin-C, L-asparaginase, azathioprine; brequinar; alkaloids, e.g. vincristine, vinblastine, vinorelbine, vindesine, etc.; podophyllotoxins, e.g. etoposide, teniposide, etc.; antibiotics, e.g. anthracycline, daunorubicin hydrochloride (daunomycin, rubidomycin, cerubidine), idarubicin, doxorubicin,

epirubicin and morpholino derivatives, etc.; phenoxizone biscyclopeptides, e.g. dactinomycin; basic glycopeptides, e.g. bleomycin; anthraquinone glycosides, e.g. plicamycin (mithramycin); anthracenediones, e.g. mitoxantrone; azirinopyrrolo indolediones, e.g. mitomycin; macrocyclic immunosuppressants, e.g. cyclosporine, FK-506 (tacrolimus, prograf), rapamycin, etc.; and the like.

[00106] Other anti-proliferative cytotoxic agents are navelbene, CPT-11, anastrazole, letrazole, capecitabine, reloxafine, cyclophosphamide, ifosamide, and droloxafine.

[00107] Microtubule affecting agents that have antiproliferative activity are also suitable for use and include, but are not limited to, allocolchicine (NSC 406042), Halichondrin B (NSC 609395), colchicine (NSC 757), colchicine derivatives (e.g., NSC 33410), dolstatin 10 (NSC 376128), maytansine (NSC 153858), rhizoxin (NSC 332598), paclitaxel (Taxol®), Taxol® derivatives, docetaxel (Taxotere®), thiocolchicine (NSC 361792), trityl cysterin, vinblastine sulfate, vincristine sulfate, natural and synthetic epothilones including but not limited to, eopthilone A, epothilone B, discodermolide; estramustine, nocodazole, and the like.

[00108] Hormone modulators and steroids (including synthetic analogs) that are suitable for use include, but are not limited to, adrenocorticosteroids, e.g. prednisone, dexamethasone, etc.; estrogens and pregestins, e.g. hydroxyprogesterone caproate, medroxyprogesterone acetate, megestrol acetate, estradiol, clomiphene, tamoxifen; etc.; and adrenocortical suppressants, e.g. aminoglutethimide; 17α-ethinylestradiol; diethylstilbestrol, testosterone, fluoxymesterone, dromostanolone propionate, testolactone, methylprednisolone, methyl-testosterone, prednisolone, triamcinolone, chlorotrianisene, hydroxyprogesterone, aminoglutethimide, estramustine, medroxyprogesterone acetate, leuprolide, Flutamide (Drogenil), Toremifene (Fareston), and Zoladex. Estrogens stimulate proliferation and differentiation, therefore compounds that bind to the estrogen receptor are used to block this activity. Corticosteroids may inhibit T cell proliferation.

[00109] Other chemotherapeutic agents include metal complexes, e.g. cisplatin (cis-DDP), carboplatin, etc.; ureas, e.g. hydroxyurea; and hydrazines, e.g. N-methylhydrazine; epidophyllotoxin; a topoisomerase inhibitor; procarbazine; mitoxantrone; leucovorin; tegafur; etc.. Other anti-proliferative agents of interest include immunosuppressants, e.g. mycophenolic acid, thalidomide, desoxyspergualin, azasporine, leflunomide, mizoribine, azaspirane (SKF 105685); Iressa® (ZD 1839, 4-(3-chloro-4-fluorophenylamino)-7-methoxy-6-(3-(4-morpholinyl)propoxy)quinazoline); etc.

[00110] "Taxanes" include paclitaxel, as well as any active taxane derivative or pro-drug. "Paclitaxel" (which should be understood herein to include analogues, formulations, and derivatives such as, for example, docetaxel, TAXOL™, TAXOTERE™ (a formulation of docetaxel), 10-desacetyl analogs of paclitaxel and 3'N-desbenzoyl-3'N-t-butoxycarbonyl analogs of paclitaxel) may be readily prepared utilizing techniques known to those skilled in the art (see also WO 94/07882, WO 94/07881, WO 94/07880, WO 94/07876, WO 93/23555, WO 93/10076; U.S. Pat. Nos. 5,294,637; 5,283,253; 5,279,949; 5,274,137; 5,202,448; 5,200,534; 5,229,529; and EP 590,267), or obtained from a variety of commercial sources, including for example, Sigma Chemical Co., St. Louis, Mo. (T7402 from *Taxus brevifolia*; or T-1912 from *Taxus yannanensis*).

[00111] Paclitaxel should be understood to refer to not only the common chemically available form of paclitaxel, but analogs and derivatives (e.g., Taxotere™ docetaxel, as noted above) and paclitaxel conjugates (e.g., paclitaxel-PEG, paclitaxel-dextran, or paclitaxel-xylose).

[00112] Also included within the term "taxane" are a variety of known derivatives, including both hydrophilic derivatives, and hydrophobic derivatives. Taxane derivatives include, but not limited to, galactose and mannose derivatives described in International Patent Application No. WO 99/18113; piperazino and other derivatives described in WO 99/14209; taxane derivatives described in WO 99/09021, WO 98/22451, and U.S. Patent No. 5,869,680; 6-thio derivatives described in WO 98/28288; sulfenamide derivatives described in U.S. Patent No. 5,821,263; and taxol derivative described in U.S. Patent No. 5,415,869. It further includes prodrugs of paclitaxel including, but not limited to, those described in WO 98/58927; WO 98/13059; and U.S. Patent No. 5,824,701.

[00113] In some instances, methods of treating a subject for cancer may further include administering an agent which enhances the activity of the treatment. Such agents that enhance the activity of the treatment will vary widely and may include but are not limited to e.g., agents that inhibit an inhibitor molecule. Suitable inhibitory molecules that may be targeted include but are not limited to e.g., PD1, PD-L1, CTLA4, TIM3, LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 and TGFR beta.

[00114] Inhibiting of inhibitory molecules may be achieved by any convenient method including but not limited to e.g., the administration of a direct inhibitor of the inhibitory molecule (e.g., an antibody that binds the inhibitory molecule, a small molecule antagonist of the inhibitory molecule, etc.), administration of an agent that inhibits expression of the inhibitory molecule

(e.g., an inhibitory nucleic acid, e.g., a dsRNA, e.g., an siRNA or shRNA targeting a nucleic acid encoding the inhibitory molecule), an indirect inhibitor of the inhibitory signaling, and the like. In some instances, an agent that may be administered may be an antibody or antibody fragment that binds to an inhibitory molecule. For example, the agent can be an antibody or antibody fragment that binds to PD1, PD-L1, PD-L2 or CTLA4 (e.g., ipilimumab (also referred to as MDX-010 and MDX-101, and marketed as Yervoy (Bristol-Myers Squibb)), Tremelimumab (Pfizer, formerly known as ticilimumab, CP-675,206)), TIM3, LAG3, or the like.

[00115] In some instances, the methods of the instant disclosure may be used without any additional conventional therapy including e.g., where the method described herein is the sole method used to treat the subject. For example, in the case of oncology, the methods described herein may, in some instances, be the sole method used to treat the subject for a GBM, including e.g., a primary GBM, a recurrent GBM, and the like.

[00116] Determining when combination therapies, e.g., involving the administration of one or more agents that ameliorates one or more side effects of a therapy described herein or involving the administration of one or more agents that enhances a therapy described herein, are indicated and the specifics of the administration of such combination therapies are within the skill of the relevant medical practitioner. In some instances, dosage regimens and treatment schedules of combination therapies may be determined through clinical trials.

Testing

[00117] As summarized above, the methods of the present disclosure may, in some instances, include testing, where such testing may include but is not limited to e.g., testing of the subject, testing of a biological sample obtained from the subject, and the like. In some instances, methods of the present disclosure may include testing and/or evaluating a subject for a heterogeneous GBM. Testing may be employed, in some instances, to determine or identify whether a subject has a heterogeneous GBM or whether a GBM, in a subject known to have such, is a heterogeneous GBM.

[00118] In some instances, a GBM of a subject may be tested or evaluated to determine, detect or identify whether the GBM expresses one or more particular antigens, including but not limited to e.g., an EGFRvIII antigen and/or a targeting antigen, including but not limited to e.g., Ephrin type-A receptor 2 (EphA2), Ephrin type-A receptor 3 (EphA3), a Interleukin-13 receptor (IL13R) (e.g., IL13RA1 or IL13RA2), Epidermal growth factor receptor (EGFR), erb-b2

receptor tyrosine kinase 2 (ERBB2), combinations thereof and the like. In some instances, whether a method of the present disclosure is employed and/or the particular combination of EGFRvIII priming antigen and targeting antigen(s) employed in a subject circuit may be determined based on testing the subject for particular antigen expression in the cells of the subject's GBM.

[00119] Subjects suitable for testing will include those that have or have not been previously treated for a GBM including a heterogeneous GBM. For example, in some instances, a subject may have been recently diagnosed with a GBM and the subject may be tested, e.g., to evaluate the presence of EGFRvIII priming antigen and/or one or more targeting antigens, before any treatment of the diagnosed GBM. In some instances, the subject may have been previously treated for a GBM and the subject may be tested, e.g., to evaluate the presence of EGFRvIII priming antigen and/or one or more targeting antigens, after treatment of the diagnosed GBM, including e.g., where the subject's GBM is responsive or refractory to the prior treatment. In some instances, the subject may be undergoing treatment for a GBM and the subject may be tested, e.g., to evaluate the presence of EGFRvIII priming antigen and/or one or more targeting antigens, during the treatment of the diagnosed GBM, including e.g., where the subject's GBM is responsive or refractory to the ongoing treatment or where the subject's response is as yet unknown.

[00120] Testing of a subject may include assaying a biological sample obtained from the subject. Useful biological samples may include but are not limited to e.g., biopsy (e.g., a GBM tumor biopsy, etc.), blood samples, and the like. Any convenient method of collecting a biological sample may find use in the herein described methods including but not limited to e.g., needle biopsy, stereotactic biopsy, open biopsy, and the like.

[00121] In a brain tumor needle biopsy, a small cut may be made and a small hole, called a burr hole, may be drilled in the skull. A narrow, hollow needle may be inserted through the hole, and tumor tissue may be removed from the core of the needle. In a stereotactic biopsy (a.k.a. a "closed" biopsy) of a brain tumor, the same general procedure may be employed as described for a needle biopsy; however, a computer-assisted guidance system that aids in the location and diagnosis of the tumor may be employed. A computer, using information from a CT or MRI scan, may provide precise information about a tumor's location and its position relative to other structures in the brain. Stereotactically guided equipment might be moved into the burr hole to remove a sample of the tumor. In an open biopsy of a brain tumor a tissue sample is taken

during an operation while the tumor is exposed. The sample, regardless of the biopsy method employed for collection, may then be sent for study and review, e.g., by a pathologist.

[00122] Any convenient method of assaying a biological sample may find use in the herein described methods including but not limited to e.g., a blood chemistry test, cancer gene mutation testing, complete blood count (CBC), cytogenetic analysis, immunophenotyping, tumor marker tests, histology, cytology (including e.g., flow cytometry, including FACS), immunohistochemistry, gene expression analysis, proteomics, in situ hybridization, and the like. For example, in some instances, immunohistochemistry and/or in situ hybridization may be performed on a biopsy sample obtained from the subject, e.g., to detect the expression of one or more antigens. In some instances, cytology may be performed on a blood sample from the subject, e.g., to detect circulating tumor cells (CTCs).

[00123] In some instances, antigen detection in a biological sample may include molecular detection of antigen transcript. Any convenient method of transcript detection may be employed including but not limited to PCR-based assays. Antigen transcript detection may find use in various embodiments of the herein described methods, including but not limited to e.g., where the methods include determining whether one of more cells from a sample of a subject express EGFRvIII, EGFR or both EGFRvIII, EGFR and/or performing quantification of the level(s) of expression thereof.

[00124] In some instances, testing of a subject may include multi-sampling. Multi-sampling, as used herein, generally refers to the process of taking multiple samples of a suspected tumor and/or multiple samples of multiple tumors present in a subject. Multi-sampling may be performed at one instance, e.g., where multiple samples are collected from various locations during one period of collection, or over multiple instances, e.g., were one or more sites are sampled over at multiple instances over a period of time. Multi-sampling may find use in subject with heterogeneous cancers, e.g., to ensure that the heterogeneity of a cancer or tumor is sufficiently sampled, e.g., to detect the cellular distribution and/or antigen distribution of a particular cancer or tumor.

[00125] In some instances, a subject may be evaluated, in certain contexts, through one or more of the following diagnostics procedures: 3D CT angiography, Angiography, Anoscopy, Autofluorescence bronchoscopy/fluorescence bronchoscopy, Barium swallow or enema, Biopsy, Bone Marrow Aspiration and Biopsy, Bone Scan, Bronchoscopy, CA-125 test, CAD for mammography, CTC Test, Chest x-ray, Colonoscopy, Complete Blood Count Test, Computed

Tomography Scan, CT-guided biopsy, DEXA scan, Digital Breast Tomosynthesis, Electrocardiogram, Endobronchial ultrasound, Endoscopic ultrasound, ERCP, Flow cytometry, Full-field digital mammography, Genetic testing, Large bore CT scanner/RT with simulation, Lumbar puncture, Magnetic Resonance Imaging, Mammography, Miraluma breast imaging, MRI-Guided Breast Biopsy, Multi-detector CT scanner, Multiple-gated acquisition (MUGA) scan, Navigational Bronchoscopy, Nuclear Medicine Imaging, Oncotype DX Test, Pap test, Pelvic exam, PET Scan, PET-CT Scan, Radiofrequency ablation, Sentinel lymph node biopsy, Spiral CT, Tumor marker testing, Tumor molecular profiling, Ultrasound, Video Capsule Endoscopy, X-ray, and the like.

[00126] Diagnostic procedures may be performed for a variety of reasons including but not limited to e.g., to screen for GBM or precancerous conditions indicative of increased risk of GBM (e.g., CMV infection) before a person has any symptoms of disease; to help diagnose GBM; to provide information about the stage of a GBM; to provide information about the malignancy of a GBM; to provide information about the size and/or extent of a primary GBM; to provide information about whether or not a GBM has metastasized; to plan treatment; to monitor a patient's general health during treatment; to check for potential side effects of the treatment; to determine whether a GBM is responding to treatment; to find out whether a GBM has recurred; etc.

Antigens

[00127] Antigens employed in the present methods include, as described above, the EGFRvIII priming antigen and one or more targeting antigens and others in some instances. In instances where the targeted cell is targeted for killing, the subject targeting antigen may be referred to herein as a "killing antigen". Such terms may, but need not necessarily, be used interchangeably where appropriate.

[00128] As described herein with regards to cancer heterogeneity, the relative presence of an antigen and/or the relative presence of cells expressing an antigen will vary. In general, less than 100% of the cells of a heterogeneous cancer treated with the described methods will express EGFRvIII antigen, including but not limited to e.g., where less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, less than 50%, less than 45%, less than 40%, less than 35%, less than 30%, less than 25%, less than 20% of cells of the heterogeneous cancer express EGFRvIII antigen.

[00129] In some instances, all cells of a heterogeneous GBM may express an employed killing antigen. Such heterogeneous GBMs may be said to be homogeneous for killing antigen expression. In some instances, a heterogeneous GBM may be heterogeneous for EGFRvIII antigen expression but homogeneous for killing antigen expression. Accordingly, in certain embodiments, certain cells of the heterogeneous GBM may express both the priming antigen and the killing antigen. In such instances, the methods of the present disclosure may be employed where the heterogeneous GBM still includes cells that express the killing antigen but not the priming antigen.

[00130] In some instances, a heterogeneous GBM may be heterogeneous for both EGFRvIII priming antigen expression and targeting/killing antigen expression, including where the targeting/killing antigen is expressed by less than 100% of the cells of the heterogeneous GBM. In some instances, the targeting/killing antigen may be expressed in a majority of the cells of the heterogeneous GBM but less than 100% of the cells, including but not limited to e.g., where more than 95%, more than 90%, more than 85%, more than 80%, more than 75%, more than 70%, more than 65%, more than 60%, more than 55%, or more than 50% of the cells of the heterogeneous GBM.

[00131] In some instances, multiple antigen-specific therapeutics targeting different targeting/killing antigens may be employed. In some instances, antigen-specific therapeutics targeting multiple different targeting/killing antigens may be employed. In some instances, multiple targeting/killing antigens may be targeted in cases where targeting/killing antigen expression is heterogeneous, including where e.g., one or more of the subject targeting/killing antigens is expressed by a majority of the cells of the GBM, where one or more of the subject targeting/killing antigens is expressed by a minority of the cells of the GBM, and the like. In some instances, the targeting of two or more different targeting/killing antigens results in combination of antigens employed targeting 100% or nearly 100% (e.g., 99% or greater, 98% or greater, 95% or greater, 90% or greater, etc.) of the cells of the GBM.

[00132] Useful antigens that may be employed as targeting antigens include but are not limited to e.g., Ephrin type-A receptor 2 (EphA2), Ephrin type-A receptor 3 (EphA3), a Interleukin-13 receptor (IL13R) (e.g., IL13RA1 or IL13RA2), Epidermal growth factor receptor (EGFR), erb-b2 receptor tyrosine kinase 2 (ERBB2) and the like. In some instances, EGFRvIII may find use as a targeting antigen. For example, in some instances, EGFRvIII may be employed as both a priming antigen and a killing antigen, including but not limited to e.g., as in a AND-OR gate

where EGFRvIII functions as a priming antigen to induce expression of one or more antigen-specific therapeutics specific for EGFRvIII as a first targeting/killing antigen and a second targeting/killing antigen. In such instances, the second targeting/killing antigen may, but need not necessarily, be selected from EphA2, EphA3, IL13RA1, IL13RA2, EGFR, and ERBB2.

[00133] In some instances, useful targeting/killing antigens include Ephrin type-A receptor 2 (EphA2). EphA2 is a receptor tyrosine kinase encoded by the EPH receptor A2 gene located at 1p36.13 in humans. EphA2 protein may be found in at least two isoforms in humans, including EphA2 Isoform 1 having the following amino acid sequence:

MELQAARACFALLWGCALAAAAAAQGKEVVLLDFAAAGGELGWLTHPYGKGWDLM
QNIMNDMPIYMYSVCNVMSGDQDNWLRTNWVYRGEAERIFIELKFTVRDCNSFPGGA
SSCKETFNLYYAESDLDYGTNFQKRLFTKIDTIAPDEITVSSDFEARHVKLNVEERSVGP
LTRKGFYLAFQDIGACVALLSVRVYYKKCPELLQGLAHFPETIAGSDAPSLATVAGTCV
DHAVVPPGGEEPRMHCAVDGEWLVPIGQCLCQAGYEKVEDACQACSPGFFKFEASESP
CLECPEHTLPSPEGATSCECEEGFFRAPQDPASMPCTRPPSAPHYLTAVGMGAKVELRW
TPPQDSGGREDIVYSVTCEQCWPESGECGPCEASVRYSEPPHGLTRTSVTVSDLEPHMN
YTFTVEARNGVSGLVTSRSFRTASVSINQTEPPKVRLEGRSTTSLSVSWSIPPPQQSRVW
KYEVTYRKKGDSNSYNVRRTEGFSVTLDDLAPDTTYLVQVQALTQEGQGAGSKVHEF
QTLSPEGSGNLAVIGGVAVGVVLLLVLAGVGFFIHRRRKNQRARQSPEDVYFSKSEQLK
PLKTYVDPHTYEDPNQAVLKFTTEIHPSCVTRQKVIGAGEFGEVYKGMLKTSSGKKEVP
VAIKTLKAGYTEKQRVDFLGEAGIMGQFSHHNIIRLEGVISKYKPMMIITEYMENGALD
KFLREKDGEFSVLQLVGMLRGIAAGMKYLANMNYVHRDLAARNILVNSNLVCKVSDF
GLSRVLEDDPEATYTTSGGKIPIRWTAPEAISYRKFTSASDVWSFGIVMWEVMTYGERP
YWELSNHEVMKAINDGFRLPTPMDCPSAIYQLMMQCWQQERARRPKFADIVSILDKLI
RAPDSLKTLADFDPRVSIRLPSTSGSEGVPFRTVSEWLESIKMQQYTEHFMAAGYTAIEK
VVQMTNDDIKRIGVRLPGHQKRIAYSLLGLKDQVNTVGIPI (SEQ ID NO:1);

and EphA2 Isoform 2 having the following amino acid sequence:

MELQAARACFALLWGCALAAAAAAQGKEVVLLDFAAAGGELGWLTHPYGKGWDLM
QNIMNDMPIYMYSVCNVMSGDQDNWLRTNWVYRGEAERIFIELKFTVRDCNSFPGGA
SSCKETFNLYYAESDLDYGTNFQKRLFTKIDTIAPDEITVSSDFEARHVKLNVEERSVGP
LTRKGFYLAFQDIGACVALLSVRVYYKKCPELLQGLAHFPETIAGSDAPSLATVAGTCV
DHAVVPPGGEEPRMHCAVDGEWLVPIGQCLCQAGYEKVEDACQACSPGFFKFEASESP
CLECPEHTLPSPEGATSCECEEGFFRAPQDPASMPCTRPPSAPHYLTAVGMGAKVELRW

TPPQDSGGREDIVYSVTCEQCWPESGECGPCEASVRYSEPPHGLTRTSVTVSDLEPHMN YTFTVEARNGVSGLVTSRSFRTASVSINQTEPPKVRLEGRSTTSLSVSWSIPPPQQSRVW KYEVTYRKKVTPRGAGLALAGPTAGDRLVT (SEQ ID NO:2).

[00134] In some instances, the methods described herein may employ an antigen-specific therapeutic that specifically binds EphA2, including e.g., human EphA2 Isoform 1, human EphA2 Isoform 2, or both human EphA2 Isoform 1 and human EphA2 Isoform 2.

[00135] In some instances, useful EphA2 binding domains may include antibody based EphA2 binding domains, including but not limited to an EphA2 scFv. In some instances, a useful EphA2 scFv may have the following amino acid sequence or a variant thereof: EIVLTQSPGTLSLSPGERATLSCRASQSVSSSNLAWYQQKPGQAPRLLIYGASSRATGVP DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQSSSYPWTFGQGTKVEIKRTGGGGSGAGG SGGGGTGGGGSEVDLLESGGGLVQPGGSLRLSCAASGFTFSRYWMHWVRQAPGKGLE WVSSISPYDGETNYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARISEWYN WAVDVFDYWGQGTLVTVSS (SEQ ID NO:3);

including e.g., where the useful EphA2 has a sequence identity of 100% or less, including e.g., at least 99%, at least 98%, at least 97%, at least 96%, at least 95%, at least 90%, at least 85%, at least 80%, etc., sequence identity with the sequence presented above.

[00136] In some instances, a useful EphA2 scFv may have the following amino acid sequence or a variant thereof:

QVQLLESGGGLVQPGGSLRLSCAASGFTFSSYTMSWVRQAPGQALEWMGTISSGGTYT YYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAREAIFTYWGRGTLVTSSGG GGSGGGGSGGGGSDIQLTQSPSSLSASVGDRVTITCKASQDINNYLSWYQQKPGQAPRL LIYRANRLVDGVPDRFSGSGYGTDFTLTINNIESEDAAYYFCLKYDVFPYTFGQGTKVEI KS (SEQ ID NO:4);

including e.g., where the useful EphA2 has a sequence identity of 100% or less, including e.g., at least 99%, at least 98%, at least 97%, at least 96%, at least 95%, at least 90%, at least 85%, at least 80%, etc., sequence identity with the sequence presented above.

[00137] In some instances, useful EphA2 binding domains include those described in Goldgur et al., Growth Factors. (2014) 32(6):214-22 and Damschroder et al., Mol Immunol. (2007) 44(11):3049-60; the disclosures of which are incorporated herein by reference in their entirety.

[00138] In some instances, useful targeting/killing antigens include Ephrin type-A receptor 3 (EphA3). EphA3 is a receptor tyrosine kinase encoded by the EPH receptor A3 gene located at

3p11.1 in humans. EphA3 protein may be found in at least two isoforms, including EphA3 Isoform 1 having the following amino acid sequence:

MDCQLSILLLLSCSVLDSFGELIPQPSNEVNLLDSKTIQGELGWISYPSHGWEEISGVDEH
YTPIRTYQVCNVMDHSQNNWLRTNWVPRNSAQKIYVELKFTLRDCNSIPLVLGTCKET
FNLYYMESDDDHGVKFREHQFTKIDTIAADESFTQMDLGDRILKLNTEIREVGPVNKKG
FYLAFQDVGACVALVSVRVYFKKCPFTVKNLAMFPDTVPMDSQSLVEVRGSCVNNSK
EEDPPRMYCSTEGEWLVPIGKCSCNAGYEERGFMCQACRPGFYKALDGNMKCAKCPP
HSSTQEDGSMNCRCENNYFRADKDPPSMACTRPPSSPRNVISNINETSVILDWSWPLDT
GGRKDVTFNIICKKCGWNIKQCEPCSPNVRFLPRQFGLTNTTVTVTDLLAHTNYTFEID
AVNGVSELSSPPRQFAAVSITTNQAAPSPVLTIKKDRTSRNSISLSWQEPEHPNGIILDYE
VKYYEKQEQETSYTILRARGTNVTISSLKPDTIYVFQIRARTAAGYGTNSRKFEFETSPD
SFSISGESSQVVMIAISAAVAIILLTVVIYVLIGRFCGYKSKHGADEKRLHFGNGHLKLPG
LRTYVDPHTYEDPTQAVHEFAKELDATNISIDKVVGAGEFGEVCSGRLKLPSKKEISVAI
KTLKVGYTEKQRRDFLGEASIMGQFDHPNIIRLEGVVTKSKPVMIVTEYMENGSLDSFL
RKHDAQFTVIQLVGMLRGIASGMKYLSDMGYVHRDLAARNILINSNLVCKVSDFGLSR
VLEDDPEAAYTTRGGKIPIRWTSPEAIAYRKFTSASDVWSYGIVLWEVMSYGERPYWE
MSNQDVIKAVDEGYRLPPPMDCPAALYQLMLDCWQKDRNNRPKFEQIVSILDKLIRNP
GSLKIITSAAARPSNLLLDQSNVDITTFRTTGDWLNGVWTAHCKEIFTGVEYSSCDTIAK
ISTDDMKKVGVTVVGPQKKIISSIKALETQSKNGPVPV (SEQ ID NO:5);

and EphA3 Isoform 2 having the following amino acid sequence:

MDCQLSILLLLSCSVLDSFGELIPQPSNEVNLLDSKTIQGELGWISYPSHGWEEISGVDEH
YTPIRTYQVCNVMDHSQNNWLRTNWVPRNSAQKIYVELKFTLRDCNSIPLVLGTCKET
FNLYYMESDDDHGVKFREHQFTKIDTIAADESFTQMDLGDRILKLNTEIREVGPVNKKG
FYLAFQDVGACVALVSVRVYFKKCPFTVKNLAMFPDTVPMDSQSLVEVRGSCVNNSK
EEDPPRMYCSTEGEWLVPIGKCSCNAGYEERGFMCQACRPGFYKALDGNMKCAKCPP
HSSTQEDGSMNCRCENNYFRADKDPPSMACTRPPSSPRNVISNINETSVILDWSWPLDT
GGRKDVTFNIICKKCGWNIKQCEPCSPNVRFLPRQFGLTNTTVTVTDLLAHTNYTFEID
AVNGVSELSSPPRQFAAVSITTNQAAPSPVLTIKKDRTSRNSISLSWQEPEHPNGIILDYE
VKYYEKQEQETSYTILRARGTNVTISSLKPDTIYVFQIRARTAAGYGTNSRKFEFETSPD
CMYYFNAV (SEQ ID NO:6).

[00139] In some instances, the methods described herein may employ an antigen-specific therapeutic that specifically binds EphA3, including e.g., human EphA3 Isoform 1, human EphA3 Isoform 2, or both human EphA3 Isoform 1 and human EphA3 Isoform 2.

[00140] In some instances, useful targeting/killing antigens include receptors for Interleukin-13 (IL13). IL13 is an immunoregulatory cytokine encoded by the interleukin 13 gene located at 5q31.1 in humans, which is a ligand for IL13R proteins: interleukin 13 receptor subunit alpha 1 (IL13RA1) and interleukin 13 receptor subunit alpha 2 (IL13RA2). An exemplary amino acid sequence of human IL13 is as follows:

MHPLLNPLLLALGLMALLLTTVIALTCLGGFASPGPVPPSTALRELIEELVNITQNQKAP
LCNGSMVWSINLTAGMYCAALESLINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHV
RDTKIEVAQFVKDLLLHLKKLFREGRFN (SEQ ID NO:7).

[00141] IL13RA1 is encoded by the interleukin 13 receptor subunit alpha 1 gene, located in humans at Xq24, and is a subunit of the interleukin 13 receptor which forms a receptor complex with IL4 receptor alpha, a subunit shared by IL13 and IL4 receptors. IL13RA1 is a primary IL13-binding subunit of the IL13 receptor. IL13RA1 protein may be found in at least two isoforms, including IL13RA1 Isoform 1 having the following amino acid sequence:

MEWPARLCGLWALLLCAGGGGGGGGAAPTETQPPVTNLSVSVENLCTVIWTWNPPEG
ASSNCSLWYFSHFGDKQDKKIAPETRRSIEVPLNERICLQVGSQCSTNESEKPSILVEKCI
SPPEGDPESAVTELQCIWHNLSYMKCSWLPGRNTSPDTNYTLYYWHRSLEKIHQCENIF
REGQYFGCSFDLTKVKDSSFEQHSVQIMVKDNAGKIKPSFNIVPLTSRVKPDPPHIKNLS
FHNDDLYVQWENPQNFISRCLFYEVEVNNSQTETHNVFYVQEAKCENPEFERNVENTS
CFMVPGVLPDTLNTVRIRVKTNKLCYEDDKLWSNWSQEMSIGKKRNSTLYITMLLIVP
VIVAGAIIVLLLYLKRLKIIIFPPIPDPGKIFKEMFGDQNDDTLHWKKYDIYEKQTKEETD
SVVLIENLKKASQ (SEQ ID NO:8);

and IL13RA1 Isoform 2 having the following amino acid sequence:

MEWPARLCGLWALLLCAGGGGGGGGAAPTETQPPVTNLSVSVENLCTVIWTWNPPEG
ASSNCSLWYFSHFGDKQDKKIAPETRRSIEVPLNERICLQVGSQCSTNESEKPSILVEKCI
SPPEGDPESAVTELQCIWHNLSYMKCSWLPGRNTSPDTNYTLYYWHRSLEKIHQCENIF
REGQYFGCSFDLTKVKDSSFEQHSVQIMVKDNAGKIKPSFNIVPLTSRVKPDPPHIKNLS
FHNDDLYVQWENPQNFISRCLFYEVEVNNSQTETHNVFYVRF (SEQ ID NO:9).

[00142] IL13RA2 is encoded by the interleukin 13 receptor subunit alpha 2 gene, located in humans at Xq23, and is a subunit of the interleukin 13 receptor complex. IL13RA2 binds IL13

with high affinity, but lacks cytoplasmic domain. IL13RA2 protein may be found in at least one isoform, including IL13RA2 having the following amino acid sequence:
MAFVCLAIGCLYTFLISTTFGCTSSSDTEIKVNPPQDFEIVDPGYLGYLYLQWQPPLSLD
HFKECTVEYELKYRNIGSETWKTIITKNLHYKDGFDLNKGIEAKIHTLLPWQCTNGSEV
QSSWAETTYWISPQGIPETKVQDMDCVYYNWQYLLCSWKPGIGVLLDTNYNLFYWYE
GLDHALQCVDYIKADGQNIGCRFPYLEASDYKDFYICVNGSSENKPIRSSYFTFQLQNIV
KPLPPVYLTFTRESSCEIKLKWSIPLGPIPARCFDYEIEIREDDTTLVTATVENETYTLKTT
NETRQLCFVVRSKVNIYCSDDGIWSEWSDKQCWEGEDLSKKTLLRFWLPFGFILILVIFV
TGLLLRKPNTYPKMIPEFFCDT (SEQ ID NO:10).

[00143] In some instances, the methods described herein may employ an antigen-specific therapeutic that specifically binds an IL13R, including IL13RA1 and/or IL13RA2, including e.g., human IL13RA1 Isoform 1, human IL13RA1 Isoform 2, human IL13RA2, or any combination thereof. In some instances, useful IL13R binding domains may be derived from IL13, including but not limited to IL13 conjugation products (e.g., wild-type or mutated IL13 conjugated to one or more moieties), derivatives or mutants of IL13, e.g., IL13 muteins, and the like. Useful muteins include but are not limited to e.g., IL13 muteins including one or more amino acid substitutions including E13K and/or K105R.

[00144] In some instances, as summarized above, useful IL13R binding domains may include a ligand-based binding domain derived from IL13, including but not limited to an IL13 mutein-based binding domain. In some instances, a useful IL13 mutein-based binding domain may have the following amino acid sequence or a variant thereof:
LTCLGGFASPGPVPPSTALRKLIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALES
LINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLHLRKLFRE
GRFN (SEQ ID NO:11);
including e.g., where the useful IL13 mutein-based binding domain has a sequence identity of 100% or less, including e.g., at least 99%, at least 98%, at least 97%, at least 96%, at least 95%, at least 90%, at least 85%, at least 80%, etc., sequence identity with the sequence presented above.

[00145] In some instances, useful IL13R binding domains include those described in Krebs et al., Cytotherapy. (2014) 16(8):1121-31; the disclosure of which is incorporated herein by reference in its entirety.

[00146] In some instances, useful targeting/killing antigens include epidermal growth factor receptor (EGFR, also known as Proto-oncogene c-ErbB-1, Receptor tyrosine-protein kinase erbB-1, ERBB, HER1, mENA, ERBB1, PIG61, and NISBD2). EGFR is a receptor tyrosine kinase encoded by the epidermal growth factor receptor gene, present at 7p11.2 in humans. Known ligands of EGFR include EGF, TGFA/TGF-alpha, amphiregulin, epigen/EPGN, BTC/betacellulin, epiregulin/EREG and HBEGF/heparin-binding EGF. EGFR protein may be found in at least four isoforms, including EGFR Isoform 1 having the following amino acid sequence:

MRPSGTAGAALLALLAALCPASRALEEKKVCQGTSNKLTQLGTFEDHFLSLQRMFNNC
EVVLGNLEITYVQRNYDLSFLKTIQEVAGYVLIALNTVERIPLENLQIIRGNMYYENSYA
LAVLSNYDANKTGLKELPMRNLQEILHGAVRFSNNPALCNVESIQWRDIVSSDFLSNMS
MDFQNHLGSCQKCDPSCPNGSCWGAGEENCQKLTKIICAQQCSGRCRGKSPSDCCHNQ
CAAGCTGPRESDCLVCRKFRDEATCKDTCPPLMLYNPTTYQMDVNPEGKYSFGATCV
KKCPRNYVVTDHGSCVRACGADSYEMEEDGVRKCKKCEGPCRKVCNGIGIGEFKDSL
SINATNIKHFKNCTSISGDLHILPVAFRGDSFTHTPPLDPQELDILKTVKEITGFLLIQAWP
ENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLNITSLGLRSLKEISDGDVIISGNKNLCYA
NTINWKKLFGTSGQKTKIISNRGENSCKATGQVCHALCSPEGCWGPEPRDCVSCRNVSR
GRECVDKCNLLEGEPREFVENSECIQCHPECLPQAMNITCTGRGPDNCIQCAHYIDGPH
CVKTCPAGVMGENNTLVWKYADAGHVCHLCHPNCTYGCTGPGLEGCPTNGPKIPSIA
TGMVGALLLLLVVALGIGLFMRRRHIVRKRTLRRLLQERELVEPLTPSGEAPNQALLRI
LKETEFKKIKVLGSGAFGTVYKGLWIPEGEKVKIPVAIKELREATSPKANKEILDEAYV
MASVDNPHVCRLLGICLTSTVQLITQLMPFGCLLDYVREHKDNIGSQYLLNWCVQIAK
GMNYLEDRRLVHRDLAARNVLVKTPQHVKITDFGLAKLLGAEEKEYHAEGGKVPIKW
MALESILHRIYTHQSDVWSYGVTVWELMTFGSKPYDGIPASEISSILEKGERLPQPPICTI
DVYMIMVKCWMIDADSRPKFRELIIEFSKMARDPQRYLVIQGDERMHLPSPTDSNFYR
ALMDEEDMDDVVDADEYLIPQQGFFSSPSTSRTPLLSSLSATSNNSTVACIDRNGLQSCP
IKEDSFLQRYSSDPTGALTEDSIDDTFLPVPEYINQSVPKRPAGSVQNPVYHNQPLNPAPS
RDPHYQDPHSTAVGNPEYLNTVQPTCVNSTFDSPAHWAQKGSHQISLDNPDYQQDFFP
KEAKPNGIFKGSTAENAEYLRVAPQSSEFIGA (SEQ ID NO:12),

EGFR Isoform 2 having the following amino acid sequence:

MRPSGTAGAALLALLAALCPASRALEEKKVCQGTSNKLTQLGTFEDHFLSLQRMFNNC
EVVLGNLEITYVQRNYDLSFLKTIQEVAGYVLIALNTVERIPLENLQIIRGNMYYENSYA

LAVLSNYDANKTGLKELPMRNLQEILHGAVRFSNNPALCNVESIQWRDIVSSDFLSNMS
MDFQNHLGSCQKCDPSCPNGSCWGAGEENCQKLTKIICAQQCSGRCRGKSPSDCCHNQ
CAAGCTGPRESDCLVCRKFRDEATCKDTCPPLMLYNPTTYQMDVNPEGKYSFGATCV
KKCPRNYVVTDHGSCVRACGADSYEMEEDGVRKCKKCEGPCRKVCNGIGIGEFKDSL
SINATNIKHFKNCTSISGDLHILPVAFRGDSFTHTPPLDPQELDILKTVKEITGLS (SEQ ID NO:13),

EGFR Isoform 3 having the following amino acid sequence:

MRPSGTAGAALLALLAALCPASRALEEKKVCQGTSNKLTQLGTFEDHFLSLQRMFNNC
EVVLGNLEITYVQRNYDLSFLKTIQEVAGYVLIALNTVERIPLENLQIIRGNMYYENSYA
LAVLSNYDANKTGLKELPMRNLQEILHGAVRFSNNPALCNVESIQWRDIVSSDFLSNMS
MDFQNHLGSCQKCDPSCPNGSCWGAGEENCQKLTKIICAQQCSGRCRGKSPSDCCHNQ
CAAGCTGPRESDCLVCRKFRDEATCKDTCPPLMLYNPTTYQMDVNPEGKYSFGATCV
KKCPRNYVVTDHGSCVRACGADSYEMEEDGVRKCKKCEGPCRKVCNGIGIGEFKDSL
SINATNIKHFKNCTSISGDLHILPVAFRGDSFTHTPPLDPQELDILKTVKEITGFLLIQAWP
ENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLNITSLGLRSLKEISDGDVIISGNKNLCYA
NTINWKKLFGTSGQKTKIISNRGENSCKATGQVCHALCSPEGCWGPEPRDCVSCRNVSR
GRECVDKCNLLEGEPREFVENSECIQCHPECLPQAMNITCTGRGPDNCIQCAHYIDGPH
CVKTCPAGVMGENNTLVWKYADAGHVCHLCHPNCTYGPGNESLKAMLFCLFKLSSC
NQSNDGSVSHQSGSPAAQESCLGWIPSLLPSEFQLGWGGCSHLHAWPSASVIITASSCH
(SEQ ID NO:14),

and EGFR Isoform 4 having the following amino acid sequence:

MRPSGTAGAALLALLAALCPASRALEEKKVCQGTSNKLTQLGTFEDHFLSLQRMFNNC
EVVLGNLEITYVQRNYDLSFLKTIQEVAGYVLIALNTVERIPLENLQIIRGNMYYENSYA
LAVLSNYDANKTGLKELPMRNLQEILHGAVRFSNNPALCNVESIQWRDIVSSDFLSNMS
MDFQNHLGSCQKCDPSCPNGSCWGAGEENCQKLTKIICAQQCSGRCRGKSPSDCCHNQ
CAAGCTGPRESDCLVCRKFRDEATCKDTCPPLMLYNPTTYQMDVNPEGKYSFGATCV
KKCPRNYVVTDHGSCVRACGADSYEMEEDGVRKCKKCEGPCRKVCNGIGIGEFKDSL
SINATNIKHFKNCTSISGDLHILPVAFRGDSFTHTPPLDPQELDILKTVKEITGFLLIQAWP
ENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLNITSLGLRSLKEISDGDVIISGNKNLCYA
NTINWKKLFGTSGQKTKIISNRGENSCKATGQVCHALCSPEGCWGPEPRDCVSCRNVSR
GRECVDKCNLLEGEPREFVENSECIQCHPECLPQAMNITCTGRGPDNCIQCAHYIDGPH
CVKTCPAGVMGENNTLVWKYADAGHVCHLCHPNCTYGS (SEQ ID NO:15).

[00147] In some instances, the methods described herein may employ an antigen-specific therapeutic that specifically binds an EGFR, including human EGFR, including e.g., human EGFR Isoform 1, human EGFR Isoform 2, human EGFR Isoform 3, EGFR Isoform 4, or any combination thereof.

[00148] In some instances, useful targeting/killing antigens include Erb-b2 receptor tyrosine kinase 2 (ERBB2; also known as Metastatic lymph node gene 19 protein, Proto-oncogene Neu, Proto-oncogene c-ErbB-2, Tyrosine kinase-type cell surface receptor HER2, NEU, NGL, HER2, TKR1, CD340, HER-2, MLN 19, and HER-2/neu). ERBB2 is a protein tyrosine kinase that is encoded by the erb-b2 receptor tyrosine kinase 2 gene, located at 17q12 in humans. ERBB2 protein may be found in various isoforms, including ERBB2 Isoform 1 having the following amino acid sequence:

MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQ
GNLELTYLPTNASLSFLQDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAV
LDNGDPLNNTTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHK
NNQLALTLIDTNRSRACHPCSPMCKGSRCWGESSEDCQSLTRTVCAGGCARCKGPLPT
DCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMPNPEGRYTF
GASCVTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYGLGME
HLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLEEITGY
LYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLALIH
HNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLACHQLCARGHCWGPGPT
QCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQC
VACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVDLDDKGC
PAEQRASPLTSIISAVVGILLVVVLGVVFGILIKRRQQKIRKYTMRRLLQETELVEPLTPS
GAMPNQAQMRILKETELRKVKVLGSGAFGTVYKGIWIPDGENVKIPVAIKVLRENTSPK
ANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQ
DLLNWCMQIAKGMSYLEDVRLVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEY
HADGGKVPIKWMALESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLL
EKGERLPQPPICTIDVYMIMVKCWMIDSECRPRFRELVSEFSRMARDPQRFVVIQNEDL
GPASPLDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSSST
RSGGGDLTLGLEPSEEEAPRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTHDPSPLQR
YSEDPTVPLPSETDGYVAPLTCSPQPEYVNQPDVRPQPPSPREGPLPAARPAGATLERPK

TLSPGKNGVVKDVFAFGGAVENPEYLTPQGGAAPQPHPPPAFSPAFDNLYYWDQDPPE
RGAPPSTFKGTPTAENPEYLGLDVPV (SEQ ID NO:16),

ERBB2 Isoform 2 having the following amino acid sequence:

MPIWKFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASPLTSIISAVVGILLVVVLGVV
FGILIKRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSG
AFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGI
CLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHR
DLAARNVLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILRRRFTHQS
DVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKCWMI
DSECRPRFRELVSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDDMGDLVDA
EEYLVPQQGFFCPDPAPGAGGMVHHRHRSSSTRSGGGDLTLGLEPSEEEAPRSPLAPSE
GAGSDVFDGDLGMGAAKGLQSLPTHDPSPLQRYSEDPTVPLPSETDGYVAPLTCSPQPE
YVNQPDVRPQPPSPREGPLPAARPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEY
LTPQGGAAPQPHPPPAFSPAFDNLYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV
(SEQ ID NO:17),

ERBB2 Isoform 3 having the following amino acid sequence:

MRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGAFGTVYKGIWIPDGE
NVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTSTVQLVTQLMPY
GCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHRDLAARNVLVKSPNHV
KITDFGLARLLDIDETEYHADGGKVPIKWMALESILRRRFTHQSDVWSYGVTVWELMT
FGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECRPRFRELVSEFSR
MARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDDMGDLVDAEEYLVPQQGFFCPDPA
PGAGGMVHHRHRSSSTRSGGGDLTLGLEPSEEEAPRSPLAPSEGAGSDVFDGDLGMGA
AKGLQSLPTHDPSPLQRYSEDPTVPLPSETDGYVAPLTCSPQPEYVNQPDVRPQPPSPRE
GPLPAARPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEYLTPQGGAAPQPHPPPA
FSPAFDNLYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV (SEQ ID NO:18),

ERBB2 Isoform 4 having the following amino acid sequence:

MPRGSWKPQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLS
FLQDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVT
GASPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSR
ACHPCSPMCKGSRCWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCAAGCTGP
KHSDCLACLHFNHSGICELHCPALVTYNTDTFESMPNPEGRYTFGASCVTACPYNYLST

DVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEF
AGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPDLSVF
QNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLALIHHNTHLCFVHTVPWDQ
LFRNPHQALLHTANRPEDECVGEGLACHQLCARGHCWGPGPTQCVNCSQFLRGQECV
EECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVAR
CPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASPLTSIISAVV
GILLVVVLGVVFGILIKRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKET
ELRKVKVLGSGAFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAG
VGSPYVSRLLGICLTSTVQLVTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGM
SYLEDVRLVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMAL
ESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDV
YMIMVKCWMIDSECRPRFRELVSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLE
DDDMGDLVDAEEYLVPQQGFFCPDPAPGAGGMVHHRHRSSSTRSGGGDLTLGLEPSEE
EAPRSPLAPSEGAGSDVFDGDLGMGAAKGLQSLPTHDPSPLQRYSEDPTVPLPSETDGY
VAPLTCSPQPEYVNQPDVRPQPPSPREGPLPAARPAGATLERPKTLSPGKNGVVKDVFA
FGGAVENPEYLTPQGGAAPQPHPPPAFSPAFDNLYYWDQDPPERGAPPSTFKGTPTAEN
PEYLGLDVPV (SEQ ID NO:19),

ERBB2 Isoform 5 having the following amino acid sequence:

MKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEVQGYVLIAH
NQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLRELQLRSLT
EILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCW
GESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGI
CELHCPALVTYNTDTFESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQE
VTAEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPES
FDGDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGA
YSLTLQGLGISWLGLRSLRELGSGLALIHHNTHLCFVHTVPWDQLFRNPHQALLHTANR
PEDECVGEGLACHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVN
ARHCLPCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIW
KFPDEEGACQPCPINCTHSCVDLDDKGCPAEQRASPLTSIISAVVGILLVVVLGVVFGILI
KRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETELRKVKVLGSGAFGT
VYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDEAYVMAGVGSPYVSRLLGICLTST
VQLVTQLMPYGCLLDHVRENRGRLGSQDLLNWCMQIAKGMSYLEDVRLVHRDLAAR

NVLVKSPNHVKITDFGLARLLDIDETEYHADGGKVPIKWMALESILRRRFTHQSDVWS
YGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQPPICTIDVYMIMVKCWMIDSECR
PRFRELVSEFSRMARDPQRFVVIQNEDLGPASPLDSTFYRSLLEDDDMGDLVDAEEYLV
PQQGFFCPDPAPGAGGMVHHRHRSSSTRSGGGDLTLGLEPSEEEAPRSPLAPSEGAGSD
VFDGDLGMGAAKGLQSLPTHDPSPLQRYSEDPTVPLPSETDGYVAPLTCSPQPEYVNQP
DVRPQPPSPREGPLPAARPAGATLERPKTLSPGKNGVVKDVFAFGGAVENPEYLTPQGG
AAPQPHPPPAFSPAFDNLYYWDQDPPERGAPPSTFKGTPTAENPEYLGLDVPV (SEQ ID
NO:20),

and ERBB2 Isoform 6 having the following amino acid sequence:

MELAALCRWGLLLALLPPGAASTQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQ
GNLELTYLPTNASLSFLQDIQEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAV
LDNGDPLNNTTPVTGASPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHK
NNQLALTLIDTNRSRACHPCSPMCKGSRCWGESSEDCQSLTRTVCAGGCARCKGPLPT
DCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNTDTFESMPNPEGRYTF
GASCVTACPYNYLSTDVGSCTLVCPLHNQEVTAEDGTQRCEKCSKPCARVCYGLGME
HLREVRAVTSANIQEFAGCKKIFGSLAFLPESFDGDPASNTAPLQPEQLQVFETLEEITGY
LYISAWPDSLPDLSVFQNLQVIRGRILHNGAYSLTLQGLGISWLGLRSLRELGSGLALIH
HNTHLCFVHTVPWDQLFRNPHQALLHTANRPEDECVGEGLACHQLCARGHCWGPGPT
QCVNCSQFLRGQECVEECRVLQGLPREYVNARHCLPCHPECQPQNGSVTCFGPEADQC
VACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEGACQPCPINCTHSPLTSIISAVVG
ILLVVVLGVVFGILIKRRQQKIRKYTMRRLLQETELVEPLTPSGAMPNQAQMRILKETEL
RKVKVLGSGAFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKANKEILDETISNLFSNFA
PRGPSACCEPTCWCHSGKGQDSLPREEWGRQRRFCLWGCRGEPRVLDTPGRSCPSAPP
SSCLQPSLRQPLLLGPGPTRAGGSTQHLQRDTYGREPRVPGSGRASVNQKAKSAEALM
CPQGAGKA (SEQ ID NO:21).

[00149] In some instances, the methods described herein may employ an antigen-specific therapeutic that specifically binds an ERBB2, including human ERBB2, including e.g., human ERBB2 Isoform 1, human ERBB2 Isoform 2, human ERBB2 Isoform 3, human ERBB2 Isoform 4, human ERBB2 Isoform 5, human ERBB2 Isoform 6, or any combination thereof.

[00150] In some instances, combinations of two or more targeting antigens may be employed, including but not limited to e.g., where such combinations include EphA2 and EphA3, EphA2 and IL13RA2 (or IL13RA1), EphA2 and EGFR, EphA2 and ERBB2, EphA3 and IL13RA2 (or

IL13RA1), EphA3 and EGFR, EphA3 and ERBB2, IL13RA2 (or IL13RA1) and EGFR, IL13RA2 (or IL13RA1) and ERBB2, or EGFR and ERBB2. In some instances, such combinations may find use in an OR gate as described herein. In some instances, a two-headed antigen-specific therapeutic may be employed, including but not limited to e.g., where the two-headed antigen-specific therapeutic binds to EphA2 and EphA3, EphA2 and IL13RA2 (or IL13RA1), EphA2 and EGFR, EphA2 and ERBB2, EphA3 and IL13RA2 (or IL13RA1), EphA3 and EGFR, EphA3 and ERBB2, IL13RA2 (or IL13RA1) and EGFR, IL13RA2 (or IL13RA1) and ERBB2, or EGFR and ERBB2.

[00151] In some instances, useful priming and/or targeting/killing antigens include Epidermal growth factor receptor variant III (EGFRvIII). EGFRvIII is a mutant of EGFR where, commonly exons 2-7 of the EGFR gene are deleted as a result of EGFR rearrangement (see e.g., Gan et al. FEBS J. (2013) 280(21):5350-70; the disclosure of which is incorporated herein in its entirety). Methods described herein may employ EGFRvIII as a priming antigen and may include the use of a BTTS (described in more detail herein) that specifically binds EGFRvIII in a circuit employed in the subject methods.

[00152] In some instances, circuits of the present disclosure may include an antigen-specific therapeutic that specifically binds an EGFRvIII, including e.g., where EGFRvIII is employed as both a priming antigen and a targeting antigen. For example, an antigen-specific therapeutic that specifically binds an EGFRvIII may be employed in an AND-OR gate where the circuit-containing cells are primed by EGFRvIII to target EGFRvIII or another antigen (including but not limited to e.g., EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR, or ERBB2). In some instances, the targeted antigens of a circuit used in a method of the present disclosure will not include EGFRvIII such that the circuit does not target EGFRvIII, including e.g., where EGFRvIII is utilized solely as a priming antigen.

[00153] The amino acid sequence of EGFRvIII may vary, e.g., depending on the particular mutation and/or rearrangement from which a particular EGFRvIII is derived. A non-limiting example of an EGFRvIII amino acid sequence is as follows:

MRPSGTAGAAFLALLAALCPASRALEEKKGNYVVTDHGSCVRACGADSYEMEEDGVR
KCKKCEGPCRKVCNGIGIGEFKDSLSINATNIKHFKNCTSISGDLHILPVAFRGDSFTHTP
PLDPQELDILKTVKEITGFLLIQAWPENRTDLHAFENLEIIRGRTKQHGQFSLAVVSLNIT
SLGLRSLKEISDGDVIISGNKNLCYANTINWKKLFGTSGQKTKIISNRGENSCKATGQVC
HALCSPEGCWGPEPRDCVSCRNVSRGRECVDKCNLLEGEPREFVENSECIQCHPECLPQ

AMNITCTGRGPDNYIQCAHYIDGPHCVKTCPAGVMGENNTLVWKYADAGHVCHLCH
PNCTYGCTGPGLEGCPTNGPKIPSIATGMVGALLLLLVVALGIGLFMRRRHIVRKRTLR
RLLQERELVEPLTPSGEAPNQALLRILKETEFKKIKVLGSGAFGTVYKGLWIPEGEKVKI
PVAIKELREATSPKANKEILDEAYVMASVDNPHVCRLLGICLTSTVQLITQLMPFGCLLD
YVREHKDNIGSQYLLNWCVQIAKGMNYLEDRRLVHRDLAARNVLVKTPQHVKITDFG
LAKLLGAEEKEYHAEGGKVPIKWMALESILHRIYTHQSDVWSYGVTVWELMTFGSKP
YDGIPASEISSILEKGERLPQPPICTIDVYMIMVKCWMIDADSRPKFRELIIEFSKMARDPQ
RYLVIQGDERMHLPSPTDSNFYRALMDEEDMDDVVDADEYLIPQQGFFSSPSTSRTPLL
SSLSATSNNSTVACIDRNGLQSCPIKEDSFLQRYSSDPTGALTEDSIDDTFLPVPEYINQS
VPKRPAGSVQNPVYHNQPLNPAPSRDPHYQDPHSTAVGNPEYLNTVQPTCVNSTFDSP
AHWAQKGSHQISLDNPDYQQDFFPKEAKPNGIFKGSTAENAEYLRVAPQSSEFIGA
(SEQ ID NO:22).

[00154] EGFRvIII proteins, and the amino acid sequences thereof, to which an antigen-binding domain of a BTTS or an antigen-specific therapeutic bind may vary from that provided above. For example, in some instances, a subject EGFRvIII variant may include one or more mutations relative to the sequence provided above, including but not limited to e.g., 1 mutation, 2 or less, 3 or less, 4 or less, 5 or less mutations, etc. In some instances, a subject EGFRvIII variant may share 80% or greater sequence identity with the amino acid sequence provided above, including but not limited to e.g., 85% or greater, 90% or greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, 99% or greater, or 100% sequence identity with the above EGFRvIII sequence.

[00155] In some instances, useful EGFRvIII binding domains may include antibody based EGFRvIII binding domains, including but not limited to an EGFRvIII scFv. In some instances, a useful EGFRvIII scFv may have the following amino acid sequence or a variant thereof:
DIQMTQSPSSLSASVGDRVTITCRASQGIRNNLAWYQQKPGKAPKRLIYAASNLQSGVP
SRFTGSGSGTEFTLIVSSLQPEDFATYYCLQHHSYPLTSGGGTKVEIKGSTSGSGKPGSGE
GSEVQVLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGG
STNYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGSSGWSEYWGQGTLVT
VSS (SEQ ID NO:23);
including e.g., where the useful EGFRvIII scFv has a sequence identity of 100% or less, including e.g., at least 99%, at least 98%, at least 97%, at least 96%, at least 95%, at least 90%, at least 85%, at least 80%, etc., sequence identity with the sequence presented above.

[00156] In some instances, useful EGFRvIII binding domains include those described in Morgan et al. Hum Gene Ther. (2012) 23(10):1043-5; the disclosure of which is incorporated herein by reference it its entirety.

Antigen-specific therapeutics

[00157] As summarized above, in the present methods a BTTS responsive to an EGFRvIII priming antigen may induce the expression of an antigen-specific therapeutic responsive to one or more targeting antigens. Useful antigen-specific therapeutics will vary and may include surfaced expressed and secreted antigen-specific therapeutics. For example, in some instances, an antigen-specific therapeutic used in the methods of the present disclosure may be expressed, in response to the activation of a BTTS, on the surface of an immune cell, i.e., the immune cell genetically modified to encode a EGFRvIII priming/targeting circuit as described herein. In some instances, an antigen-specific therapeutic used in the methods of the present disclosure may be secreted, in response to the activation of a BTTS, from an immune cell, i.e., the immune cell genetically modified to encode a EGFRvIII priming/targeting circuit as described herein.

[00158] In general, except where described otherwise, the antigen-specific therapeutic of a herein described circuit will not be expressed in the absence of the activation of the BTTS that induces its expression. Also, except where described otherwise, an antigen-specific therapeutic of a herein described circuit will not be active in the absence of the antigen to which it binds, i.e., without binding the antigen to which the antigen-specific therapeutic is specific. Binding of its respective antigen, or antigens in the case of multi- or bispecific agents, results in activation of the antigen-specific therapeutic. When expressed by, or otherwise engaged with, an immune cell and bound to antigen(s) the antigen-specific therapeutic may activate the immune cell. Activated immune cells may mediate one or more beneficial effects with respect to a heterogeneous GBM of a subject, including those described herein such as but not limited to e.g., cancer cell killing, cytokine release, and the like.

[00159] The term "antigen", with respect to the herein described antigen-specific binding domains, is used in a broad sense to refer to essentially any specific binding partner to which the antigen-specific therapeutic binds. As such, any convenient specific binding pair, i.e., specific binding member and specific binding partner pair, may find use in the antigen-specific therapeutics of the instant methods including but not limited to e.g., antigen-antibody pairs, ligand receptor pairs, scaffold protein pairs, etc. In some instances, the specific binding member may be an antibody and its binding partner may be an antigen to which the antibody specifically

binds. In some instances, the specific binding member may be a receptor and its binding partner may be a ligand to which the receptor specifically binds. In some instances, the specific binding member may be a ligand and its binding partner may be a receptor to which the ligand specifically binds.

[00160] In some instances, useful ligand-receptor specific binding pairs may include where the specific binding member is a mutein of a ligand having at least one mutation relative to the wild-type ligand, including but not limited to e.g., one or more mutations, two or more mutations, three or more mutations, four or more mutations, five or more mutations, etc. In some instances, useful muteins will have at least 90% sequence identity with the relevant wild-type amino acid sequence, including but not limited to e.g., at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, etc., sequence identity with the relevant wild-type amino acid sequence. In some instances, a mutein employed in the subject polypeptide may have higher affinity for the receptor as compared to the affinity between the receptor and the wild-type ligand.

[00161] Antigen-specific therapeutics useful in the methods of the present disclosure will vary and may include but are not limited to e.g., chimeric antigen receptors (CARs), T cell receptors (TCRs), chimeric bispecific binding members, and the like.

[00162] Useful CARs include essentially any CAR useful in the treatment of cancer, including single-chain and multi-chain CARs, directed to one or more targeting antigens. A CAR used in the instant methods will generally include, at a minimum, an antigen binding domain, a transmembrane domain and an intracellular signaling domain. An employed CAR may further include one or more costimulatory domains.

[00163] Non-limiting examples of CARs that may be employed include those used in commercialized CAR T cell (CART) therapies that are directed to one or more appropriate targeting antigens or have been modified to be directed to one or more appropriate targeting antigens. For example, in some instances, one or more CARs may be employed that target one or more targeting antigens, including but not limited to e.g., EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR, and ERBB2. In some instances, a CAR may be employed that targets EGFRvIII, including where EGFRvIII is the sole antigen targeted by the CAR or one of two or more antigens targeted by the CAR.

[00164] Useful CARs that may be modified to be directed to one or more appropriate targeting antigens include but are not limited to those CARs directed to CD19 and BCMA, including

e.g., the anti-CD19—4-1BB—CD3ζ CAR expressed by lentivirus loaded CTL019 (Tisagenlecleucel-T) CAR-T cells, also referred to as Kymriah(TM)(tisagenlecleucel) as commercialized by Novartis (Basel, Switzerland) and the anti-BCMA—4-1BB—CD3ζ CAR expressed by lentivirus loaded CAR-T cells called "bb2121" as commercialized by bluebird bio, Inc. (Cambridge, MA) and Celgene Corporation (Summit, NJ).

[00165] Useful CARs, e.g., that may be modified to be directed to an appropriate targeting antigen, or useful domains thereof, e.g., that may be employed in a CAR directed to an appropriate targeting antigens, in some instances may include those described in U.S. Patent Nos.: 9,914,909; 9,821,012; 9,815,901; 9,777,061; 9,662,405; 9,657,105; 9,629,877; 9,624,276; 9,598,489; 9,587,020; 9,574,014; 9,573,988; 9,499,629; 9,446,105; 9,394,368; 9,328,156; 9,233,125; 9,175,308 and 8,822,647; the disclosures of which are incorporated herein by reference in their entirety. In some instances, useful CARs may include or exclude heterodimeric, also referred to as dimerizable or switchable, CARs and/or include or exclude one or more of the domains thereof. Useful heterodimeric CARs and/or useful domains thereof may, in some instances, include those described in U.S. Patent Nos. 9,587,020 and 9,821,012 as well as U.S. Pub. Nos. US20170081411A1, US20160311901A1, US20160311907A1, US20150266973A1 and PCT Pub. Nos. WO2014127261A1, WO2015142661A1, WO2015090229A1 and WO2015017214A1; the disclosures of which are incorporated herein by reference in their entirety.

[00166] As summarized above, in some instances, the antigen binding domain of a CAR, such but not limited to e.g., those described in any one of the documents referenced above, may be substituted or amended with an alternative or additional antigen binding domain directed to a different antigen, such as but not limited to one or more of the antigens described herein, for use in the herein described methods. In such instances, the intracellular portions (i.e., the intracellular signaling domain or the one or more co-stimulatory domains) of the antigen-domain-substituted CAR may or may not be modified.

[00167] Useful CARs and/or useful domains thereof may, in some instances, include those that have been or are currently being investigated in one or more clinical trials, including but not limited to the CARs directed to the following antigens (listed with an exemplary corresponding clinical trial number, further information pertaining to which may be retrieved by visiting www(dot)clinicaltrials(dot)gov): AFP, e.g., in NCT03349255; BCMA, e.g., in NCT03288493; CD10, e.g., in NCT03291444; CD117, e.g., in NCT03291444; CD123, e.g., in NCT03114670;

CD133, e.g., in NCT02541370; CD138 , e.g., in NCT01886976; CD171, e.g., in NCT02311621; CD19, e.g., in NCT02813252; CD20, e.g., in NCT03277729; CD22, e.g., in NCT03244306; CD30, e.g., in NCT02917083; CD33, e.g., in NCT03126864; CD34, e.g., in NCT03291444; CD38, e.g., in NCT03291444; CD5, e.g., in NCT03081910; CD56, e.g., in NCT03291444; CD7, e.g., in NCT02742727; CD70, e.g., in NCT02830724; CD80, e.g., in NCT03356808; CD86, e.g., in NCT03356808; CEA, e.g., in NCT02850536; CLD18, e.g., in NCT03159819; CLL-1, e.g., in NCT03312205; cMet, e.g., in NCT01837602; EGFR, e.g., in NCT03182816; EGFRvIII, e.g., in NCT02664363; EpCAM, e.g., in NCT03013712; EphA2, e.g., in NCT02575261; GD-2, e.g., in NCT01822652; Glypican 3, e.g., in NCT02905188; GPC3, e.g., in NCT02723942; HER-2, e.g., in NCT02547961; kappa immunoglobulin, e.g., in NCT00881920; LeY, e.g., in NCT02958384; LMP1, e.g., in NCT02980315; mesothelin, e.g., in NCT02930993; MG7, e.g., in NCT02862704; MUC1, e.g., in NCT02587689; NKG2D-ligands, e.g., in NCT02203825; PD-L1, e.g., in NCT03330834; PSCA, e.g., in NCT02744287; PSMA, e.g., in NCT03356795; ROR1, e.g., in NCT02706392; ROR1R, e.g., in NCT02194374; TACI, e.g., in NCT03287804; and VEGFR2, e.g., in NCT01218867.

[00168] Useful TCRs include essentially any TCR useful in the treatment of cancer, including single-chain and multi-chain TCRs, directed to a targeting antigen. A TCR used in the instant methods will generally include, at a minimum, an antigen binding domain and a modified or unmodified TCR chain, or portion thereof, including but not limited to e.g., a modified or unmodified α-chain, a modified or unmodified β-chain, etc.. An employed TCR may further include one or more costimulatory domains. In some instances, a TCR employed herein will include an alpha chain and a beta chain and recognize antigen when presented by a major histocompatibility complex.

[00169] Essentially any TCR can be induced by a BTTS using a method of the present disclosure including e.g., TCRs that are specific for any of a variety of epitopes, including, e.g., an epitope expressed on the surface of a cancer cell, a peptide-MHC complex on the surface of cancer cell, and the like. In some cases, the TCR is an engineered TCR.

[00170] Non-limiting examples of engineered TCRs, including those having immune cell activation function and that may be modified to include an antigen-binding domain specific for a suitable targeting antigen, useful in the methods described herein include, e.g., antigen-specific TCRs, Monoclonal TCRs (MTCRs), Single chain MTCRs, High Affinity CDR2 Mutant TCRs, CD1-binding MTCRs, High Affinity NY-ESO TCRs, VYG HLA-A24 Telomerase

TCRs, including e.g., those described in PCT Pub Nos. WO 2003/020763, WO 2004/033685, WO 2004/044004, WO 2005/114215, WO 2006/000830, WO 2008/038002, WO 2008/039818, WO 2004/074322, WO 2005/113595, WO 2006/125962; Strommes et al. Immunol Rev. 2014; 257(1):145-64; Schmitt et al. Blood. 2013; 122(3):348-56; Chapuls et al. Sci Transl Med. 2013; 5(174):174ra27; Thaxton et al. Hum Vaccin Immunother. 2014; 10(11):3313-21 (PMID:25483644); Gschweng et al. Immunol Rev. 2014; 257(1):237-49 (PMID:24329801); Hinrichs et al. Immunol Rev. 2014; 257(1):56-71 (PMID:24329789); Zoete et al. Front Immunol. 2013; 4:268 (PMID:24062738); Marr et al. Clin Exp Immunol. 2012; 167(2):216-25 (PMID:22235997); Zhang et al. Adv Drug Deliv Rev. 2012; 64(8):756-62 (PMID:22178904); Chhabra et al. Scientific World Journal. 2011; 11:121-9 (PMID:21218269); Boulter et al. Clin Exp Immunol. 2005; 142(3):454-60 (PMID:16297157); Sami et al. Protein Eng Des Sel. 2007; 20(8):397-403; Boulter et al. Protein Eng. 2003; 16(9):707-11; Ashfield et al. IDrugs. 2006; 9(8):554-9; Li et al. Nat Biotechnol. 2005; 23(3):349-54; Dunn et al. Protein Sci. 2006; 15(4):710-21; Liddy et al. Mol Biotechnol. 2010; 45(2); Liddy et al. Nat Med. 2012; 18(6):980-7; Oates, et al. Oncoimmunology. 2013; 2(2):e22891; McCormack, et al. Cancer Immunol Immunother. 2013 Apr;62(4):773-85; Bossi et al. Cancer Immunol Immunother. 2014; 63(5):437-48 and Oates, et al. Mol Immunol. 2015 Oct;67(2 Pt A):67-74; the disclosures of which are incorporated herein by reference in their entirety.

[00171] Useful TCRs include those having wild-type affinity for their respective antigen as well as those having enhanced affinity for their respective antigen. TCRs having enhanced affinity for their respective antigen may be referred to as "affinity enhanced" or "enhanced affinity" TCRs. The affinity of a TCR may be enhanced by any convenient means, including but not limited to binding-site engineering (i.e., rational design), screening (e.g., TCR display), or the like. Non-limiting examples of affinity enhanced TCRs and methods of generating enhanced affinity TCRs include but are not limited to e.g., those described in PCT Pub. Nos. 20150118208, 2013256159, 20160083449; 20140349855, 20100113300, 20140371085, 20060127377, 20080292549, 20160280756, 20140065111, 20130058908, 20110038842, 20110014169, 2003276403 and the like; the disclosures of which are incorporated herein by reference in their entirety. Further engineered TCRs, modified to be directed to an appropriate targeting antigen, that may be expressed in response to release of an intracellular domain of a BTTS of the present disclosure include e.g., those described in PCT Application No. US2017/048040; the disclosure of which is incorporated herein by reference in its entirety.

[00172] Useful TCRs, which may be modified to be directed to an appropriate targeting antigen, may, in some instances, also include those described in U.S. Patent Nos.: 9,889,161; 9,889,160; 9,868,765; 9,862,755; 9,717,758; 9,676,867; 9,409,969; 9,115,372; 8,951,510; 8,906,383; 8,889,141; 8,722,048; 8,697,854; 8,603,810; 8,383,401; 8,361,794; 8,283,446; 8,143,376; 8,003,770; 7,998,926; 7,666,604; 7,456,263; 7,446,191; 7,446,179; 7,329,731; 7,265,209; and 6,770,749; the disclosures of which are incorporated herein by reference in their entirety.

[00173] As described above, in some instances, the antigen binding domain of a TCR, such as but not limited to e.g., those described or referenced above, may be substituted or amended with an alternative or additional antigen binding domain directed to a different antigen, such as but not limited to one or more of the antigens described herein, for use in the herein described methods. In such instances, the other portions (i.e., the transmembrane domain, any intracellular signaling domains, etc.) of the antigen-domain-substituted TCR may or may not be modified.

[00174] As summarized above, in some instances, useful antigen-specific therapeutics may include those that, upon induction by an activated BTTS, are expressed and secreted from the producing cell, including e.g., where the secreting cell is an immune cell. For example, upon binding of a BTTS expressed by an immune cell, the BTTS may induce expression and secretion of an encoded antigen-specific therapeutic specific for a targeting antigen. The secreted antigen-specific therapeutic may target a target antigen expressing cancer cell in *trans*, thereby mediating killing of the target cell. As described herein, in some instances, a secreted antigen-specific therapeutic may increase the zone of targeting or the zone of killing of a subject circuit as compared to a similar circuit encoding a non-secreted (e.g., membrane expressed) antigen-specific therapeutic.

[00175] Useful secreted antigen-specific therapeutics will vary and in some instances may include but are not limited to e.g., chimeric bispecific binding members. In some instances, useful chimeric bispecific binding members may include those that target a protein expressed on the surface of an immune cell, including but not limited to e.g., a component of the T cell receptor (TCR), e.g., one or more T cell co-receptors. Chimeric bispecific binding members that bind to a component of the TCR may be referred to herein as a TCR-targeted bispecific binding agent. Chimeric bispecific binding members useful in the instant methods will generally be specific for a targeting antigen and may, in some instances, be specific for a targeting antigen and a protein expressed on the surface of an immune cell (e.g.,. a component of a TCR such as e.g., a CD3 co-receptor).

[00176] Non-limiting examples of useful chimeric bispecific binding members include those that bind Ephrin type-A receptor 2 (EphA2), Ephrin type-A receptor 3 (EphA3), an Interleukin-13 receptor (IL13R) (e.g., IL13RA2 or IL13RA1), Epidermal growth factor receptor (EGFR) or erb-b2 receptor tyrosine kinase 2 (ERBB2). Non-limiting examples of useful chimeric bispecific binding members also include those that have been modified to bind EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR or ERBB2.

[00177] In some instances, useful chimeric bispecific binding members may include a bispecific T cell engager (BiTE). A BiTE is generally made by fusing a specific binding member (e.g., a scFv) that binds an immune cell antigen to a specific binding member (e.g., a scFv) that binds a cancer antigen (e.g., a tumor associated antigen, a tumor specific antigen, etc.). For example, an exemplary BiTE includes an anti-CD3 scFv fused to an anti-tumor associated antigen (e.g., EpCAM, CD19, etc.) scFv via a short peptide linker (e.g., a five amino acid linker, e.g., GGGGS).

[00178] In some instances, a BiTE, suitable for use in the herein described methods may include e.g., an anti-CD3 × anti-CD19 BiTE (e.g., Blinatumomab) that has been modified to bind a suitable targeting antigen (including but not limited to e.g., EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR or ERBB2), an anti-EpCAM × anti-CD3 BiTE (e.g., MT110) that has been modified to bind a suitable targeting antigen (including but not limited to e.g., EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR or ERBB2), an anti-CEA × anti-CD3 BiTE (e.g., MT111/MEDI-565) that has been modified to bind a suitable targeting antigen (including but not limited to e.g., EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR or ERBB2), an anti-CD33 × anti-CD3 BiTE that has been modified to bind a suitable targeting antigen (including but not limited to e.g., EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR or ERBB2), an anti-HER2 BiTE that has been modified to bind a suitable targeting antigen (including but not limited to e.g., EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR or ERBB2), an anti-EGFR BiTE, an anti-IgE BiTE that has been modified to bind a suitable targeting antigen (including but not limited to e.g., EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR or ERBB2), and the like.

[00179] As summarized above, in some instances, the antigen binding domain of a chimeric bispecific binding member, such as but not limited to e.g., those described or referenced above, may be substituted or amended with an alternative or additional antigen binding domain directed to a different antigen, such as but not limited to one or more of the antigens described herein, for use in the herein described methods. In such instances, the other portions (i.e., linker domain,

any immune cell targeting domains, etc.) of the antigen-domain-substituted chimeric bispecific binding member may or may not be modified.

[00180] In some instances, a payload induced by binding of a BTTS to its respective priming antigen in a herein described method may include a secreted bio-orthogonal adapter molecule. Such bio-orthogonal adapter molecules may, in some instances, be configured to target and bind a targeting antigen and also bind or be bound by a heterologous polypeptide expressed by an immune cell.

[00181] For example, in some instances, a subject circuit employed in the herein described methods may encode, within an immune cell: a BTTS responsive to an EGFRvIII priming antigen; a bio-orthogonal adapter molecule specific for a targeting antigen; and a therapeutic, or portion thereof, which binds the bio-orthogonal adapter molecule. In such a circuit, expression and secretion of the bio-orthogonal adapter molecule is induced upon binding of the BTTS to EGFRvIII. Then, in the presence of both (1) a cancer cell expressing the targeting antigen and (2) the therapeutic that binds the bio-orthogonal adapter molecule, the therapeutic binds the bio-orthogonal adapter molecule which then binds the targeting antigen, thereby activating the therapeutic. The activated therapeutic may then mediate a therapeutic effect (e.g., a cytotoxic effect) on the cancer cell expressing the targeting antigen, including where the targeting antigen is expressed in *trans* with respect to the EGFRvIII priming antigen. As described herein, in some instances, a secreted bio-orthogonal adapter molecule may increase the zone of targeting or the zone of killing of a subject circuit as compared to a similar circuit encoding a non-secreted (e.g., membrane expressed) antigen-specific therapeutic.

[00182] Bio-orthogonal adapter molecules may be employed in various contexts within the herein described methods. For example, in some instances, a bio-orthogonal adapter molecule may be employed that includes a diffusible antigen binding portion of an antigen-specific therapeutic, such as e.g., a diffusible antigen binding portion of a CAR, a diffusible antigen binding portion of a TCR, or the like. In some instances, such diffusible antigen binding portion of antigen-specific therapeutics may be referred to a "diffusible head", including e.g., a "diffusible CAR head", a "diffusible TCR head", and the like. In some instances, a diffusible antigen binding portion may be specific for one or more of EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR and/or ERBB2.

[00183] In some instances, the therapeutic may bind directly to the bio-orthogonal adapter molecule. Strategies for direct binding of the therapeutic to the bio-orthogonal adapter molecule

may vary. For example, in some instances, the therapeutic may include a binding domain (e.g., such as an orthogonal antibody or fragment thereof) that binds a binding moiety (e.g., an orthogonal epitope to which an antibody may be directed) covalently attached to the bio-orthogonal adapter. As a non-limiting example, a therapeutic may include a binding domain to a non-naturally occurring epitope, e.g., an anti-fluorescein antibody or a fragment thereof, and the bio-orthogonal adapter molecule may include the epitope, e.g., a fluorescein, covalently attached thereto. In some instances, the configuration of the bio-orthogonal adapter molecule and therapeutic interaction may be reversed as compared to that previously described, including e.g., where the therapeutic includes a covalently attached epitope and the bio-orthogonal adapter molecule includes a binding domain to the epitope. Useful epitopes will vary and may include but are not limited to e.g., small molecule-based epitopes, peptide-based epitopes (e.g., peptide neo-epitopes), oligonucleotide-based epitopes, and the like. The epitope-binding domains will vary correspondingly and may include but are not limited to e.g., small molecule binding domains, peptide binding domains, oligonucleotide binding domains, and the like.

[00184] Non-limiting examples of useful bio-orthogonal adapter molecules, and the domains that bind thereto, include but are not limited to e.g., the peptide neo-epitopes and the antibody binding domains that bind thereto as used in switchable CAR (sCAR) T cells, including but not limited to e.g., those described in Rodgers et al. Proc Natl Acad Sci USA. (2016) 113(4):E459-68 and Cao et al., Angew Chem Int Ed Engl. 2016 Jun 20;55(26):7520-4 as well as PCT Pub. No. WO2016168773; the disclosures of which are incorporated herein by reference in their entirety.

[00185] In some instances, the therapeutic may bind indirectly to the bio-orthogonal adapter molecule, including e.g., where binding is mediated by a diffusible dimerizing agent. Non-limiting examples of suitable dimerizing agents, and the dimerizing domains that bind thereto, include protein dimerizers.

[00186] Protein dimerizers generally include polypeptide pairs that dimerize, e.g., in the presence of or when exposed to a dimerizing agent. The dimerizing polypeptide pairs of a protein dimerizer may homo-dimerize or hetero-dimerize (i.e., the dimerizing polypeptide pairs may include two of the same polypeptide that form a homodimer or two different polypeptides that form a heterodimer). Non-limiting pairs of protein dimerizers (with the relevant dimerizing agent in parentheses) include but are not limited to e.g., FK506 binding protein (FKBP) and FKBP (rapamycin); FKBP and calcineurin catalytic subunit A (CnA) (rapamycin); FKBP and

cyclophilin (rapamycin); FKBP and FKBP-rapamycin associated protein (FRB) (rapamycin); gyrase B (GyrB) and GyrB (coumermycin); dihydrofolate reductase (DHFR) and DHFR (methotrexate); DmrB and DmrB (AP20187); PYL and ABI (abscisic acid); Cry2 and CIB1 (blue light); GAI and GID1 (gibberellin); and the like. Further description, including the amino acid sequences, of such protein dimerizers is provided in U.S. Patent Application Publication No. US 2015-0368342 A1; the disclosure of which is incorporated herein by reference in its entirety.

[00187] Useful protein dimerizers also include those nuclear hormone receptor derived protein dimerizers that dimerize in the presence of a dimerizing agent described in PCT Pub. No. WO 2017/120546 and U.S. Patent Pub. No. US 2017/0306303 A1; the disclosures of which are incorporated by reference herein in their entirety, and the like. Such nuclear hormone receptor derived dimerizers will generally include a first member of the dimerization pair that is a co-regulator of a nuclear hormone receptor and a second member of the dimerization pair comprises an LBD of the nuclear hormone receptor.

[00188] Where a bio-orthogonal adapter molecule is employed in a subject circuit, the expression of the therapeutic, which binds the bio-orthogonal adapter molecule to mediate targeting antigen recognition, may or may not be controlled by the circuit. Put another way, the expression of the therapeutic may or may not be tied to the activation of the BTTS (e.g., the binding of the BTTS to EGFRvIII or another antigen) of the circuit. In some instances, the circuit may be configured such that binding of a BTTS to its antigen induces expression of a therapeutic which binds a bio-orthogonal adapter molecule. In some instances, the BTTS that induces expression of the therapeutic is the same BTTS that induces expression of the bio-orthogonal adapter molecule. In some instance, the therapeutic is induced by a BTTS that is different (i.e., separate) from the BTTS that induces expression of the bio-orthogonal adapter molecule.

[00189] In some instances, expression of a therapeutic which binds a bio-orthogonal adapter molecule may not be induced by a BTTS. For example, in some instances, rather than being induced by a BTTS, such a therapeutic is expressed under the control of a separate regulatory element or sequence, including but not limited to e.g., where the expression of the therapeutic is constitutive, inducible, conditional, tissue specific, cell type specific, or the like. In some instances, for example, independent expression (e.g., constitutive expression, inducible expression, etc.) of the therapeutic by introduced immune cells allows for a diffusible bio-

orthogonal adapter molecule to mediate the activation of the therapeutic in immune cells that are distant from the site of priming.

[00190] In some instances, expression of a bio-orthogonal adapter molecule, bound by a therapeutic, may not be induced by a BTTS, including where the corresponding therapeutic is induced by a BTTS. For example, in some instances, rather than being induced by a BTTS, such a bio-orthogonal adapter molecule is expressed under the control of a separate regulatory element or sequence, including but not limited to e.g., where the expression of the bio-orthogonal adapter molecule is constitutive, inducible, conditional, tissue specific, cell type specific, or the like. In some instances, the bio-orthogonal adapter molecule may be externally provided.

[00191] In some instances, an antigen-specific therapeutic may have an extracellular domain that includes a first member of a specific binding pair that binds a second member of the specific binding pair, wherein the extracellular domain does not include any additional first or second member of a second specific binding pair. For example, in some instances, an antigen-specific therapeutic may have an extracellular domain that includes a first antigen-binding domain that binds an antigen, wherein the extracellular domain does not include any additional antigen-binding domains and does not bind any other antigens. A subject antigen-specific therapeutic may, in some instances, include only a single extracellular domain. Accordingly, an employed antigen-specific therapeutic may be specific for a single antigen and only specific for the single antigen. Such, antigen-specific therapeutics may be referred to as a "single antigen antigen-specific therapeutic".

[00192] In some instances, an antigen-specific therapeutic may have an extracellular domain that includes the first or second members of two or more specific binding pairs. For example, in some instances, an antigen-specific therapeutic may have an extracellular domain that includes a first antigen-binding domain and a second antigen-binding domain that are different such that the extracellular domain is specific for two different antigens. In some instances, an antigen-specific therapeutic may have two or more extracellular domains that each includes the first or second members of two different specific binding pairs. For example, in some instances, an antigen-specific therapeutic may have a first extracellular domain that includes a first antigen-binding domain and a second extracellular domain that includes a second antigen-binding domain where the two different antigen binding domains are each specific for a different antigen. As such, the antigen-specific therapeutic may be specific for two different antigens.

[00193] An antigen-specific therapeutic specific for two or more different antigens, containing either two extracellular domains or one extracellular domain specific for two different antigens, may be configured such that the binding of either antigen to the antigen-specific therapeutic is sufficient to active the antigen-specific therapeutic. Such an antigen-specific therapeutic, capable of being activated by any of two or more antigens, may find use in the described circuits as a component of a logic gate containing OR functionality. In some instances, an antigen-specific therapeutic specific for two different antigens may be referred to as a "two-headed antigen-specific therapeutic". Antigen-specific therapeutics specific for multiple antigens will not be limited to only two antigens and may, e.g., be specific for and/or activated by more than two antigens, including e.g., three or more, four or more, five or more, etc.

[00194] For example, in some instances, an antigen-specific therapeutic specific for two or more different antigens may bind, and/or be activated by, EphA2 or EphA3, EphA2 or IL13RA1, EphA2 or IL13RA2, EphA2 or EGFR, EphA2 or ERBB2, EphA3 or IL13RA1, EphA3 or IL13RA2, EphA3 or EGFR, EphA3 or ERBB2, IL13RA1 or IL13RA2, IL13RA1 or EGFR, IL13RA1 or ERBB2, IL13RA2 or EGFR, IL13RA2 or ERBB2, or EGFR or ERBB2.

[00195] An example of an antigen-specific therapeutic specific for two or more different antigens is a tandem CAR (also referred to as "tan CAR" or "tanCAR"). A "tandem CAR" is a bispecific CAR that includes two or more non-identical antigen recognition domains. Non-limiting examples of tandem CARs include those described in U.S. Patent Nos. 9,447,194; 10,155,038; 10,189,903; and 10,239,948; U.S. Patent Application Pub. No. 20130280220 and PCT Application Pub. No. WO/2013/123061; the disclosures of which are incorporated herein by reference in their entirety. Tandem CARs may be configured to bind a variety of different antigens, including but not limited to e.g., two or more or the antigens described herein and/or two or more of the antigens described in U.S. Patent Nos. 9,447,194; 10,155,038; 10,189,903; and 10,239,948; U.S. Patent Application Pub. No. 20130280220 and PCT Application Pub. No. WO/2013/123061.

Binding triggered transcriptional switches (BTTS)

[00196] The methods of the instant disclosure include the use of circuits employing a BTTS to induce expression of an encoded antigen-specific therapeutic. As used herein, a "binding-triggered transcriptional switch" or BTTS generally refers to a synthetic modular polypeptide or system of interacting polypeptides having an extracellular domain that includes a first member of a specific binding pair, a binding-transducer and an intracellular domain. Upon binding of the

second member of the specific binding pair to the BTTS the binding signal is transduced to the intracellular domain such that the intracellular domain becomes activated and performs some function within the cell that it does not perform in the absence of the binding signal. Binding triggered transcriptional switches are described in e.g., PCT Pub. No. WO 2016/138034 as well as U.S. Patent Nos. 9,670,281 and 9,834,608; the disclosures of which are incorporated herein by reference in their entirety.

[00197] The specific binding member of the extracellular domain generally determines the specificity of the BTTS. In some instances, a BTTS may be referred according to its specificity as determined based on its specific binding member. For example, a specific binding member having binding partner "X" may be referred to as an X-BTTS or an anti-X BTTS.

[00198] Any convenient specific binding pair, i.e., specific binding member and specific binding partner pair, may find use in the BTTS of the instant methods including but not limited to e.g., antigen-antibody pairs, ligand receptor pairs, scaffold protein pairs, etc. In some instances, the specific binding member may be an antibody and its binding partner may be an antigen to which the antibody specifically binds. In some instances, the specific binding member may be a receptor and its binding partner may be a ligand to which the receptor specifically binds. In some instances, the specific binding member may be a scaffold protein and its binding partner may be a protein to which the scaffold protein specifically binds. Useful specific binding pairs include those specific for EGFRvIII priming antigen and/or one or more targeting/killing antigens, including those described herein.

[00199] In some cases, the specific binding member is an antibody. The antibody can be any antigen-binding antibody-based polypeptide, a wide variety of which are known in the art. In some instances, the specific binding member is or includes a monoclonal antibody, a single chain Fv (scFv), a Fab, etc. Other antibody based recognition domains (cAb VHH (camelid antibody variable domains) and humanized versions, IgNAR VH (shark antibody variable domains) and humanized versions, sdAb VH (single domain antibody variable domains) and "camelized" antibody variable domains are suitable for use. In some instances, T-cell receptor (TCR) based recognition domains such as single chain TCR (scTv, single chain two-domain TCR containing VαVβ) are also suitable for use.

[00200] Where the specific binding member of a BTTS is an antibody-based binding member, the BTTS can be activated in the presence of a binding partner to the antibody-based binding member, including e.g., an antigen specifically bound by the antibody-based binding member.

In some instances, antibody-based binding member may be defined, as is commonly done in the relevant art, based on the antigen bound by the antibody-based binding member, including e.g., where the antibody-based binding member is described as an "anti-" antigen antibody, e.g., an anti-EGFRvIII antibody. Accordingly, antibody-based binding members suitable for inclusion in a BTTS or an antigen-specific therapeutic of the present methods can have a variety of antigen-binding specificities.

[00201] The components of BTTS's, employed in the described methods, and the arrangement of the components of the switch relative to one another will vary depending on many factors including but not limited to e.g., the desired binding trigger, the activity of the intracellular domain, the overall function of the BTTS, the broader arrangement of a molecular circuit comprising the BTTS, etc. The first binding member may include but is not limited to e.g., those agents that bind an antigen described herein. The intracellular domain may include but is not limited e.g., those intracellular domains that activate or repress transcription at a regulatory sequence, e.g., to induce or inhibit expression of a downstream component of a particular circuit.

[00202] The binding transducer of BTTS's will also vary depending on the desired method of transduction of the binding signal. Generally, binding transducers may include those polypeptides and/or domains of polypeptides that transduce an extracellular signal to intracellular signaling e.g., as performed by the receptors of various signal transduction pathways. Transduction of a binding signal may be achieved through various mechanisms including but not limited to e.g., binding-induced proteolytic cleavage, binding-induced phosphorylation, binding-induced conformational change, etc. In some instances, a binding-transducer may contain a ligand-inducible proteolytic cleavage site such that upon binding the binding-signal is transduced by cleavage of the BTTS, e.g., to liberate an intracellular domain. For example, in some instances, a BTTS may include a Notch derived cleavable binding transducer, such as, e.g., a chimeric notch receptor polypeptide as described herein.

[00203] In other instances, the binding signal may be transduced in the absence of inducible proteolytic cleavage. Any signal transduction component or components of a signaling transduction pathway may find use in a BTTS whether or not proteolytic cleavage is necessary for signal propagation. For example, in some instances, a phosphorylation-based binding transducer, including but not limited to e.g., one or more signal transduction components of the Jak-Stat pathway, may find use in a non-proteolytic BTTS.

[00204] For simplicity, BTTS's, including but not limited to chimeric notch receptor polypeptides, are described primarily as single polypeptide chains. However, BTTS's, 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.

[00205] Useful BTTS's 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.

[00206] Useful BTTS's 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.

[00207] Useful BTTS's that may be employed in the subject methods include, but are not limited to von Willebrand Factor (vWF) cleavage domain-based BTTS's, 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 BTTS's are described in

Langridge & Struhl (Cell (2017) 171(6):1383-1396); the disclosure of which is incorporated herein by reference in its entirety.

[00208] Useful BTTS's 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.

[00209] SynNotch polypeptides are generally proteolytically cleavable chimeric polypeptides that generally include: a) an extracellular domain comprising a specific binding member; b) a proteolytically cleavable Notch receptor polypeptide comprising one or more proteolytic cleavage sites; and c) an intracellular domain. Binding of the specific binding member by its binding partner generally induces cleavage of the synNotch at the one or more proteolytic cleavage sites, thereby releasing the intracellular domain. In some instances, the instant methods may include where release of the intracellular domain triggers (i.e., induces) the production of an encoded payload, the encoding nucleic acid sequence of which is contained within the cell. Depending on the particular context, the produced payload is then generally expressed on the cell surface or secreted. SynNotch polypeptides generally include at least one sequence that is heterologous to the Notch receptor polypeptide (i.e., is not derived from a Notch receptor), including e.g., where the extracellular domain is heterologous, where the intracellular domain is heterologous, where both the extracellular domain and the intracellular domain are heterologous to the Notch receptor, etc.

[00210] Useful synNotch BTTS's will vary in the domains employed and the architecture of such domains. SynNotch polypeptides will generally include a Notch receptor polypeptide that includes one or more ligand-inducible proteolytic cleavage sites. The length of Notch receptor polypeptides will vary and may range in length from about 50 amino acids or less to about 1000 amino acids or more.

[00211] In some cases, the Notch receptor polypeptide present in a synNotch polypeptide has a length of from 50 amino acids (aa) to 1000 aa, e.g., from 50 aa to 75 aa, from 75 aa to 100 aa, from 100 aa to 150 aa, from 150 aa to 200 aa, from 200 aa to 250 aa, from 250 a to 300 aa, from 300 aa to 350 aa, from 350 aa to 400 aa, from 400 aa to 450 aa, from 450 aa to 500 aa, from 500 aa to 550 aa, from 550 aa to 600 aa, from 600 aa to 650 aa, from 650 aa to 700 aa, from 700 aa

to 750 aa, from 750 aa to 800 aa, from 800 aa to 850 aa, from 850 aa to 900 aa, from 900 aa to 950 aa, or from 950 aa to 1000 aa. In some cases, the Notch receptor polypeptide present in a synNotch polypeptide has a length of from 300 aa to 400 aa, from 300 aa to 350 aa, from 300 aa to 325 aa, from 350 aa to 400 aa, from 750 aa to 850 aa, from 50 aa to 75 aa. In some cases, the Notch receptor polypeptide has a length of from 310 aa to 320 aa, e.g., 310 aa, 311 aa, 312 aa, 313 aa, 314 aa, 315 aa, 316 aa, 317 aa, 318 aa, 319 aa, or 320 aa. In some cases, the Notch receptor polypeptide has a length of 315 aa. In some cases, the Notch receptor polypeptide has a length of from 360 aa to 370 aa, e.g., 360 aa, 361 aa, 362 aa, 363 aa 364 aa, 365 aa, 366 aa, 367 aa, 368 aa, 369 aa, or 370 aa. In some cases, the Notch receptor polypeptide has a length of 367 aa.

[00212] In some cases, a Notch receptor polypeptide comprises an amino acid sequence having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence of a Notch receptor. In some instances, the Notch regulatory region of a Notch receptor polypeptide is a mammalian Notch regulatory region, including but not limited to e.g., a mouse Notch (e.g., mouse Notch1, mouse Notch2, mouse Notch3 or mouse Notch4) regulatory region, a rat Notch regulatory region (e.g., rat Notch1, rat Notch2 or rat Notch3), a human Notch regulatory region (e.g., human Notch1, human Notch2, human Notch3 or human Notch4), and the like or a Notch regulatory region derived from a mammalian Notch regulatory region and having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, amino acid sequence identity to the amino acid sequence of a mammalian Notch regulatory region of a mammalian Notch receptor amino acid sequence.

[00213] Subject Notch regulatory regions may include or exclude various components (e.g., domains, cleavage sites, etc.) thereof. Examples of such components of Notch regulatory regions that may be present or absent in whole or in part, as appropriate, include e.g., one or more EGF-like repeat domains, one or more Lin12/Notch repeat domains, one or more heterodimerization domains (e.g., HD-N or HD-C), a transmembrane domain, one or more proteolytic cleavage sites (e.g., a furin-like protease site (e.g., an S1 site), an ADAM-family protease site (e.g., an S2 site) and/or a gamma-secretase protease site (e.g., an S3 site)), and the like. Notch receptor polypeptides may, in some instances, exclude all or a portion of one or more Notch extracellular domains, including e.g., Notch-ligand binding domains such as Delta-

binding domains. Notch receptor polypeptides may, in some instances, include one or more non-functional versions of one or more Notch extracellular domains, including e.g., Notch-ligand binding domains such as Delta-binding domains. Notch receptor polypeptides may, in some instances, exclude all or a portion of one or more Notch intracellular domains, including e.g., Notch Rbp-associated molecule domains (i.e., RAM domains), Notch Ankyrin repeat domains, Notch transactivation domains, Notch PEST domains, and the like. Notch receptor polypeptides may, in some instances, include one or more non-functional versions of one or more Notch intracellular domains, including e.g., non-functional Notch Rbp-associated molecule domains (i.e., RAM domains), non-functional Notch Ankyrin repeat domains, non-functional Notch transactivation domains, non-functional Notch PEST domains, and the like.

[00214] Non-limiting examples of particular synNotch BTTS's, the domains thereof, and suitable domain arrangements are described in PCT Pub. Nos. WO 2016/138034, WO 2017/193059, WO 2018/039247 and U.S. Patent Nos. 9,670,281 and 9,834,608; the disclosures of which are incorporated herein by reference in their entirety.

[00215] Domains of a useful BTTS, e.g., the extracellular domain, the binding-transducer domain, the intracellular domain, etc., may be joined directly, i.e., with no intervening amino acid residues or may include a peptide linker that joins two domains. Peptide linkers may be synthetic or naturally derived including e.g., a fragment of a naturally occurring polypeptide.

[00216] A peptide linker can vary in length of from about 3 amino acids (aa) or less to about 200 aa or more, including but not limited to e.g., from 3 aa to 10 aa, from 5 aa to 15 aa, from 10 aa to 25 aa, from 25 aa to 50 aa, from 50 aa to 75 aa, from 75 aa to 100 aa, from 100 aa to 125 aa, from 125 aa to 150 aa, from 150 aa to 175 aa, or from 175 aa to 200 aa. A peptide linker can have a length of from 3 aa to 30 aa, e.g., 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 aa. A peptide linker can have a length of from 5 aa to 50 aa, e.g., from 5 aa to 40 aa, from 5 aa to 35 aa, from 5 aa to 30 aa, from 5 aa to 25 aa, from 5 aa to 20 aa, from 5 aa to 15 aa or from 5 aa to 10 aa.

[00217] In some instances, a BTTS may have an extracellular domain that includes a first member of a specific binding pair that binds a second member of the specific binding pair, wherein the extracellular domain does not include any additional first or second member of a second specific binding pair. For example, in some instances, a BTTS may have an extracellular domain that includes a first antigen-binding domain that binds an antigen, wherein the extracellular domain does not include any additional antigen-binding domains and does not bind

any other antigens. A subject BTTS may, in some instances, include only a single extracellular domain. Accordingly, an employed BTTS may be specific for a single antigen and only specific for the single antigen. Such, BTTS's may be referred to as a "single antigen BTTS".

[00218] In some instances, a BTTS may have an extracellular domain that includes the first or second members of two or more specific binding pairs. For example, in some instances, a BTTS may have an extracellular domain that includes a first antigen-binding domain and a second antigen-binding domain that are different such that the extracellular domain is specific for two different antigens. In some instances, a BTTS may have two or more extracellular domains that each includes the first or second members of two different specific binding pairs. For example, in some instances, a BTTS may have a first extracellular domain that includes a first antigen-binding domain and a second extracellular domain that includes a second antigen-binding domain where the two different antigen binding domains are each specific for a different antigen. As such, the BTTS may be specific for two different antigens.

[00219] A BTTS specific for two or more different antigens, containing either two extracellular domains or one extracellular domain specific for two different antigens, may be configured such that the binding of either antigen to the BTTS is sufficient to trigger activation of the BTTS, e.g., proteolytic cleavage of a cleavage domain of the BTTS, e.g., releasing an intracellular domain of the BTTS. Such a BTTS, capable of being triggered by any of two or more antigens, may find use in the described circuits as a component of a logic gate containing OR functionality. In some instances, a BTTS specific for two different antigens may be referred to as a "two-headed BTTS" or a tandem BTTS (or tanBTTS). For example, in some instances, a synNotch BTTS configured to bind two or more different antigens may be referred to as a tandem SynNotch or tanSynNotch. BTTS specific for multiple antigens will not be limited to only two antigens and may, e.g., be specific for and/or triggered by more than two antigens, including e.g., three or more, four or more, five or more, etc.

Methods of Making

[00220] The present disclosure further includes methods of making the nucleic acids, circuits, and cells employed in the herein described methods. In making the subject nucleic acids and circuits, and components thereof, any convenient methods of nucleic acid manipulation, modification and amplification (e.g., collectively referred to as "cloning") may be employed. In making the subject cells, containing the nucleic acids encoding the described circuits, convenient methods of transfection, transduction, culture, etc., may be employed.

[00221] A nucleotide sequence encoding all or a portion of the components of a circuit of the present disclosure can be present in an expression vector and/or a cloning vector. Where a subject circuit or component thereof is split between two or more separate polypeptides, nucleotide sequences encoding the two or more polypeptides can be cloned in the same or separate vectors. An expression vector can include a selectable marker, an origin of replication, and other features that provide for replication and/or maintenance of the vector. Suitable expression vectors include, e.g., plasmids, viral vectors, and the like.

[00222] Large numbers of suitable vectors and promoters are known to those of skill in the art; many are commercially available for generating a subject recombinant construct. The following vectors are provided by way of example. Bacterial: pBs, phagescript, PsiX174, pBluescript SK, pBs KS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene, La Jolla, Calif., USA); pTrc99A, pKK223-3, pKK233-3, pDR540, and pRIT5 (Pharmacia, Uppsala, Sweden). Eukaryotic: pWLneo, pSV2cat, pOG44, PXR1, pSG (Stratagene) pSVK3, pBPV, pMSG and pSVL (Pharmacia).

[00223] Expression vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences encoding heterologous proteins. A selectable marker operative in the expression host may be present. Suitable expression vectors include, but are not limited to, viral vectors (e.g. viral vectors based on vaccinia virus; poliovirus; adenovirus (see, e.g., Li et al., Invest Opthalmol Vis Sci 35:2543 2549, 1994; Borras et al., Gene Ther 6:515 524, 1999; Li and Davidson, PNAS 92:7700 7704, 1995; Sakamoto et al., H Gene Ther 5:1088 1097, 1999; WO 94/12649, WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655); adeno-associated virus (see, e.g., Ali et al., Hum Gene Ther 9:81 86, 1998, Flannery et al., PNAS 94:6916 6921, 1997; Bennett et al., Invest Opthalmol Vis Sci 38:2857 2863, 1997; Jomary et al., Gene Ther 4:683 690, 1997, Rolling et al., Hum Gene Ther 10:641 648, 1999; Ali et al., Hum Mol Genet 5:591 594, 1996; Srivastava in WO 93/09239, Samulski et al., J. Vir. (1989) 63:3822-3828; Mendelson et al., Virol. (1988) 166:154-165; and Flotte et al., PNAS (1993) 90:10613-10617); SV40; herpes simplex virus; human immunodeficiency virus (see, e.g., Miyoshi et al., PNAS 94:10319 23, 1997; Takahashi et al., J Virol 73:7812 7816, 1999); a retroviral vector (e.g., Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus); and the like.

[00224] As noted above, in some embodiments, a nucleic acid comprising a nucleotide sequence encoding a circuit or component thereof of the present disclosure will in some embodiments be DNA or RNA, e.g., *in vitro* synthesized DNA, recombinant DNA, *in vitro* synthesized RNA, recombinant RNA, etc. Methods for *in vitro* synthesis of DNA/RNA are known in the art; any known method can be used to synthesize DNA/RNA comprising a desired sequence. Methods for introducing DNA/RNA into a host cell are known in the art. Introducing DNA/RNA into a host cell can be carried out *in vitro* or *ex vivo* or *in vivo*. For example, a host cell (e.g., an NK cell, a cytotoxic T lymphocyte, etc.) can be transduced, transfected or electroporated *in vitro* or *ex vivo* with DNA/RNA comprising a nucleotide sequence encoding all or a portion of a circuit of the present disclosure.

[00225] Methods of the instant disclosure may further include culturing a cell genetically modified to encode a circuit of the instant disclosure including but not limited to e.g., culturing the cell prior to administration, culturing the cell in vitro or ex vivo (e.g., the presence or absence of one or more antigens), etc. Any convenient method of cell culture may be employed whereas such methods will vary based on various factors including but not limited to e.g., the type of cell being cultured, the intended use of the cell (e.g., whether the cell is cultured for research or therapeutic purposes), etc. In some instances, methods of the instant disclosure may further include common processes of cell culture including but not limited to e.g., seeding cell cultures, feeding cell cultures, passaging cell cultures, splitting cell cultures, analyzing cell cultures, treating cell cultures with a drug, harvesting cell cultures, etc.

[00226] Methods of the instant disclosure may, in some instances, further include receiving and/or collecting cells that are used in the subject methods. In some instances, cells are collected from a subject. Collecting cells from a subject may include obtaining a tissue sample from the subject and enriching, isolating and/or propagating the cells from the tissue sample. Isolation and/or enrichment of cells may be performed using any convenient method including e.g., isolation/enrichment by culture (e.g., adherent culture, suspension culture, etc.), cell sorting (e.g., FACS, microfluidics, etc.), and the like. Cells may be collected from any convenient cellular tissue sample including but not limited to e.g., blood (including e.g., peripheral blood, cord blood, etc.), bone marrow, a biopsy, a skin sample, a cheek swab, etc. In some instances, cells are received from a source including e.g., a blood bank, tissue bank, etc. Received cells may have been previously isolated or may be received as part of a tissue sample thus isolation/enrichment may be performed after receiving the cells and prior to use. In certain

instances, received cells may be non-primary cells including e.g., cells of a cultured cell line. Suitable cells for use in the herein described methods are further detailed herein.

NUCLEIC ACIDS

[00227] As summarized above, the present disclosure provides nucleic acids encoding a circuit for treating a subject for a heterogeneous EGFRvIII(+) GBM and components thereof. The subject nucleic acids may include, e.g., a sequence encoding a BTTS specific for EGFRvIII and a sequence encoding a targeting antigen-specific therapeutic, including e.g., a targeting antigen-specific therapeutic specific for one or more of EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR and/or ERBB2. Such nucleic acids may be configured such that the sequence encoding the targeting antigen-specific therapeutic is operably linked to a regulatory sequence responsive to activation of the BTTS. Provided are nucleic acids encoding essentially any circuit employing *trans*-targeting utilizing recognition of an EGFRvIII priming antigen expressed on a first GBM cell to target a second GBM cell expressing a targeting antigen, including but not limited to those circuits specifically described herein. Encompassed are isolated nucleic acids encoding the subject circuits as well as various configurations containing such nucleic acids, such as vectors, e.g., expression cassettes, recombinant expression vectors, viral vectors, and the like.

[00228] Recombinant expression vectors of the present disclosure include those comprising one or more of the described nucleic acids. A nucleic acid comprising a nucleotide sequence encoding all or a portion of the components of a circuit of the present disclosure will in some embodiments be DNA, including, e.g., a recombinant expression vector. A nucleic acid comprising a nucleotide sequence encoding all or a portion of the components of a circuit of the present disclosure will in some embodiments be RNA, e.g., *in vitro* synthesized RNA.

[00229] As summarized above, in some instances, the subject circuits may make use of an encoding nucleic acid (e.g., a nucleic acid encoding a BTTS or an antigen-specific therapeutic) that is operably linked to a regulatory sequence such as a transcriptional control element (e.g., a promoter; an enhancer; etc.). In some cases, the transcriptional control element is inducible. In some cases, the transcriptional control element is constitutive. In some cases, the promoters are functional in eukaryotic cells. In some cases, the promoters are cell type-specific promoters. In some cases, the promoters are tissue-specific promoters.

[00230] Depending on the host/vector system utilized, any of a number of suitable transcription and translation control elements, including constitutive and inducible promoters, transcription

enhancer elements, transcription terminators, etc. may be used in the expression vector (see e.g., Bitter et al. (1987) *Methods in Enzymology*, 153:516-544).

[00231] A promoter can be a constitutively active promoter (i.e., a promoter that is constitutively in an active/"ON" state), it may be an inducible promoter (i.e., a promoter whose state, active/"ON" or inactive/"OFF", is controlled by an external stimulus, e.g., the presence of a particular temperature, compound, or protein.), it may be a spatially restricted promoter (i.e., transcriptional control element, enhancer, etc.)(e.g., tissue specific promoter, cell type specific promoter, etc.), and it may be a temporally restricted promoter (i.e., the promoter is in the "ON" state or "OFF" state during specific stages of embryonic development or during specific stages of a biological process, e.g., hair follicle cycle in mice).

[00232] Suitable promoter and enhancer elements are known in the art. For expression in a bacterial cell, suitable promoters include, but are not limited to, lacI, lacZ, T3, T7, gpt, lambda P and trc. For expression in a eukaryotic cell, suitable promoters include, but are not limited to, light and/or heavy chain immunoglobulin gene promoter and enhancer elements; cytomegalovirus immediate early promoter; herpes simplex virus thymidine kinase promoter; early and late SV40 promoters; promoter present in long terminal repeats from a retrovirus; mouse metallothionein-I promoter; and various art-known tissue specific promoters.

[00233] In some instances, a transcriptional control element of a herein described nucleic acid may include a cis-acting regulatory sequence. Any suitable cis-acting regulatory sequence may find use in the herein described nucleic acids. For example, in some instances a cis-acting regulatory sequence may be or include an upstream activating sequence or upstream activation sequence (UAS). In some instances, a UAS of a herein described nucleic acid may be a Gal4 responsive UAS.

[00234] Suitable reversible promoters, including reversible inducible promoters are known in the art. Such reversible promoters may be isolated and derived from many organisms, e.g., eukaryotes and prokaryotes. Modification of reversible promoters derived from a first organism for use in a second organism, e.g., a first prokaryote and a second a eukaryote, a first eukaryote and a second a prokaryote, etc., is well known in the art. Such reversible promoters, and systems based on such reversible promoters but also comprising additional control proteins, include, but are not limited to, alcohol regulated promoters (e.g., alcohol dehydrogenase I (alcA) gene promoter, promoters responsive to alcohol transactivator proteins (AlcR), etc.), tetracycline regulated promoters, (e.g., promoter systems including TetActivators, TetON, TetOFF, etc.),

steroid regulated promoters (e.g., rat glucocorticoid receptor promoter systems, human estrogen receptor promoter systems, retinoid promoter systems, thyroid promoter systems, ecdysone promoter systems, mifepristone promoter systems, etc.), metal regulated promoters (e.g., metallothionein promoter systems, etc.), pathogenesis-related regulated promoters (e.g., salicylic acid regulated promoters, ethylene regulated promoters, benzothiadiazole regulated promoters, etc.), temperature regulated promoters (e.g., heat shock inducible promoters (e.g., HSP-70, HSP-90, soybean heat shock promoter, etc.), light regulated promoters, synthetic inducible promoters, and the like.

[00235] Inducible promoters suitable for use include any inducible promoter described herein or known to one of ordinary skill in the art. Examples of inducible promoters include, without limitation, chemically/biochemically-regulated and physically-regulated promoters such as alcohol-regulated promoters, tetracycline-regulated promoters (e.g., anhydrotetracycline (aTc)-responsive promoters and other tetracycline-responsive promoter systems, which include a tetracycline repressor protein (tetR), a tetracycline operator sequence (tetO) and a tetracycline transactivator fusion protein (tTA)), steroid-regulated promoters (e.g., promoters based on the rat glucocorticoid receptor, human estrogen receptor, moth ecdysone receptors, and promoters from the steroid/retinoid/thyroid receptor superfamily), metal-regulated promoters (e.g., promoters derived from metallothionein (proteins that bind and sequester metal ions) genes from yeast, mouse and human), pathogenesis-regulated promoters (e.g., induced by salicylic acid, ethylene or benzothiadiazole (BTH)), temperature/heat-inducible promoters (e.g., heat shock promoters), and light-regulated promoters (e.g., light responsive promoters from plant cells).

[00236] In some cases, the promoter is an immune cell promoter such as a CD8 cell-specific promoter, a CD4 cell-specific promoter, a neutrophil-specific promoter, or an NK-specific promoter. For example, a CD4 gene promoter can be used; see, e.g., Salmon et al. (1993) *Proc. Natl. Acad. Sci. USA* 90: 7739; and Marodon et al. (2003) *Blood* 101:3416. As another example, a CD8 gene promoter can be used. NK cell-specific expression can be achieved by use of an *Ncr1* (*p46*) promoter; see, e.g., Eckelhart et al. (2011) *Blood* 117:1565.

[00237] In some instances, an immune cell specific promoter of a nucleic acid of the present disclosure may be a promoter of a B29 gene promoter, a CD14 gene promoter, a CD43 gene promoter, a CD45 gene promoter, a CD68 gene promoter, a IFN-β gene promoter, a WASP

gene promoter, a T-cell receptor β-chain gene promoter, a V9 γ (TRGV9) gene promoter, a V2 δ (TRDV2) gene promoter, and the like.

[00238] In some cases, a nucleic acid comprising a nucleotide sequence encoding a circuit of the present disclosure, or one or more components thereof, is a recombinant expression vector or is included in a recombinant expression vector. In some embodiments, the recombinant expression vector is a viral construct, e.g., a recombinant adeno-associated virus (AAV) construct, a recombinant adenoviral construct, a recombinant lentiviral construct, a recombinant retroviral construct, etc. In some cases, a nucleic acid comprising a nucleotide sequence encoding a circuit of the present disclosure, or one or more components thereof, is a recombinant lentivirus vector. In some cases, a nucleic acid comprising a nucleotide sequence encoding a circuit of the present disclosure, or one or more components thereof, is a recombinant AAV vector.

[00239] Suitable expression vectors include, but are not limited to, viral vectors (e.g. viral vectors based on vaccinia virus; poliovirus; adenovirus (see, e.g., Li et al., Invest Opthalmol Vis Sci 35:2543 2549, 1994; Borras et al., Gene Ther 6:515 524, 1999; Li and Davidson, PNAS 92:7700 7704, 1995; Sakamoto et al., Hum Gene Ther 5:1088 1097, 1999; WO 94/12649, WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655); adeno-associated virus (see, e.g., Ali et al., Hum Gene Ther 9:81 86, 1998, Flannery et al., PNAS 94:6916 6921, 1997; Bennett et al., Invest Opthalmol Vis Sci 38:2857 2863, 1997; Jomary et al., Gene Ther 4:683 690, 1997, Rolling et al., Hum Gene Ther 10:641 648, 1999; Ali et al., Hum Mol Genet 5:591 594, 1996; Srivastava in WO 93/09239, Samulski et al., J. Vir. (1989) 63:3822-3828; Mendelson et al., Virol. (1988) 166:154-165; and Flotte et al., PNAS (1993) 90:10613-10617); SV40; herpes simplex virus; human immunodeficiency virus (see, e.g., Miyoshi et al., PNAS 94:10319 23, 1997; Takahashi et al., J Virol 73:7812 7816, 1999); a retroviral vector (e.g., Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, a lentivirus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus); and the like. In some cases, the vector is a lentivirus vector. Also suitable are transposon-mediated vectors, such as piggyback and sleeping beauty vectors.

[00240] In some instances, nucleic acids of the present disclosure may have a single sequence encoding two or more polypeptides where expression of the two or more polypeptides is made possible by the presence of a sequence element between the individual coding regions that facilitates separate expression of the individual polypeptides. Such sequence elements, may be

referred to herein as bicistronic-facilitating sequences, where the presence of a bicistronic-facilitating sequence between two coding regions makes possible the expression of a separate polypeptide from each coding region present in a single nucleic acid sequence. In some instances, a nucleic acid may contain two coding regions encoding two polypeptides present in a single nucleic acid with a bicistronic-facilitating sequence between the coding regions. Any suitable method for separate expression of multiple individual polypeptides from a single nucleic acid sequence may be employed and, similarly, any suitable method of bicistronic expression may be employed.

[00241] In some instances, a bicistronic-facilitating sequence may allow for the expression of two polypeptides from a single nucleic acid sequence that are temporarily joined by a cleavable linking polypeptide. In such instances, a bicistronic-facilitating sequence may include one or more encoded peptide cleavage sites. Suitable peptide cleavage sites include those of self-cleaving peptides as well as those cleaved by a separate enzyme. In some instances, a peptide cleavage site of a bicistronic-facilitating sequence may include a furin cleavage site (i.e., the bicistronic-facilitating sequence may encode a furin cleavage site).

[00242] In some instances, the bicistronic-facilitating sequence may encode a self-cleaving peptide sequence. Useful self-cleaving peptide sequences include but are not limited to e.g., peptide 2A sequences, including but not limited to e.g., the T2A sequence.

[00243] In some instances, a bicistronic-facilitating sequence may include one or more spacer encoding sequences. Spacer encoding sequences generally encode an amino acid spacer, also referred to in some instances as a peptide tag. Useful spacer encoding sequences include but are not limited to e.g., V5 peptide encoding sequences, including those sequences encoding a V5 peptide tag.

[00244] Multi- or bicistronic expression of multiple coding sequences from a single nucleic acid sequence may make use of but is not limited to those methods employing furin cleavage, T2A, and V5 peptide tag sequences. For example, in some instances, an internal ribosome entry site (IRES) based system may be employed. Any suitable method of bicistronic expression may be employed including but not limited to e.g., those described in Yang et al. (2008) Gene Therapy. 15(21):1411-1423; Martin et al. (2006) BMC Biotechnology. 6:4; the disclosures of which are incorporated herein by reference in their entirety.

CELLS

[00245] As summarized above, the present disclosure also provides immune cells. Immune cells of the present disclosure include those that contain one or more of the described nucleic acids, expression vectors, etc., encoding a described circuit. Immune cells of the present disclosure include mammalian immune cells including e.g., those that are genetically modified to produce the components of a circuit of the present disclosure or to which a nucleic acid, as described above, has been otherwise introduced. In some instances, the subject immune cells have been transduced with one or more nucleic acids and/or expression vectors to express one or more components of a circuit of the present disclosure.

[00246] Suitable mammalian immune cells include primary cells and immortalized cell lines. Suitable mammalian cell lines include human cell lines, non-human primate cell lines, rodent (e.g., mouse, rat) cell lines, and the like. In some instances, the cell is not an immortalized cell line, but is instead a cell (e.g., a primary cell) obtained from an individual. For example, in some cases, the cell is an immune cell, immune cell progenitor or immune stem cell obtained from an individual. As an example, the cell is a lymphoid cell, e.g., a lymphocyte, or progenitor thereof, obtained from an individual. As another example, the cell is a cytotoxic cell, or progenitor thereof, obtained from an individual. As another example, the cell is a stem cell or progenitor cell obtained from an individual.

[00247] As used herein, the term "immune cells" generally includes white blood cells (leukocytes) which are derived from hematopoietic stem cells (HSC) produced in the bone marrow. "Immune cells" includes, e.g., lymphoid cells, i.e., lymphocytes (T cells, B cells, natural killer (NK) cells), and myeloid-derived cells (neutrophil, eosinophil, basophil, monocyte, macrophage, dendritic cells). "T cell" includes all types of immune cells expressing CD3 including T-helper cells (CD4+ cells), cytotoxic T-cells (CD8+ cells), T-regulatory cells (Treg) and gamma-delta T cells. A "cytotoxic cell" includes CD8+ T cells, natural-killer (NK) cells, and neutrophils, which cells are capable of mediating cytotoxicity responses. "B cell" includes mature and immature cells of the B cell lineage including e.g., cells that express CD19 such as Pre B cells, Immature B cells, Mature B cells, Memory B cells and plasmablasts. Immune cells also include B cell progenitors such as Pro B cells and B cell lineage derivatives such as plasma cells.

[00248] Immune cells encoding a circuit of the present disclosure may be generated by any convenient method. Nucleic acids encoding one or more components of a subject circuit may be

stably or transiently introduced into the subject immune cell, including where the subject nucleic acids are present only temporarily, maintained extrachromosomally, or integrated into the host genome. Introduction of the subject nucleic acids and/or genetic modification of the subject immune cell can be carried out *in vivo*, *in vitro*, or *ex vivo*.

[00249] In some cases, the introduction of the subject nucleic acids and/or genetic modification is carried out *ex vivo*. For example, a T lymphocyte, a stem cell, or an NK cell is obtained from an individual; and the cell obtained from the individual is modified to express components of a circuit of the present disclosure. The modified cell can thus be redirected to one or more antigens of choice, as defined by the one or more antigen binding domains present on the introduced components of the circuit. In some cases, the modified cell is modulated *ex vivo*. In other cases, the cell is introduced into (e.g., the individual from whom the cell was obtained) and/or already present in an individual; and the cell is modulated *in vivo*, e.g., by administering a nucleic acid or vector to the individual *in vivo*.

CIRCUITS

[00250] As summarized above, the present disclosure also provides circuits encoded by nucleic acid sequences, also referred to in some instances as molecular circuits. Such circuits may, in some instances, be present and/or configured in expression vectors and/or expression cassettes. The subject nucleic acids of the present circuits may, in some instances, be contained within a vector, including e.g., viral and non-viral vectors. Such circuits may, in some instances, be present in cells, such as immune cells, or may be introduced into cells by various means, including e.g., through the use of a viral vector. Cells may, in some instances, be genetically modified to encode a subject circuit, where such modification may be effectively permanent (e.g., integrated) or transient as desired.

[00251] Encoded components of the circuits of the present disclosure will generally include at a minimum at least one encoded BTTS and at least one encoded antigen-specific therapeutic. Circuits of the present disclosure integrate multiple inputs, where such inputs include antigens, such as EGFRvIII priming antigen, one or more targeting antigens (e.g., EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR, ERBB2 and/or combinations thereof) and the like. The expression of a component of a circuit of the present disclosure may be dependent upon the state (i.e., active/inactive state) of another component of the circuit. For example, the expression of an antigen-specific therapeutic may be dependent upon the activation of a BTTS, where the BTTS is activated by binding to an antigen for which the BTTS is specific (e.g., EGFRvIII). In

some instances, dependency of one component of the circuit on another may be mediated by a regulatory sequence. For example, a sequence encoding a second component of a circuit may be operably linked to a regulatory sequence that is responsive to the activation of a first component of the circuit, thus linking the expression of the second component to the activation of the first.

[00252] The use of a BTTS in a circuit of the present disclosure facilitates the linking of expression and/or activity to molecular binding events. Systems involving binding-triggered transcriptional switches, and components thereof, have been described in PCT Publication No. WO 2016/138034, US Patent Application Pub. No. US 2016-0264665 A1 and issued U.S. Patent Nos. 9,670,281 and 9,834,608; the disclosures of which are incorporated by reference herein in their entirety.

[00253] Circuits of the present disclosure may be configured in various ways. In some instances, the independent activities and/or induced expression of two or more polypeptides or domains of a single polypeptide may generate a logic gated circuit. Such logic gated circuits may include but are not limited to e.g., "AND gates", "OR gates", "NOT gates" and combinations thereof including e.g., higher order gates including e.g., higher order AND gates, higher order OR gates, higher order NOT gates, higher order combined gates (i.e., gates using some combination of AND, OR and/or NOT gates). In some instances, useful circuits may further include IF/THEN gates.

[00254] "AND" gates include where two or more inputs are required for propagation of a signal. For example, in some instances, an AND gate allows signaling through a first input of a first polypeptide or a first polypeptide domain and a second input dependent upon the output of the first input. In an AND gate two inputs, e.g., two antigens, are required for signaling through the circuit.

[00255] "OR" gates include where either of two or more inputs may allow for the propagation of a signal. For example, in some instances, an OR gate allows signaling through binding of either of two different antigens. In an OR gate any one input, e.g., either of two antigens, may induce the signaling output of the circuit. In one embodiment, an OR gate may be achieved through the use of two separate molecules or constructs. In another embodiment, an OR gate may be achieved through the use of a single construct that recognizes two antigens, including e.g., a BTTS or an antigen-specific therapeutic (e.g., a CAR or TCR) having two different antigen binding domains that each bind a different antigen and each binding event can independently

propagate the signal (e.g., induce expression of a downstream component of the circuit, activate an immune cell, etc.).

[00256] "NOT" gates include where an input is capable of preventing the propagation of a signal. For example, in some instances, a NOT gate inhibits signaling through a circuit of the instant disclosure. In one embodiment, a NOT gate may prevent the expression of a component of a circuit, or activation of a particular component of the circuit, e.g., a CAR or a TCR.

[00257] "IF/THEN" gates include where the output of the gate depends upon a first input. For example, in some instances, IF a first input is present THEN signaling may proceed through a second input, and where the first input is absent signaling may not proceed. A non-limiting example of a circuit that includes an IF/THEN gate is a circuit having at least two receptors where the first receptor, in response to an input, induces expression of the second receptor, which has some output in response to a second input. As such, IF the first input of the first receptor is present, THEN the second receptor is expressed and signaling can proceed through the second receptor via the second input to produce the output. IF/THEN gates may or may not include an OR component (e.g., a receptor with OR functionality).

[00258] Non-limiting examples of IF/THEN gates, including examples with OR functionality, are depicted in FIG. 16. The circuit depicted in the first (top) cell of FIG. 16 includes a BTTS responsive to antigen "A" and an antigen-specific therapeutic that binds antigen "C". Note that although the antigen-specific therapeutic is depicted as a CAR, the disclosure is not so limited and other antigen-specific therapeutics may be readily substituted. In the first (top) circuit, IF antigen A is present THEN cell killing is induced based on the presence of antigen C.

[00259] In various embodiments, OR functionality may be employed, including where one or more components of a subject circuit include an OR functionality. As shown in the second, third and fourth cells depicted in FIG. 16, OR functionality may be provided by a BTTS, an antigen-specific therapeutic, or both having specificity for, and being triggered or activated by, two or more antigens.

[00260] For example, in the second (from the top) cell depicted in FIG. 16, a circuit is employed that includes a BTTS responsive to antigen "A" and an antigen-specific therapeutic that binds to, and is activated by, antigen "C" or antigen "D". In such a circuit, IF antigen A is present THEN cell killing is induced based on the presence of antigen C OR antigen D. Note that killing of cells expressing antigen C and antigen D may also be induced, as well as killing of cells that express antigen C alone or antigen D alone.

[00261] In the third (from the top) cell depicted in FIG. 16, a circuit is employed that includes a BTTS responsive to antigen "A" or antigen "B" and an antigen-specific therapeutic that binds to, and is activated by, antigen "C". In such a circuit, IF antigen A OR antigen B is present THEN cell killing is induced based on the presence of antigen C. Note that the immune cells encoding the subject circuit may be primed to kill by a cell expressing only antigen A, only antigen B, or both antigens A and B.

[00262] In the fourth (bottom) cell depicted in FIG. 16, a circuit is employed that includes a BTTS responsive to antigen "A" or antigen "B" and an antigen-specific therapeutic that binds to, and is activated by, antigen "C" or antigen "D". In such a circuit, IF antigen A OR antigen B is present THEN cell killing is induced based on the presence of antigen C or antigen D. Note that the immune cells encoding the subject circuit may be primed to kill by a cell expressing only antigen A, only antigen B, or both antigens A and B. Also note that killing of cells expressing antigen C and antigen D may also be induced, as well as killing of cells that express antigen C alone or antigen D alone.

[00263] In some instances, the use of OR functionality may have certain advantages. For example, the above described circuits having OR gate functionality (i.e., the second, third and fourth cells of FIG. 16) and variations thereof provide resistance to escape and improved efficacy for heterogeneous cancers because, without being bound by theory, to escape a cancer (or tumor) would need to contain, or evolve/produce, a cell that does not express either of the two priming and/or killing antigens.

[00264] In some instances, multiple antigen binding domains present on a BTTS or antigen-specific therapeutic may provide an OR gate capability to the herein described molecular circuits. For example, in some instances, a BTTS having two different antigen binding domains may be responsive to a first antigen (e.g., a first priming antigen) OR a second antigen (e.g., a second priming antigen). In some instances, an antigen-specific therapeutic (e.g., a CAR, a TCR, etc.) having two different antigen binding domains may be responsive to a first antigen (e.g., a first targeting antigen) OR a second antigen (e.g., a second targeting antigen).

[00265] In some instances, such OR gates may be combined with other gates, including an AND gate. For example, a nucleic acid encoding an OR-gate antigen-specific therapeutic having two different antigen binding domains may be operably linked to a promoter that is responsive to a BTTS which is responsive to a first antigen (e.g., EGFRvIII). As such, upon binding the first

antigen (e.g., EGFRvIII), the BTTS drives expression of the antigen-specific therapeutic which is responsive to two different antigens, resulting in an AND-OR gate.

[00266] In some instances, OR gates may find use in the circuits of the present disclosure to produce an OR gate for two or more targeting antigens (or two or more killing antigens). For example, in some instances, the circuit may be configured such that the cell genetically modified with the circuit contains a nucleic acid sequence encoding an antigen-specific therapeutic that binds to a first targeting/killing antigen or a second targeting/killing antigen expressed by a targeted cancer cell (or expressed by two different targeted cancer cells), thereby producing a cell that is activated, e.g., activated for cell killing, by either the first targeting/killing antigen or the second targeting/killing antigen. In some instances, a circuit of the present disclosure may include nucleic acid sequence encoding a first antigen-specific therapeutic and second antigen-specific therapeutic that each bind to a different targeting/killing antigen. Useful antigens in such dual antigen-specific therapeutic OR gates include but are not limited to e.g., EphA2, EphA3, IL13RA2 (or IL13RA1), EGFR and ERBB2.

[00267] In some instances, an OR gate may be employed to allow for simultaneous targeting of cells both in *trans* and in *cis*. For example, in some instances, a second killing antigen to which an OR gate is directed may be expressed by the priming cell. In some instances, an OR gate for targeting may be employed to target two antigens that that are not mutually exclusively expressed within cells of the GBM (i.e., GBM cells with overlapping, but not completely coincident, expression of two antigens). For example, in some instances, the second killing antigen to which an OR gate is targeted may be expressed by a subpopulation of GBM cells that also expresses the first killing antigen. However, the cancer may further include a subpopulation of cells that express the second killing antigen but not the first killing antigen. In some instances, the first and second killing antigens employed in an OR gate will not have overlapping expression in the cells of the heterogeneous cancer. As such, in some instances, the second killing antigen may be expressed by a cell of the heterogeneous GBM other than the priming cell and/or the GBM cell that expresses the first killing antigen.

KITS

[00268] The present disclosure provides a kit for carrying out a method as described herein and/or constructing one or more circuits, components thereof, nucleic acids encoding a circuit or a component thereof, etc. In some cases, a subject kit comprises a vector, e.g., an expression vector or a delivery vector, comprising a nucleotide sequence encoding a circuit of the present

disclosure or one or more portions thereof. Delivery vectors may be provided in a delivery device or may be provided separately, e.g., as a kit that includes the delivery vector and the delivery device as separate components of the kit.

[00269] In some cases, a subject kit comprises a cell, e.g., a host cell or host cell line, that is or is to be genetically modified with a nucleic acid comprising nucleotide sequence encoding a circuit of the present disclosure or a portion thereof. In some cases, a subject kit comprises a cell, e.g., a host cell, that is or is to be genetically modified with a recombinant expression vector comprising a nucleotide sequence encoding a circuit of the present disclosure. Kit components can be in the same container, or in separate containers.

[00270] Any of the above-described kits can further include one or more additional reagents, where such additional reagents can be selected from: a dilution buffer; a reconstitution solution; a wash buffer; a control reagent; a control expression vector; a nucleic acid encoding a negative control (e.g., a circuit that lacks the one or more critical elements); a nucleic acid encoding a positive control polypeptide; and the like.

[00271] In addition to above-mentioned components, a subject kit can further include instructions for using the components of the kit to practice the subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or subpackaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, flash drive, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

Examples of Non-Limiting Aspects of the Disclosure

[00272] Aspects, including embodiments, of the present subject matter described above may be beneficial alone or in combination, with one or more other aspects or embodiments. Without limiting the foregoing description, certain non-limiting aspects of the disclosure are provided

below. As will be apparent to those of skill in the art upon reading this disclosure, each of the individually numbered aspects may be used or combined with any of the preceding or following individually numbered aspects. This is intended to provide support for all such combinations of aspects and is not limited to combinations of aspects explicitly provided below:

1. A method of treating a subject for an epidermal growth factor receptor variant III (EGFRvIII) positive glioblastoma, the method comprising:

administering to the subject an immune cell genetically modified with:

(a) a nucleic acid sequence encoding a binding triggered transcriptional switch (BTTS) that binds to EGFRvIII;

(b) a nucleic acid sequence encoding an antigen-specific therapeutic that binds to a killing antigen expressed by the glioblastoma; and

(c) a regulatory sequence operably linked to (b) that is responsive to the BTTS;

wherein binding of the BTTS to EGFRvIII activates expression of the antigen-specific therapeutic which binds the killing antigen thereby inducing killing of glioblastoma cells expressing the killing antigen.

2. The method according to aspect 1, wherein the EGFRvIII positive glioblastoma comprises cells that express either EGFRvIII or the killing antigen.

3. The method according to aspect 1 or aspect 2, wherein the glioblastoma comprises cells that express both EGFRvIII and the killing antigen.

4. The method according to aspect 1, wherein the killing antigen is expressed by all cells of the glioblastoma.

5. The method according to any of the preceding aspects, wherein the killing antigen is expressed by non-glioblastoma cells in the subject.

6. The method according to any of the preceding aspects, wherein less than 95% of the glioblastoma cells express EGFRvIII.

7. The method according to any of the preceding aspects, wherein the killing antigen is selected from the group consisting of: Ephrin type-A receptor 2 (EphA2), Ephrin type-A receptor 3 (EphA3), Interleukin-13 receptor subunit alpha-1 (IL13RA1), Interleukin-13 receptor subunit alpha-2 (IL13RA2),, Epidermal growth factor receptor (EGFR) and erb-b2 receptor tyrosine kinase 2 (ERBB2).

8. The method according to any of the preceding aspects, wherein the antigen-specific therapeutic, when expressed, is expressed on the surface of the immune cell.

9. The method according to aspect 8, wherein the antigen-specific therapeutic is a chimeric antigen receptor (CAR) or a T cell receptor (TCR).

10. The method according to any of aspects 1 to 7, wherein the antigen-specific therapeutic, when expressed, is secreted by the immune cell.

11. The method according to aspect 10, wherein the antigen-specific therapeutic is a chimeric bispecific binding member.

12. The method according to aspect 11, wherein the chimeric bispecific binding member is a TCR-targeted bispecific binding agent.

13. The method according to aspect 11 or aspect 12, wherein the chimeric bispecific binding member is specific for the killing antigen and a protein expressed on the surface of an immune cell.

14. The method according to aspect 13, wherein the protein expressed on the surface of an immune cell is CD3.

15. The method according to any of the preceding aspects, wherein the antigen-specific therapeutic comprises a bio-orthogonal adapter molecule.

16. The method according to aspect 15, wherein the bio-orthogonal adapter molecule is bound by an extracellular domain of a switchable CAR.

17. The method according to aspect 16, wherein the bio-orthogonal adapter molecule comprises a peptide neo-epitope (PNE) and the extracellular domain of the switchable CAR binds the PNE.

18. The method according to any of aspects 15 to 17, wherein the bio-orthogonal adapter molecule binds an antigen selected from the group consisting of: EphA2, EphA3, IL13RA1, IL13RA2, EGFR and ERBB2.

19. The method according to any of the preceding aspects, wherein the antigen-specific therapeutic binds two different killing antigens expressed by the glioblastoma.

20. The method according to aspect 19, wherein the two different killing antigens are expressed by EGFRvIII positive glioblastoma cells.

21. The method according to aspect 19, wherein the two different killing antigens are expressed by glioblastoma cells other than EGFRvIII positive glioblastoma cells.

22. The method according to aspect 19, wherein the two different killing antigens are expressed in the same glioblastoma cells.

23. The method according to aspect 19, wherein the two different killing antigens are expressed in different glioblastoma cells.

24. The method according to any of aspects 19 to 23, wherein the two different killing antigens are selected from the group consisting of: EphA2, EphA3, IL13RA1, IL13RA2, EGFR and ERBB2.

25. The method according to aspect 24, wherein the two different killing antigens are EphA2 and IL13RA2.

26. The method according to any of the preceding aspects, wherein the immune cell is further genetically modified with a nucleic acid sequence encoding a second antigen-specific therapeutic that binds to a second killing antigen expressed by the glioblastoma.

27. The method according to aspect 26, wherein the second killing antigen is expressed by EGFRvIII positive glioblastoma cells.

28. The method according to aspect 26, wherein the second killing antigen is expressed by glioblastoma cells expressing the first killing antigen.

29. The method according to aspect 26, wherein the second killing antigen is expressed by a cell of the glioblastoma other than the EGFRvIII positive glioblastoma cells or the glioblastoma cells expressing the first killing antigen.

30. The method according to any of aspects 26 to 29, wherein the second killing antigen is selected from the group consisting of: EphA2, EphA3, IL13RA1, IL13RA2, EGFR and ERBB2.

31. The method according to any of aspects 26 to 30, wherein the second killing antigen is expressed by all cells of the glioblastoma.

32. The method according to any of aspects 26 to 31, wherein the second killing antigen is expressed by non-glioblastoma cells in the subject.

33. The method according to any of the preceding aspects, wherein the BTTS is a SynNotch polypeptide.

34. The method according to any of the preceding aspects, wherein the immune cell is a myeloid cell.

35. The method according to any of aspects 1 to 33, wherein the immune cell is a lymphoid cell.

36. The method according to aspect 35, wherein the lymphoid cell is selected from the group consisting of: a T lymphocyte, a B lymphocyte and a Natural Killer cell.

37. The method according to any of the preceding aspects, wherein the method further comprises identifying that the glioblastoma comprises EGFRvIII positive cells and cells that express the killing antigen.

38. The method according to aspect 37, wherein the identifying comprises assaying a sample of the glioblastoma obtained from the subject for cellular expression of EGFRvIII and the killing antigen.

39. The method according to aspect 38, wherein the sample is a biopsy.

EXAMPLES

[00273] The following examples are put forth so as to provide those of ordinary skill in the art 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 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.

Example 1: Treatment of glioblastoma using EGFRviii prime/kill circuit encoding therapeutic cells in a mouse model

[00274] Certain forms of glioblastoma are associated with a unique EGFR splice form called EGFRviii. This unique neoepitope can be targeted by a specific CAR T cell. However, although these CAR T cells have been shown to infiltrate tumors and kill the EGFRviii+ cells in clinical trials, clinical benefit has been limited because EGFRviii expression is highly heterogeneous (expressed in 25-70% of tumor cells) and cancer cells lacking the antigen escape and survive. Thus, the EGFRviii neoantigen has not been effectively harnessed as a target for adoptive CAR T cell therapy.

[00275] Conversely, other antigens that could potentially be targeted in GBM are not absolutely specific, and although some are expressed throughout the tumor, they are also be expressed in other normal tissues. Thus, targeting both EGFRviii and one or more other GBM antigens using combined independent therapies could be either ineffective or yield toxic cross-reactivity.

[00276] In this example, a novel way to use the targeting specificity of the EGFRviii neoantigen was developed by using it to prime the expression of a second molecule that targets and kills tumor cells based on a second, homogenously expressed antigen (or combination of antigens). This approach is effective even if the second antigen(s) are not perfectly tumor-specific. Without being bound by theory, in essence this approach harnesses two or more imperfect antigens (EGFRviii and the second antigen(s)) to develop a combinatorial T cell that shows both high selectivity and is insensitive to antigen expression heterogeneity.

[00277] Circuits were designed in which a therapeutic cell is primed based on the tumor specific antigen EGFRviii, inducing expression of killing agent (e.g., a CAR, a BiTE, etc.) that then kills based on a homogenous antigen (see **FIG. 1A**). In other words, the circuit is primed based on the cancer-specific but heterogeneous antigen, but is then activated to kill in a "killing zone" around the priming antigen cells by targeting a homogeneously expressed antigen (see **FIG. 1B**). The killing zone size is tunable based on a variety of factors such as, but not limited to, killing receptor (e.g., CAR) stability or the use of extracellular diffusible agents as killing payload (e.g. bispecific adapters) (see **FIG. 1C** and **FIG. 1D**).

[00278] As depicted in **FIG. 1A-1D**, priming of therapeutic cells, such as a cell engineered with a circuit as depicted in **FIG. 1A**, creates a killing zone around the therapeutic cell such that tumor cells expressing the killing antigen are targeted even when such tumor cells do not express the priming antigen. An example of this scenario is schematized in **FIG. 1B**, which shows a therapeutic cell, shown as a T cell, primed by a tumor heterogeneously expressing the priming antigen, EGFRviii. The primed therapeutic cell targets and kills tumor cells in its proximity, including those expressing the killing antigen but not the priming antigen. In this way, cells in the proximity of the tumor prime the therapeutic cells to create a killing zone around the primed cell, leading to effective clearance of all tumor cells.

[00279] The size of the killing zone may be widened or tuned as desired, e.g., through the use of a diffusible payload, stability of the therapeutic employed (e.g., CAR stability). For example, **FIG. 1C** depicts a circuit that includes a synNotch binding-triggered transcriptional switch configured to bind a priming antigen (EGFRviii, circle) which induces expression of a diffusible

CAR head. The diffusible CAR head is specific for a killing antigen (triangle) and is bound by a portion of a CAR, referred to in **FIG. 1C** as a "split CAR", that includes the intracellular signaling components necessary for T cell activation upon antigen binding. Accordingly, by diffusing away from the primed cell, the diffusible CAR head serves to mediate antigen recognition and target cell killing in more distant T cells that express the split CAR, but do not necessarily express the diffusible CAR head.

[00280] As depicted in the left panel of **FIG. 1D**, by using a circuit that includes a synNotch driving expression of a traditional CAR (i.e., a single continuous chain having an antigen recognition domain and the intracellular signaling components), the killing radius of non-priming cancer cells that express the killing antigen is kept relatively short. In comparison, as depicted in the right panel of **FIG. 1D**, by using a circuit that includes a diffusible orthogonal bispecific adapter, such as a diffusible CAR head, the killing radius of non-priming cancer cells that express the killing antigen is widened. Accordingly, the desired killing radius may be controlled as desired. In some instances, e.g., a short killing radius may be desired where a killing antigen is expressed in non-cancerous tissues (i.e., bystander tissues). In other instances, a wide killing radius may be desired where, e.g., relatively few cells expressing the priming antigen are present diffusely throughout a cancerous area of a subject.

[00281] Whether this type of circuit could function in a GBM model was tested. For this purpose a GBM specific antigen, Epidermal Growth Factor Receptor Variant III (EGFRvIII), as the priming antigen and GBM associated antigen, Ephrin type-A receptor 2 (EphA2) and IL-13 receptor α2, as the target antigen, were used. Tumor cell lines were mixed in varying ratios to recapitulate the heterogeneity observed in GBM patients (10-100% priming surface EGFRvIII U87 mixed with the requisite percentage of target U87 cells) (**FIG. 2**). Such heterogeneous tumor cell line mixtures were utilized in cytotoxicity assay with an engineered primary human CD8 T cells. These synNotch CAR T cells only express the inducible CAR when the cell engages EGFRvIII antigen, thus, the T cells that are cultured with only EGFRvIII negative U87 cells should not express any CAR. When the synNotch CAR T cell engages the priming antigen, EGFRvIII, the corresponding CAR expresses, and the T cells are 'primed' to kill surrounding target tumor cells (**FIG. 3**).

[00282] Since clinical models of glioblastoma have shown antigen escape to be a problem in tumor recurrence after CAR T cell treatment, testing to determine whether a tandem CAR IL13 mutein-EphA2 CAR could be effectively employed in the context of prime-and-kill circuit was

performed. By targeting multiple antigens rather than a single antigen the risk of tumor escape is reduced.

[00283] The cytotoxic capability of a constitutive EphA2 CAR or constitutive IL13 Mutein-EphA2 CAR against U87 cells was first tested. The constitutive IL13 Mutein-EphA2 CAR had more efficient cytotoxicity as compared to the constitutive EphA2 CAR alone.

[00284] The IL13 Mutein-EphA2 CAR and the EphA2 CAR were tested under the control of anti-EGFRvIII synNotch to evaluate inducibility of expression as well as cytotoxic capacity under the prime-and-kill circuit. Both the IL13 Mutein-EphA2 CAR and the EphA2 CAR were only expressed when synNotch CAR T cells engaged with the priming antigen, EGFRvIII. Therefore, the circuits have minimal to no cytotoxicity to target cells when no priming cells are present. However, unlike the increased cytotoxicity in the constitutive IL13 Mutein-EphA2 CAR when compared against the constitutive EphA2 CAR, no significant difference in cytotoxic activity under the prime-and-kill circuit was observed. Since no decrease in cytotoxic activity was seen and because targeting two different antigen mitigates the risk for tumor escape, the IL13 mutein-EphA2 CAR was used for further characterization in both *in vitro* and *in vivo* assay.

[00285] Given the above described results, it was expected that the percentage of cells that express the priming antigen would be an important parameter that regulates the effectiveness of the 'prime and kill' circuit with the U87 system. Therefore, the induction of CAR expression and the kinetics of the elimination of two target tumor cell populations over 72 hours were assessed. The results showed that tumor cell populations with a lower percentage of priming tumor cells were more difficult to eliminate since both the level of CAR expression and percentage of T cells positive for CAR expression were lower. Even so, the 'prime and kill' circuit was effective at driving the full eradication of a heterogeneous population of tumor cells where only 10% of the tumor cells expressed the priming antigen (**FIG. 4**). Furthermore, it was observed that decreasing the ratio of priming antigen led to delayed killing kinetics (**FIG. 5**). All together, these findings show that synNotch CAR T cell prime/kill circuits may be employed to address heterogeneous tumors in glioblastoma and such circuits reduce both the chances of tumor escape and off-target toxicity.

[00286] The effectiveness of a prime/kill circuit based on priming with EGFRviii was also demonstrated using an *in vivo* preclinical heterogeneous GBM model, with 50% of GBM cells of the heterogeneous tumor expressing EGFRviii and 50% of GBM cells of the heterogeneous

tumor negative for EGFRviii. Mice carrying the heterogeneous GBM model tumors were administered therapeutic T cells encoding a prime/kill circuit employing a synNotch receptor detecting EGFRviii to trigger expression of a two headed CAR (with domains that recognize EphA2 and IL13R antigens).

[00287] A time course displaying tumor reduction in five (5) of the treated mice is provided in **FIG. 6A**. Tumor size and model animal survival was also quantified. As shown in **FIG. 6B**, animals treated with cells encoding the EGFRviii priming and EphA2/IL13R killing circuit (refereed to "Smart-CAR-T") showed a reduction in tumor growth as compared to both vehicle ("PBS") and "Non-CAR-T" treated controls. As shown in **FIG. 6C**, mice treated with Smart-CAR-T cells also showed prolonged survival as compared to controls.

[00288] In a further experiment U87wt (EphA2 and IL13rα2 expressing only) cells were mixed with U87-EGFRvIII cells in a 1:1 ratio and engrafted orthotopically into immunodeficient mice (see **FIG. 7**). *In vivo* heterogeneity of the SynNotch priming antigen, EGFRviii, was confirmed by histological examination of the brain on day 7. Mice were distributed according to tumor burden into equivalent treatment and control groups before receiving a single intravenous injection of 6 million of either EGFRviii-synCART or non-transduced T cells one week after establishment of heterogeneous GBM xenograft (**FIG. 8**).

[00289] Marked reduction in tumor burden was observed in the cohort of mice treated with synCART as assessed by longitudinal bioluminescence imaging (**FIG. 9**). In addition, substantial improvement in the survival was seen in the synCART-treated mice compared with non-transduced T cell treated controls (**FIG. 10**). Systemic delivery of synCART achieved potent and lasting tumor clearance as seen in follow-up histologic analysis of the brains of treated mice censored due to non-tumor related issues at endpoint triggered by the onset of GVHD.

[00290] To study the *in vivo* dynamics of CAR expression following recognition of priming antigen EGFRviii, tumors were examined two days after administration of synCART. Upregulation of IL13 mutein-anti-EphA2 CAR (killing CAR) was observed, measured by a c-terminally tagged GFP, in the brain but not spleen (**FIG. 11**). This finding shows that expression of killing CAR is localized to the microenvironment bearing priming antigen. Furthermore, the induced CAR expression in the brain was downregulated with reduction in tumor burden.

[00291] The ability of prime-and-kill T cells to ignore off-target/bystander cells was also investigated using a flank control tumor expressing killing antigen (EphA2/IL13RA2) but

lacking priming antigen. Put another way, given the observed success of EGFRviii-synCART in reducing tumor burden, whether prime-and-kill T cells activated in heterogeneous tumors would migrate out and attack cells expressing killing antigen alone was investigated. For these experiments, a dual tumor model was employed: a heterogeneous (1:1 U87-EGFRviii/U87wt) intracranial tumor and a flank tumor (U87wt – lacking priming antigen) (**FIG. 12**).

[00292] Tumors were implanted and on day 6 CD4+ and CD8+ T cells engineered to express EGFRviii-synCAR were injected (**FIG. 13**). Tumor burden was assessed by longitudinal bioluminescence imaging. Mice treated with control untransduced T cells reached euthanasia endpoint rapidly while synCART treated animals displayed marked reduction in intracranial tumor burden without any impact on the growth of flank tumor (**FIG. 14**). This *in vivo* data shows that SynNotch driven IL13mutein/EphA2 CAR expression and T cell functionality is confined to tumors expressing both priming and killing antigen. No evidence of priming of synCART in flank tumors was observed, showing that the activate/kill behavior of synCART was absent in EphA2+ bystander tissues.

[00293] Collectively, these data demonstrate the effectiveness of prime/kill circuits using EGFRviii as the priming antigen for treating heterogeneous GBM.

Materials & Methods

[00294] SynNotch Receptor and Response Element Construct Design: SynNotch receptors were built by fusing the LaG17 (lower affinity GFP), or EGFRvIII 139 scFv (Johnson et al. Sci Transl Med. (2015) 7(275):275ra2; the disclosure of which is incorporated herein by reference in its entirety) to the mouse Notch1 (NM_008714) minimal regulatory region (Ile1427 to Arg1752) and Gal4 DBD VP64. All synNotch receptors contain an n-terminal CD8a signal peptide (MALPVTALLLPLALLLHAARP; SEQ ID NO:24) for membrane targeting and a myc-tag (EQKLISEEDL; SEQ ID NO:25) or flag-tag (DYKDDDDK; SEQ ID NO:26) for easy determination of surface expression with a-myc A647 (cell-signaling) or a-flag A647 (RND systems); see Morsut et al., Cell. (2016) 164(4):780-91 (the disclosure of which is incorporated herein by reference in its entirety) for synNotch receptor peptide sequences). The receptors were cloned into a modified pHR'SIN:CSW vector containing a PGK or SFFV promoter for all primary T cell experiments. The pHR'SIN:CSW vector was also modified to make the response element plasmids. Five copies of the Gal4 DNA binding domain target sequence (GGAGCACTGTCCTCCGAACG; SEQ ID NO:27) were cloned 5' to a minimal CMV

promoter. Also included in the response element plasmids is a PGK promoter that constitutively drives mCherry or BFP expression to easily identify transduced T cells. Inducible EphA2 CAR was built by fusing the CD19 scFv (Porter et al. N Engl J Med. (2011) 365(8):725-33; the disclosure of which is incorporated herein by reference in its entirety), EphA2 scFv (Goldgur et al., Growth Factors. (2014) 32(6):214-22; the disclosure of which is incorporated herein by reference in its entirety), IL13 Mutein [E13K, K105R] (Krebs et al., Cytotherapy. (2014) 16(8):1121-3; the disclosure of which is incorporated herein by reference in its entirety)-G4Sx4-EphA2 scFv (Goldgur et al.) to the hinge region of the human CD8α chain and transmembrane and cytoplasmic regions of the human 4-1BB, and CD3z signaling endodomains. The inducible CAR constructs were cloned via a BamHI site in the multiple cloning site 3' to the Gal4 response elements. For some inducible CAR vectors, the CARs were tagged c-terminally with GFP/BFP or contain myc/flag tag to verify surface expression. All constructs were cloned via in fusion cloning (Clontech/Takara).

[00295] Primary Human T Cell Isolation and Culture: Primary CD4+ and CD8+ T cells were isolated from anonymous donor blood after apheresis by negative selection (STEMCELL Technologies). T cells were cryopreserved in RPMI-1640 with 20% human AB serum (Valley Biomedical) and 10% DMSO. After thawing, T cells were cultured in human T cell medium consisting of X-VIVO 15 (Lonza), 5% Human AB serum, and 10 mM neutralized N-acetyl L-Cysteine (Sigma-Aldrich) supplemented with 30 units/mL IL-2 (NCI BRB Preclinical Repository) for all experiments.

[00296] Lentiviral Transduction of Human T Cells: Pantropic VSV-G pseudotyped lentivirus was produced via transfection of Lenti-X 293T cells (Clontech/Takara) with a pHR'SIN:CSW transgene expression vector and the viral packaging plasmids pCMVdR8.91 and pMD2.G using Fugene HD (Promega). Primary T cells were thawed the same day and, after 24 hr in culture, were stimulated with Human T-Activator CD3/CD28 Dynabeads (Life Technologies) at a 1:3 cell:bead ratio. At 48 hr, viral supernatant was harvested and the primary T cells were exposed to the virus for 24 hr. At day 4 after T cell stimulation, the Dynabeads were removed, and the T cells expanded until day 9 when they were rested and could be used in assays. T cells were sorted for assays with a Beckton Dickinson (BD) FACs ARIA Fusion. AND-gate T cells exhibiting basal CAR expression were gated out during sorting.

[00297] Cancer Cell Lines: The cancer cell lines used were K562 myelogenous leukemia cells (ATCC) and U87 MG glioblastoma cells (ATCC). K562s were lentivirally transduced to stably

express human CD19 at equivalent levels as Daudi tumors. CD19 levels were determined by staining the cells with a-CD19 PE-Cy7 (BD Biosciences). K562s cells were also transduced to stably express surface GFP (GFP fused to the PDGF transmembrane domain). The CD19 and surface-GFP peptide sequences can be found in Morsut et al., (2016). U87 MG were lentivirally transduced to stably express GFP or mCherry under control of the spleen focus-forming virus (SFFV) promoter. At 72 hours after transductions, cells were sorted on an Aria Fusion cell sorter (BD Biosciences) on the basis of GFP expression to be 100% GFP or mCherry positive and subsequently expanded. All cell lines were sorted for expression of the transgenes.

[00298] In Vitro Stimulation of SynNotch T cells: For all in vitro synNotch T cell stimulations, 1 x 10^5 T cells were co-cultured with 1 x 10^5 K562 cells in complete human T cell media. After mixing the T cells and cancer cells in round bottom 96-well tissue culture plates, the cells were centrifuged for 1 min at 400 x g to force interaction of the cells, and the cultures were analyzed at 24-72 hr for activation and specific lysis of target tumor cells. For all in vitro synNotch T cell stimulations co-cultured with U87, 1 x 10^4 U87s were cultured overnight in a flat bottom 96-well tissue culture plate. Next morning, 1 x 10^4 – 5 x 10^4 T cells were added to the flat bottom 96-well tissue culture plate and the co-cultures were analyzed at 24-96 hr for activation and specific lysis of target tumor cells. All flow cytometry was performed using BD LSR II or Attune NxT Flow Cytometer and the analysis was performed in FlowJo software (TreeStar).

[00299] Assessment of SynNotch AND-Gate T Cell Cytotoxicity: CD8+ synNotch AND-Gate T cells were stimulated for 24-96 hr as described above with target cells expressing the indicated antigens. The level of specific lysis of target cancer cells was determined by comparing the fraction of target cells alive in the culture compared to treatment with untransduced T cell controls. Cell death was monitored by shifting of the target cells out of the side scatter and forward scatter region normally populated by the target cells. Alternatively, cell viability was analyzed using the IncuCyte Zoom system (Essen Bioscience). The target cells and T cells were co-cultured as described above. 2 fields of view were taken per well every 15 minutes. The mean florescence intensity (MFI) was calculated using IncuCyte Zoom software (Essen BioScience) in order to determine the target cell survival.

[00300] Mouse Models: For orthotropic heterogeneous model, mix of 1.5 × 10^4 U87-luc-mCherry and 1.5 × 10^4 U87-luc-EGFRvIII-GFP cells were implanted intracranially into 6- to 8-week-old female NCG mice (Charles River), with 6-10 mice per group. The surgical implants were done using a stereotactic surgical setup with tumor cells implanted 2 mm right and 1 mm

anterior to the bregma and 3 mm into the brain. Before surgery and for 3 days after surgery, mice were treated with an analgesic and monitored for adverse symptoms. In subcutaneous model, NCG mice were injected with 1.0×10^6 U87-Luc-mcherry tumors subcutaneously in 100 µl of HBSS on day 0. Tumor progression was evaluated by luminescence emission on a Xenogen IVIS Spectrum after intraperitoneal D-luciferin injection according to the manufacturer's directions (GoldBio). Prior to the treatment, mice were randomized such that initial tumor burden in the control and treatment groups were equivalent. Mice were treated with 6.0×10^6 CART or a matched number of untransduced T cells intravenously via tail vein in 100 µl of PBS. Survival was followed over time until predetermined endpoint was reached ($n = 6$ to 10 mice per group).

[00301] Immunofluorescence and Confocal Microscopy: Mice were euthanized before being perfused transcardially with cold PBS. Brains were then removed and fixed overnight in 4% PFA–PBS before being transferred to 30% sucrose and were allowed to sink (1-2 d). Subsequently, the brains were embedded in O.C.T. Compound (Tissue-Tek; 4583; Sakura Finetek). Serial 10-µm coronal sections were then cut on freezing microtome and stored at -20 ^{0}C. Images were acquired using a Zeiss Axio Imager 2 microscope (×20 magnification) and TissueFAXS scanning software (TissueGnostics). Identical exposure times and threshold settings were used for each channel on all sections of similar experiments.

Examples of relevant amino acid sequences (and domains thereof) employed in the examples and/or employable in the described methods:

[00302] EGFRvIII synNotch receptor:

DIQMTQSPSSLSASVGDRVTITCRASQGIRNNLAWYQQKPGKAPKRLIYAASNLQSGVP
SRFTGSGSGTEFTLIVSSLQPEDFATYYCLQHHSYPLTSGGGTKVEIKGSTSGSGKPGSGE
GSEVQVLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGG
STNYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAGSSGWSEYWGQGTLVT
VSS**ILDYSFTGGAGRDIPPPQIEEACELPECQVDAGNKVCNLQCNNHACGWDGGD
CSLNFNDPWKNCTQSLQCWKYFSDGHCDSQCNSAGCLFDGFDCQLTEGQCNPLY
DQYCKDHFSDGHCDQGCNSAECEWDGLDCAEHVPERLAAGTLVLVVLLPPDQLR
NNSFHFLRELSHVLHTNVVFKRDAQGQQMIFPYYGHEEELRKHPIKRSTVGWATS
SLLPGTSGGRQRRELDPMDIRGSIVYLEIDNRQCVQSSSQCFQSATDVAAFLGALAS
LGSLNIPYKIEAVKSEPVEPPLPSQLHLMYVAAAAFVLLFFVGCGVLLSRKRRR**MK

LLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVESRL
ERLEQLFLLIFPREDLDMILKMDSLQDIKALLTGLFVQDNVNKDAVTDRLASVETDMPL
TLRQHRISATSSSEESSNKGQRQLTVSAAAGGSGGSGGSDALDDFDLDMLGSDALDDF
DLDMLGSDALDDFDLDMLGSDALDDFDLDMLGS (SEQ ID NO:28)
(*EGFRvIII scFv;* **Notch Core**; Gal4VP64)

[00303] EphA2 CAR:
EIVLTQSPGTLSLSPGERATLSCRASQSVSSSNLAWYQQKPGQAPRLLIYGASSRATGVP
DRFSGSGSGTDFTLTISRLEPEDFAVYYCQQSSSYPWTFGQGTKVEIKRTGGGGSGAGG
SGGGGTGGGGSEVDLLESGGGLVQPGGSLRLSCAASGFTFSRYWMHWVRQAPGKGLE
WVSSISPYDGETNYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARISEWYN
WAVDVFDYWGQGTLVTVSS**TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHT**
RGLDFACDIYIWAPLAGTCGVLLLSLVITLYCGSGSGSGSGSKRGRKKLLYIFKQPFM
RPVQTTQEEDGCSCRFPEEEEGGCELGSGSGSRVKFSRSADAPAYKQGQNQLYNELNL
GRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRR
GKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:29)
(*EphA2 scFv;* **CD8alpha hinge Transmembrane Domain;** GSlinker-41BB-CD3Zeta Domain)

[00304] IL13Ra2 CAR:
LTCLGGFASPGPVPPSTALRKLIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALESLINVS
*GCSAIEKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLHLRKLFREGRFN***TTTP**
APRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLL
LSLVITLYCGSGSGSGSGSKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGC
ELGSGSGSRVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP
RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH
MQALPPR (SEQ ID NO:30)
(*IL13 Mutein;* **CD8alpha hinge Transmembrane Domain**; GSlinker-41BB-CD3Zeta Domain)

[00305] EphA2/IL13Ra2 CAR:
LTCLGGFASPGPVPPSTALRKLIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALESLINVS
*GCSAIEKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKDLLLHLRKLFREGRFN***GGG**
SGGGSGGGSGGGSEIVLTQSPGTLSLSPGERATLSCRASQSVSSSNLAWYQQKPGQAP

RLLIYGASSRATGVPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQSSSYPWTFGQGTKV
EIKRTGGGGSGAGGSGGGGTGGGGSEVDLLESGGGLVQPGGSLRLSCAASGFTFSRYW
MHWVRQAPGKGLEWVSSISPYDGETNYADSVKGRFTISRDNSKNTLYLQMNSLRAED
TAVYYCARISEWYNWAVDVFDYWGQGTLVTVSS***TTTPAPRPPTPAPTIASQPLSLRPE***
ACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCGSGSGSGSGSKRGRK
KLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELGSGSGSRVKFSRSADAPAYKQG
QNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYS
EIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:31)

(*IL13 Mutein;* **GSLinker;** EphA2 scFv; ***CD8alpha hinge Transmembrane Domain;*** GSlinker-41BB-CD3Zeta Domain)

[00306] Bi-specific EphA2:

QVQLLESGGGLVQPGGSLRLSCAASGFTFSSYTMSWVRQAPGQALEWMGTISSGGTYT
YYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAREAIFTYWGRGTLVTSSGG
GGSGGGGSGGGGSDIQLTQSPSSLSASVGDRVTITCKASQDINNYLSWYQQKPGQAPRL
LIYRANRLVDGVPDRFSGSGYGTDFTLTINNIESEDAAYYFCLKYDVFPYTFGQGTKVEI
KS**GGGG**SDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGY
INPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDY
WGQGTTLTVSSGGGGSGGGGSGGGGSDIQLTQSPAIMSASPGEKVTMTCRASSSVSYM
NWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQW
SSNPLTFGAGTKLELKS (SEQ ID NO:32)

(*EphA2 4H5 scFv*; **GSLinker**; CD3 OKT3 scFv)

[00307] PNE Peptide Orthogonal Bispecific EphA2:

*NYHLENEVARLKKL***LVGEAAAKEAAAKA**QVQLLESGGGLVQPGGSLRLSCAASGFTFS
SYTMSWVRQAPGQALEWMGTISSGGTYTYYPDSVKGRFTISRDNAKNSLYLQMNSLR
AEDTAVYYCAREAIFTYWGRGTLVTSSGGGGSGGGGSGGGGSDIQLTQSPSSLSASVG
DRVTITCKASQDINNYLSWYQQKPGQAPRLLIYRANRLVDGVPDRFSGSGYGTDFTLTI
NNIESEDAAYYFCLKYDVFPYTFGQGTKVEIKS (SEQ ID NO:33)

(*PNE Peptide*; **EAAAK Linker**; EphA2 4H5 scFv)

[00308] PNE Peptide Cognate CAR:

DAVVTQESALTSSPGETVTLTCRSSTGAVTTSNYASWVQEKPDHLFTGLIGGTNNRAPG VPARFSGSLIGDKAALTITGAQTEDEAIYFCVLWYSDHWVFGGGTKLTVLGGGGGSGG GGSGGGGSGGGGSDVQLQESGPGLVAPSQSLSITCTVSGFLLTDYGVNWVRQSPGKGL EWLGVIWGDGITDYNSALKSRLSVTKDNSKSQVFLKMNSLQSGDSARYYCVTGLFDY WGQGTTLTVSS**ESKYGPPCPPCP**IYIWAPLAGTCGVLLLSLVITLYC**KRGRKKLLYIF KQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMK GERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR** (SEQ ID NO:34)
(*PNE 52SR4 scFv*; **IgG4m hinge**; CD8alpha Transmembrane Domain; ***41BB-CD3Zeta Domain***)

Example 2: Multi-receptor circuits for IF/THEN gated EGFRvIII dependent T cell activation

[00309] A molecular circuit was designed to demonstrate the use of an EGFRvIII dependent IF/THEN gate. A schematic depiction of the designed 2-receptor circuit engineered in primary human CD8 T cells to generate a 2-input IF/THEN gate controlling T cell activation is shown in FIG. 15A. In the depicted circuit a synNotch receptor specific for anti-EGFRvIII is expressed in CD8 T cells to control the expression of a split CAR through the release of the a Gal4VP64 transcription factor (TF) domain upon EGFRvIII antigen binding. The split CAR is made up of an anti-PNE-CAR (CAR_{in}) portion and a secreted portion (CAR_{out}) containing an anti-Epha2 scFv linked to the PNE peptide. The anti-EGFRvIII Gal4-VP64 synNotch controls expression of the CAR_{out}, and once CAR_{out} is expressed, engineered CD8 T cells become capable of recognizing and killing EphA2+ target cells.

[00310] To test the above described circuit the heterogeneity observed in GBM was mimicked using engineered U87 GBM cell lines (FIG. 15B). U87 cells express EphA2, but not EGFRvIII ("U87 EGFRvIII-" cells – here also referred to as "target" cells). U87 cells that also express the EGFRvIII priming antigen ("U87 EGFRvIII+" cells – here also referred to as "priming" cells) were also used. Different levels of heterogeneity were systematically generated by mixing these different U87 cells at different ratios. Tumor cells were labeled with different fluorescent proteins to allow tracking of cell survival for each individual cell type.

[00311] T cells engineered to contain the circuit described above were incubated with priming cells only, various mixtures of priming cells and target cells, or target cells only. Specifically, primary CD8+ synNotch split CAR T cells (schematically depicted in FIG. 15A) were co-

cultured with the U87 cells described in FIG. 15B. FIG. 15C provides forward (FSC) and side scatter (SSC) flow cytometry plots after 96 hr co-culture of CD8+ synNotch split CAR T cells with the U87 priming cells (EGFRvIII+) only, target cells (EGFRvIII-) only and various mixtures of priming and target cells. The live U87 (i.e., "GBM") cells are identified in the indicated circled gates. FACS histograms showed only minimal killing of U87 cells in the absence of priming cells. However, significant killing of both priming and target cells was observed with as low as 10% priming cells (EGFRvIII+) in the priming/target cell mixture. FIG. 15D provides quantification related to FIG. 15C, specifically, quantification of CD8+ anti-EGFRvIII synNotch split CAR T cell killing as a function of priming/target cell ratio as shown in FIG. 15C.

[00312] Collectively, the data provided in this example demonstrates killing of GBM cells through the specific activation of a 2-receptor IF/THEN gate dependent upon the presence of EGFRvIII+ priming cells. In this embodiment, relatively small numbers (e.g., 10% of the population) of EGFRvIII+ priming cells were sufficient to induce widespread killing of targeted GBM cells whether or not the targeted cells express EGFRvIII.

[00313] 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 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 method of treating a subject for an epidermal growth factor receptor variant III (EGFRvIII) positive glioblastoma, the method comprising:

administering to the subject an immune cell genetically modified with:

(a) a nucleic acid sequence encoding a binding triggered transcriptional switch (BTTS) that binds to EGFRvIII;

(b) a nucleic acid sequence encoding an antigen-specific therapeutic that binds to a killing antigen expressed by the glioblastoma; and

(c) a regulatory sequence operably linked to (b) that is responsive to the BTTS;

wherein binding of the BTTS to EGFRvIII activates expression of the antigen-specific therapeutic which binds the killing antigen thereby inducing killing of glioblastoma cells expressing the killing antigen.

2. The method according to claim 1, wherein the EGFRvIII positive glioblastoma comprises cells that express either EGFRvIII or the killing antigen.

3. The method according to claims 1 or 2, wherein the glioblastoma comprises cells that express both EGFRvIII and the killing antigen.

4. The method according to claim 1, wherein the killing antigen is expressed by all cells of the glioblastoma.

5. The method according to any of the preceding claims, wherein the killing antigen is expressed by non-glioblastoma cells in the subject.

6. The method according to any of the preceding claims, wherein less than 95% of the glioblastoma cells express EGFRvIII.

7. The method according to any of the preceding claims, wherein the killing antigen is selected from the group consisting of: Ephrin type-A receptor 2 (EphA2), Ephrin type-A receptor 3 (EphA3), Interleukin-13 receptor subunit alpha-1 (IL13RA1), Interleukin-13 receptor

subunit alpha-2 (IL13RA2),, Epidermal growth factor receptor (EGFR) and erb-b2 receptor tyrosine kinase 2 (ERBB2).

8. The method according to any of the preceding claims, wherein the antigen-specific therapeutic, when expressed, is expressed on the surface of the immune cell.

9. The method according to claim 8, wherein the antigen-specific therapeutic is a chimeric antigen receptor (CAR) or a T cell receptor (TCR).

10. The method according to any of claims 1 to 7, wherein the antigen-specific therapeutic, when expressed, is secreted by the immune cell.

11. The method according to claim 10, wherein the antigen-specific therapeutic is a chimeric bispecific binding member.

12. The method according to claim 11, wherein the chimeric bispecific binding member is a TCR-targeted bispecific binding agent.

13. The method according to claims 11 or 12, wherein the chimeric bispecific binding member is specific for the killing antigen and a protein expressed on the surface of an immune cell.

14. The method according to claim 13, wherein the protein expressed on the surface of an immune cell is CD3.

15. The method according to any of the preceding claims, wherein the antigen-specific therapeutic comprises a bio-orthogonal adapter molecule.

16. The method according to claim 15, wherein the bio-orthogonal adapter molecule is bound by an extracellular domain of a switchable CAR.

17. The method according to claim 16, wherein the bio-orthogonal adapter molecule comprises a peptide neo-epitope (PNE) and the extracellular domain of the switchable CAR binds the PNE.

18. The method according to any of claims 15 to 17, wherein the bio-orthogonal adapter molecule binds an antigen selected from the group consisting of: EphA2, EphA3, IL13RA1, IL13RA2, EGFR and ERBB2.

19. The method according to any of the preceding claims, wherein the antigen-specific therapeutic binds two different killing antigens expressed by the glioblastoma.

20. The method according to claim 19, wherein the two different killing antigens are expressed by EGFRvIII positive glioblastoma cells.

21. The method according to claim 19, wherein the two different killing antigens are expressed by glioblastoma cells other than EGFRvIII positive glioblastoma cells.

22. The method according to claim 19, wherein the two different killing antigens are expressed in the same glioblastoma cells.

23. The method according to claim 19, wherein the two different killing antigens are expressed in different glioblastoma cells.

24. The method according to any of claims 19 to 23, wherein the two different killing antigens are selected from the group consisting of: EphA2, EphA3, IL13RA1, IL13RA2, EGFR and ERBB2.

25. The method according to claim 24, wherein the two different killing antigens are EphA2 and IL13RA2.

26. The method according to any of the preceding claims, wherein the immune cell is further genetically modified with a nucleic acid sequence encoding a second antigen-specific therapeutic that binds to a second killing antigen expressed by the glioblastoma.

27. The method according to claim 26, wherein the second killing antigen is expressed by EGFRvIII positive glioblastoma cells.

28. The method according to claim 26, wherein the second killing antigen is expressed by glioblastoma cells expressing the first killing antigen.

29. The method according to claim 26, wherein the second killing antigen is expressed by a cell of the glioblastoma other than the EGFRvIII positive glioblastoma cells or the glioblastoma cells expressing the first killing antigen.

30. The method according to any of claims 26 to 29, wherein the second killing antigen is selected from the group consisting of: EphA2, EphA3, IL13RA1, IL13RA2, EGFR and ERBB2.

31. The method according to any of claims 26 to 30, wherein the second killing antigen is expressed by all cells of the glioblastoma.

32. The method according to any of claims 26 to 31, wherein the second killing antigen is expressed by non-glioblastoma cells in the subject.

33. The method according to any of the preceding claims, wherein the BTTS is a SynNotch polypeptide.

34. The method according to any of the preceding claims, wherein the immune cell is a myeloid cell.

35. The method according to any of claims 1 to 33, wherein the immune cell is a lymphoid cell.

36. The method according to claim 35, wherein the lymphoid cell is selected from the group consisting of: a T lymphocyte, a B lymphocyte and a Natural Killer cell.

37. The method according to any of the preceding claims, wherein the method further comprises identifying that the glioblastoma comprises EGFRvIII positive cells and cells that express the killing antigen.

38. The method according to claim 37, wherein the identifying comprises assaying a sample of the glioblastoma obtained from the subject for cellular expression of EGFRvIII and the killing antigen.

39. The method according to claim 38, wherein the sample is a biopsy.

FIG. 1A

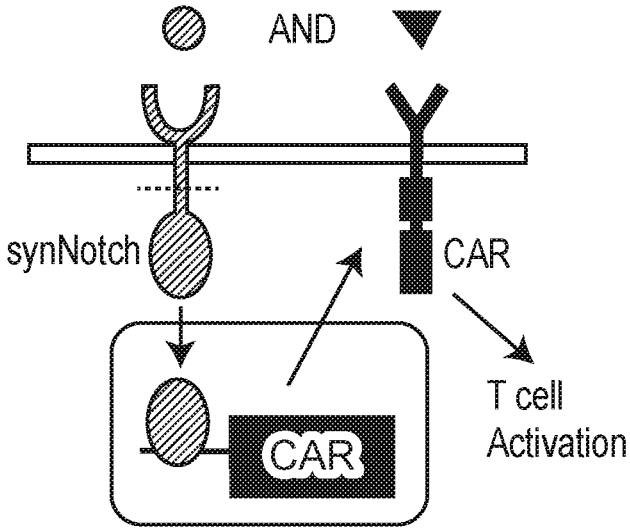

AND
synNotch
CAR
CAR
T cell
Activation

FIG. 1B

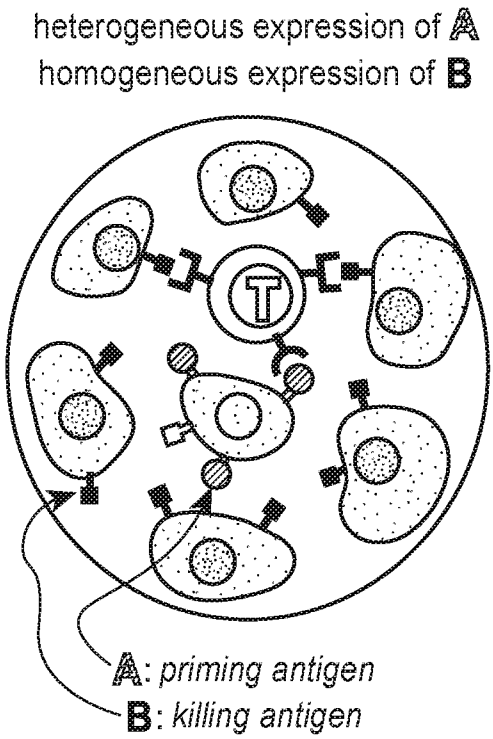

heterogeneous expression of A
homogeneous expression of B
T
A: priming antigen
B: killing antigen

FIG. 1C

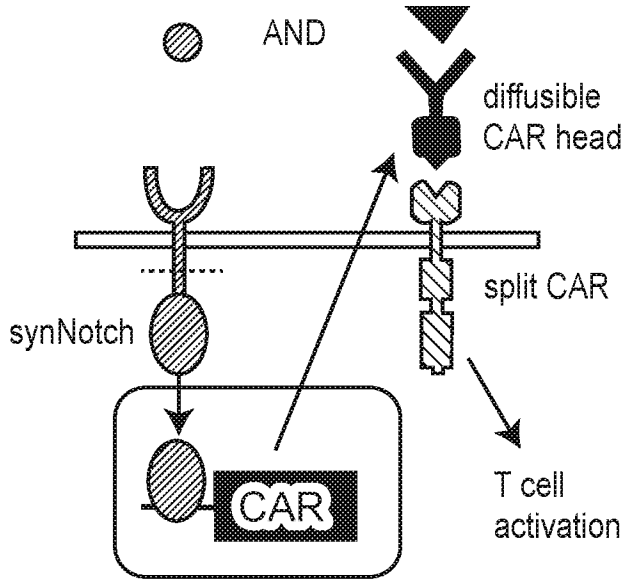

AND
diffusible
CAR head
split CAR
synNotch
CAR
T cell
activation

FIG. 1D

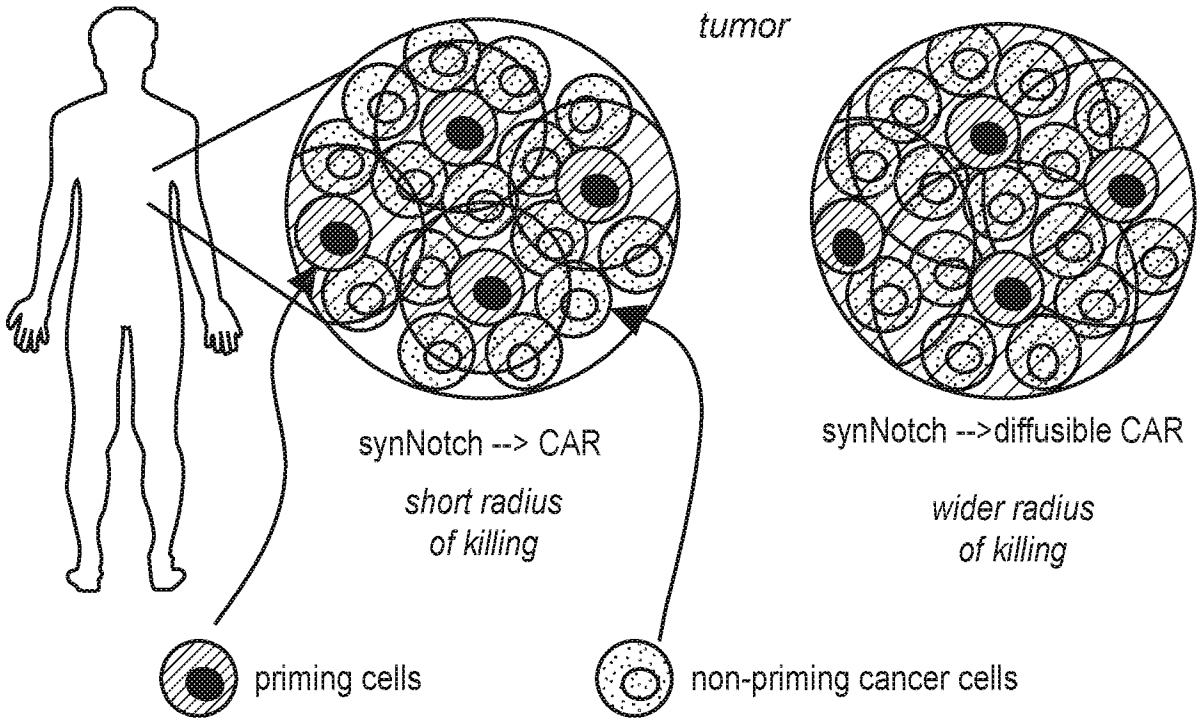

tumor
synNotch --> CAR
short radius
of killing
synNotch -->diffusible CAR
wider radius
of killing
priming cells
non-priming cancer cells

FIG. 2

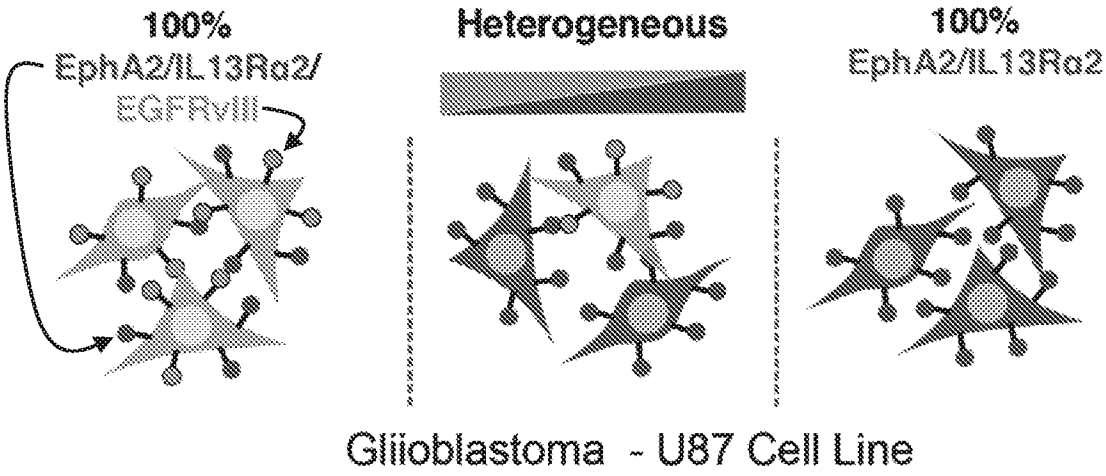

100%
EphA2/IL13Rα2/
EGFRvIII
Heterogeneous
100%
EphA2/IL13Rα2
Gliioblastoma - U87 Cell Line

FIG. 3

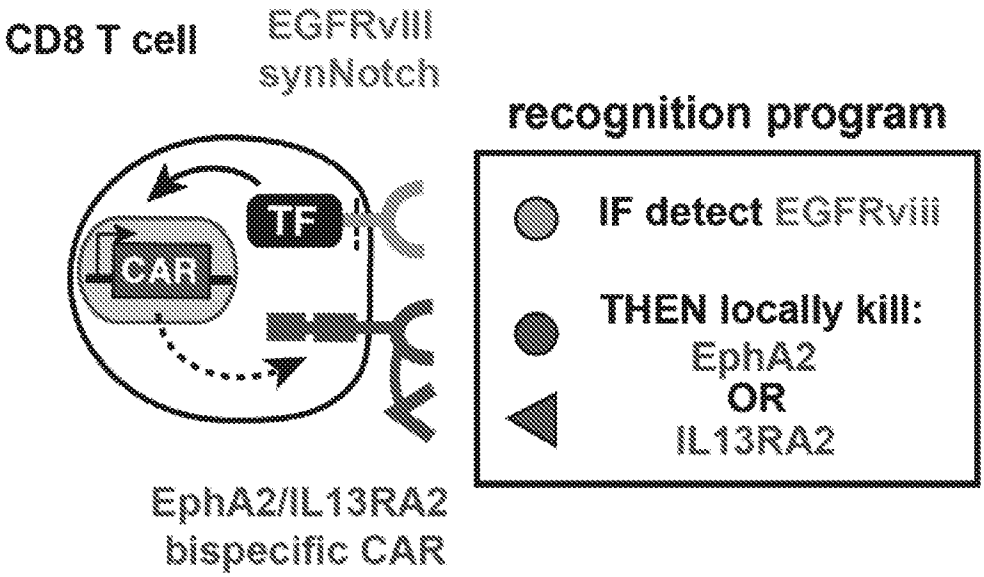

CD8 T cell
EGFRvIII
synNotch
TF
CAR
EphA2/IL13RA2
bispecific CAR
recognition program
IF detect EGFRviii
THEN locally kill:
EphA2
OR
IL13RA2

FIG. 4

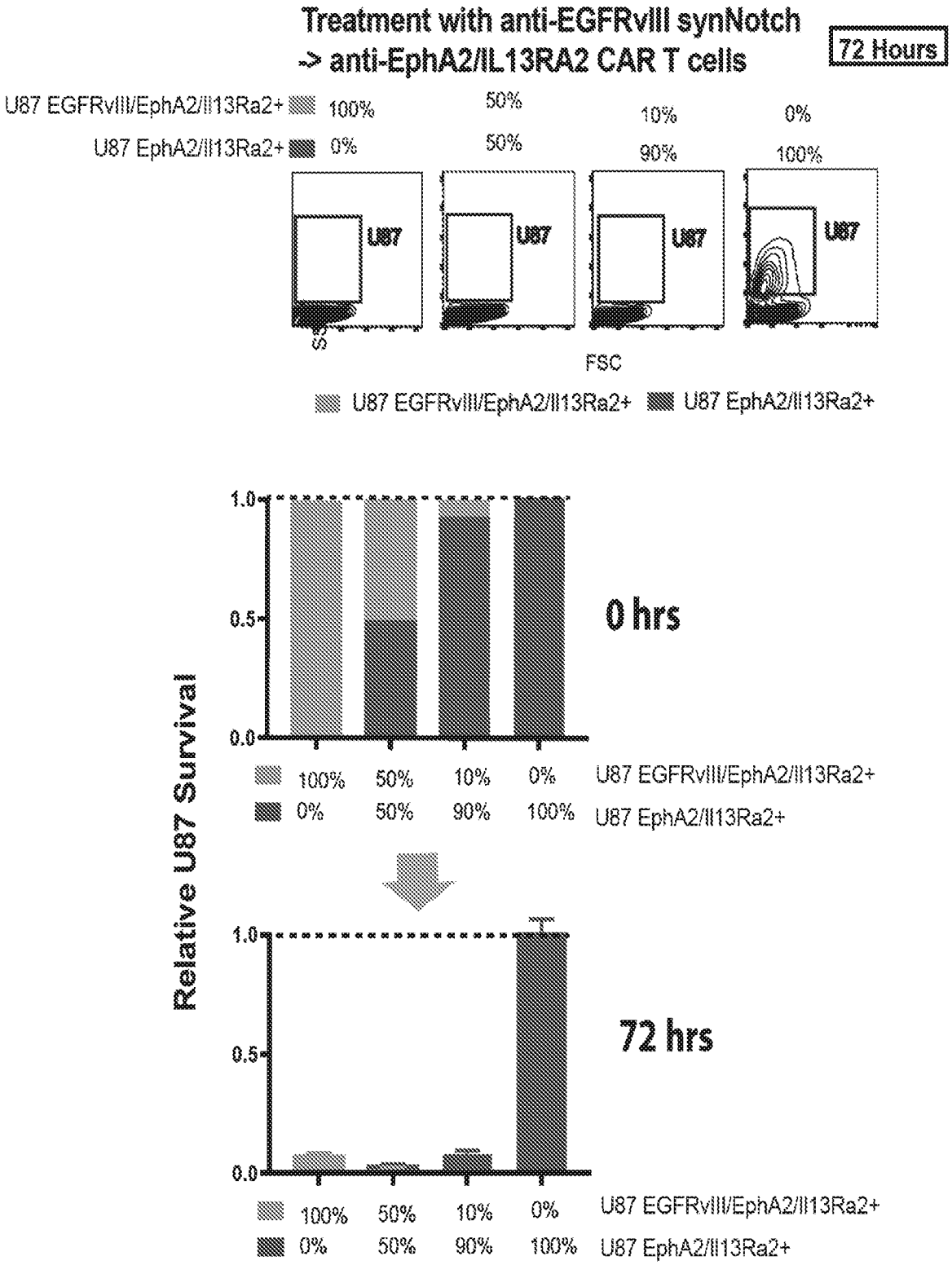

Treatment with anti-EGFRvIII synNotch
-> anti-EphA2/IL13RA2 CAR T cells
72 Hours
U87 EGFRvIII/EphA2/Il13Ra2+
U87 EphA2/Il13Ra2+
100%
0%
50%
50%
10%
90%
0%
100%
U87
SSC
FSC
U87 EGFRvIII/EphA2/Il13Ra2+
U87 EphA2/Il13Ra2+
Relative U87 Survival
1.0
0.5
0.0
0 hrs
72 hrs
100%
50%
10%
0%
U87 EGFRvIII/EphA2/Il13Ra2+
U87 EphA2/Il13Ra2+

FIG. 5

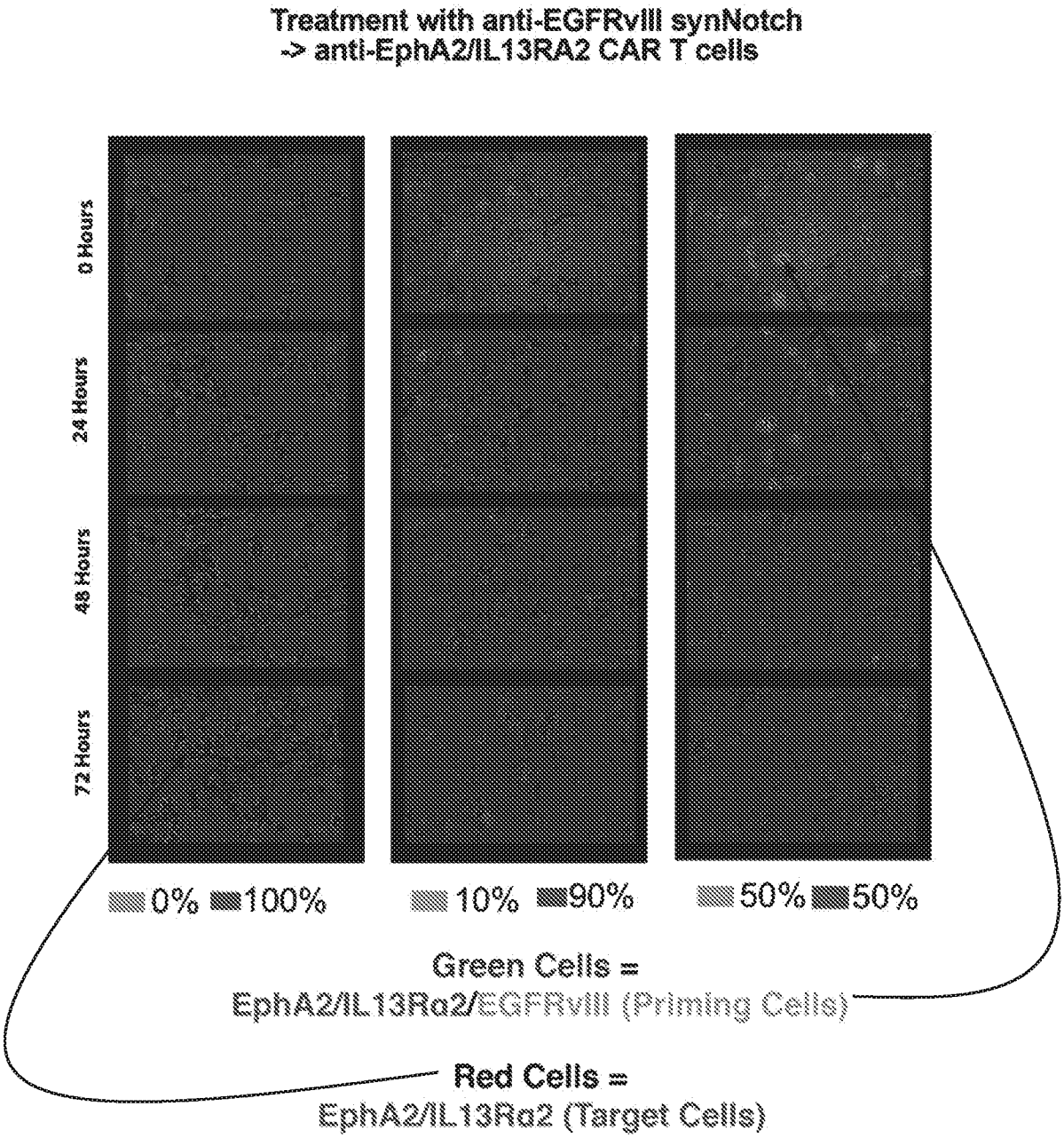

Treatment with anti-EGFRvIII synNotch
-> anti-EphA2/IL13RA2 CAR T cells
0 Hours
24 Hours
48 Hours
72 Hours
0% 100%
10% 90%
50% 50%
Green Cells =
EphA2/IL13Ra2/EGFRvIII (Priming Cells)
Red Cells =
EphA2/IL13Ra2 (Target Cells)

FIG. 6A

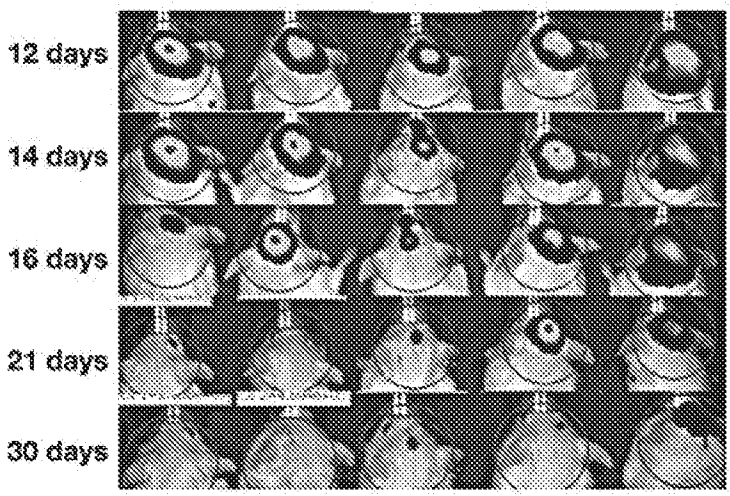


FIG. 6B

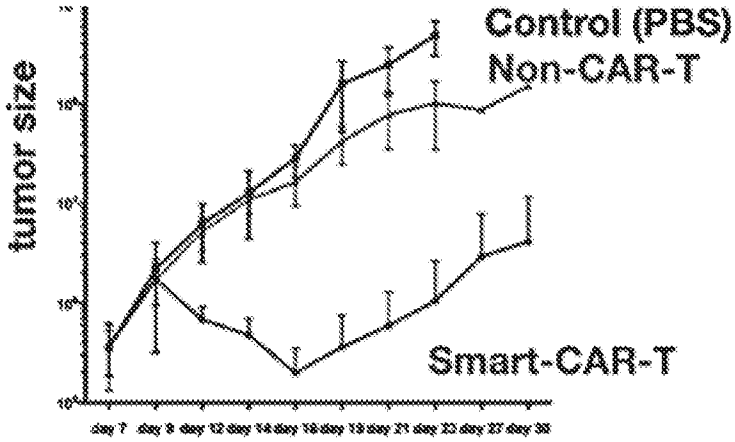


FIG. 6C

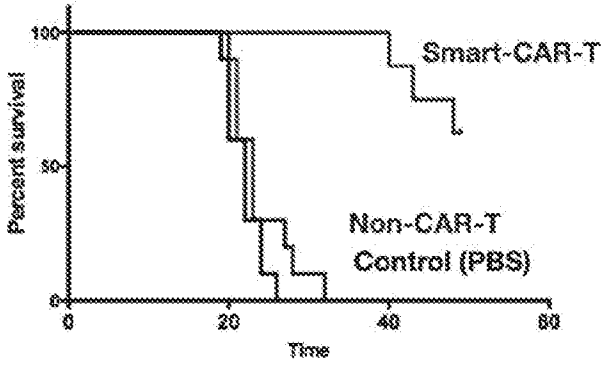


FIG. 7

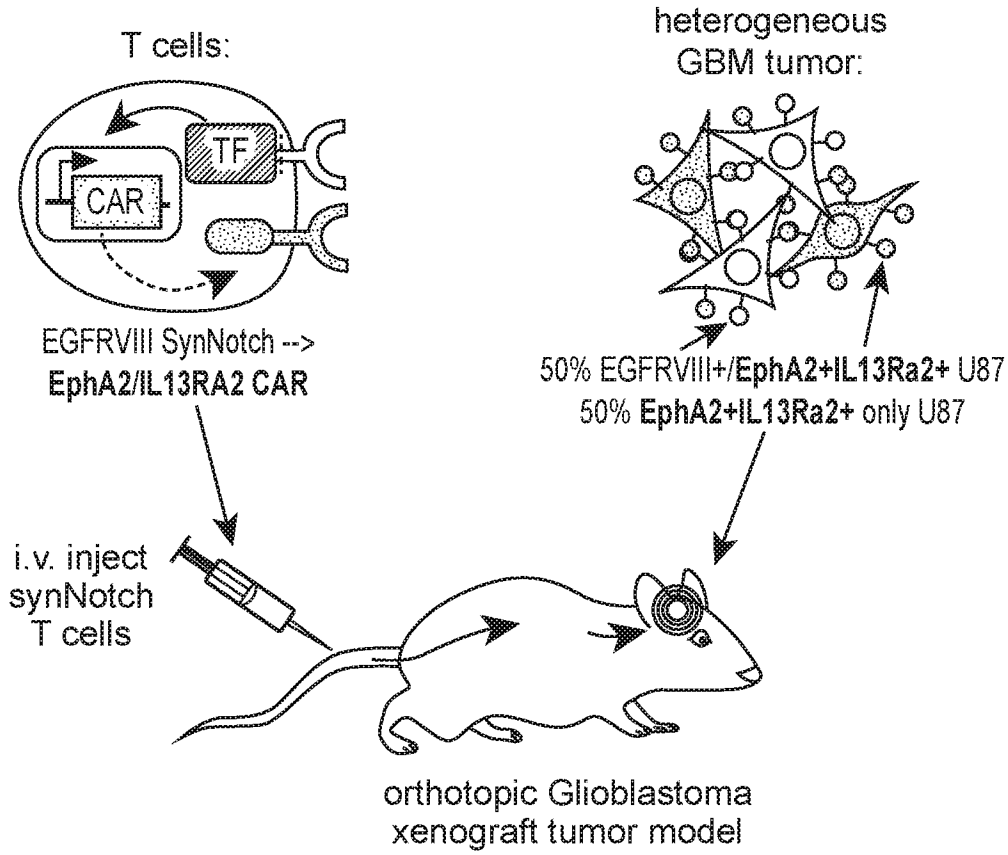

T cells:
TF
CAR
EGFRVIII SynNotch -->
EphA2/IL13RA2 CAR
heterogeneous
GBM tumor:
50% EGFRVIII+/EphA2+IL13Ra2+ U87
50% EphA2+IL13Ra2+ only U87
i.v. inject
synNotch
T cells
orthotopic Glioblastoma
xenograft tumor model

FIG. 8

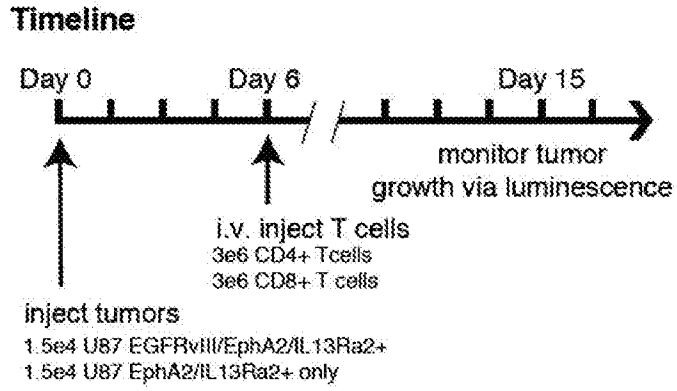


FIG. 9

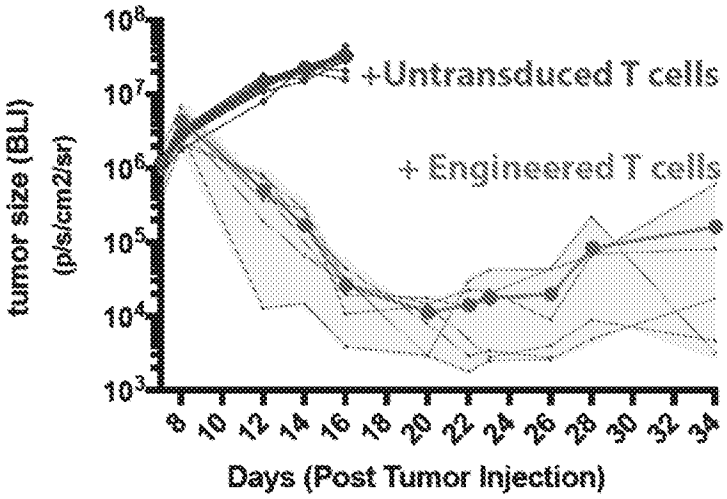


FIG. 10

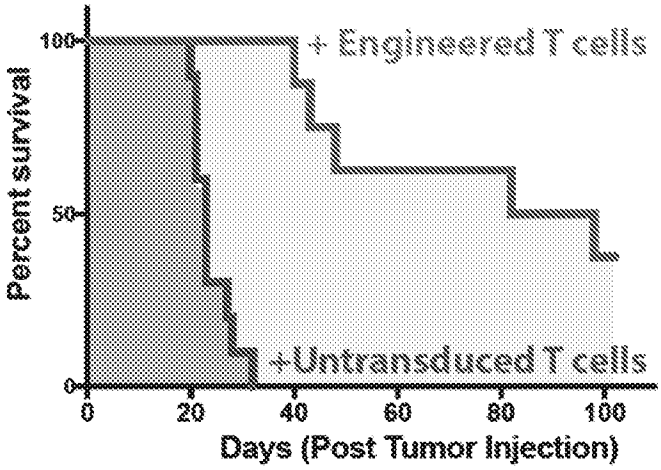


FIG. 11

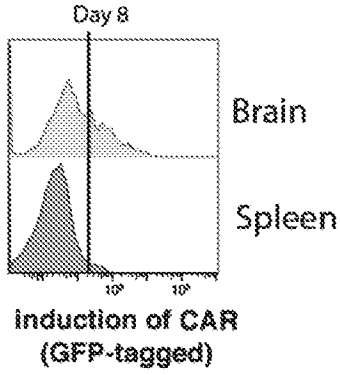


FIG. 12

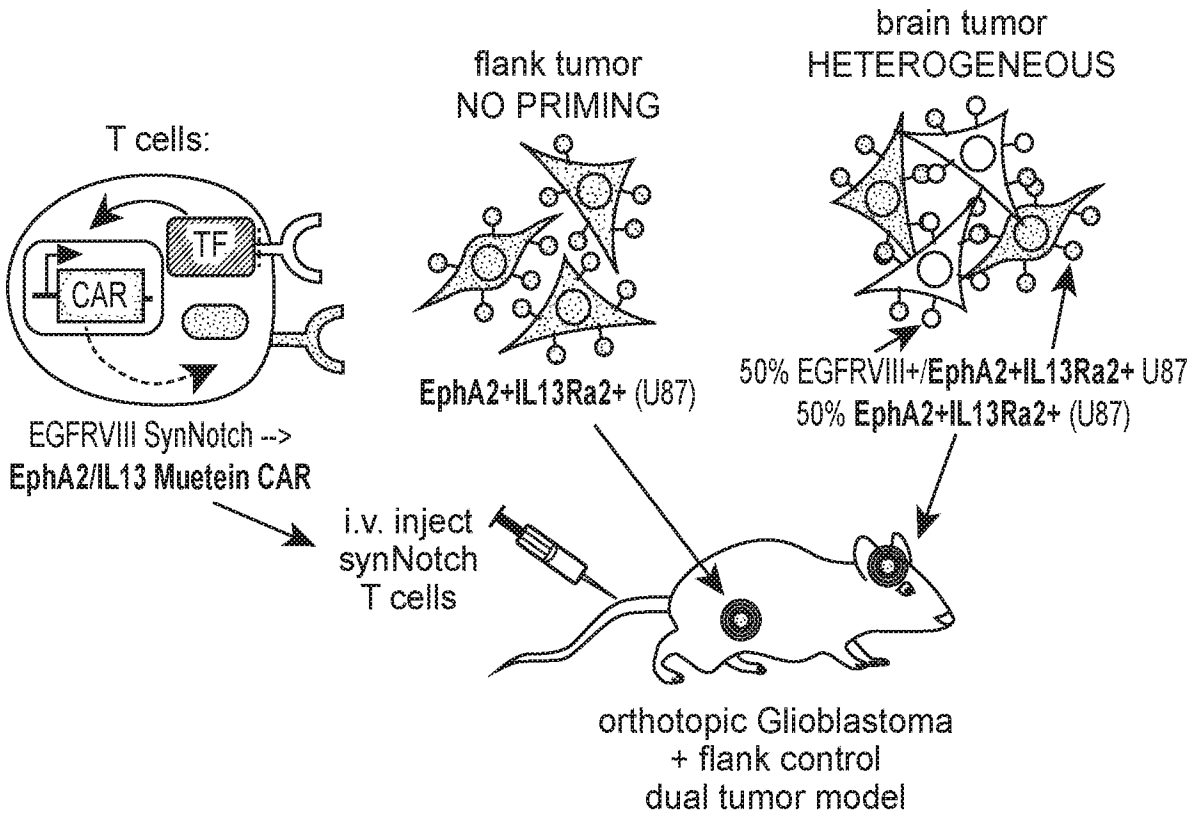
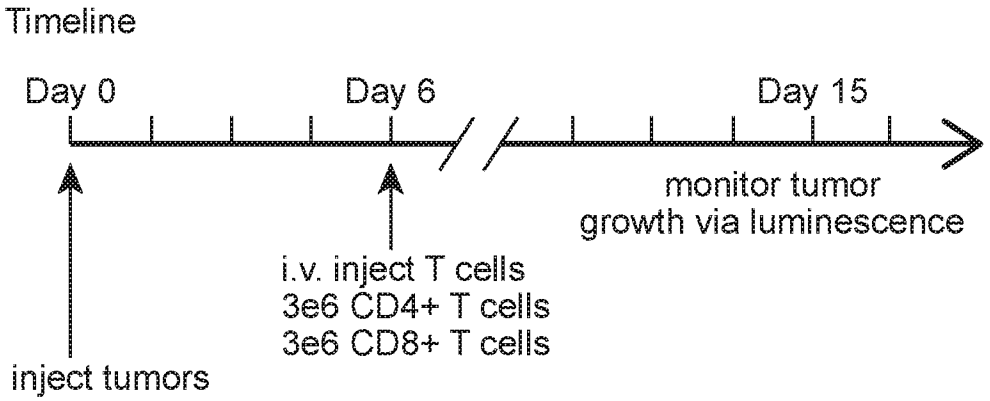


FIG. 13



Brain
1.5e4 U87 EGFRvIII/EphA2/IL13Ra2+
1.5e4 U87 EphA2/IL13Ra2+ only

Flank
1e6U87 EphA2/IL13Ra2+

FIG. 14

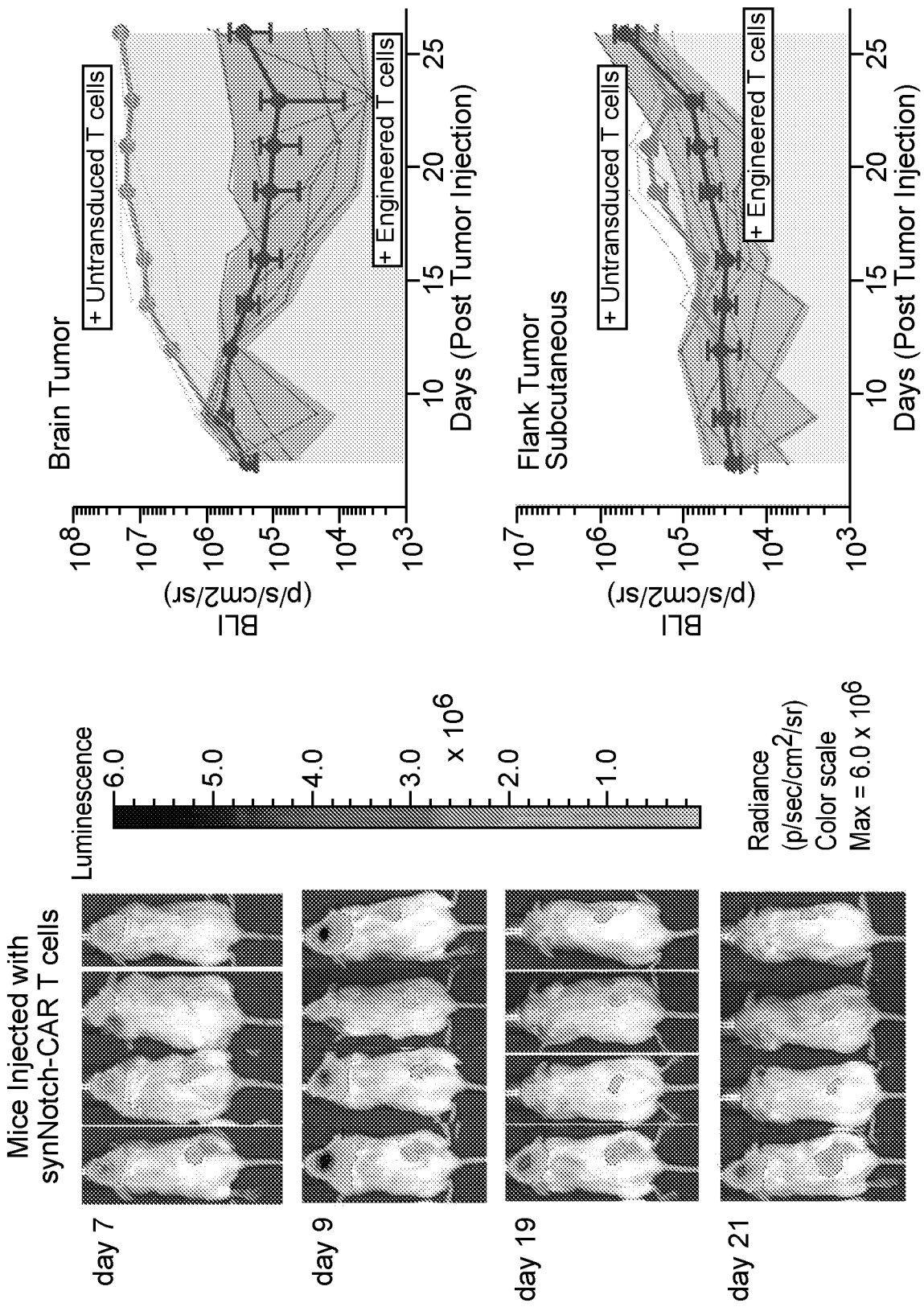

Mice Injected with synNotch-CAR T cells
day 7
day 9
day 19
day 21
Luminescence
6.0
5.0
4.0
3.0
x 10^6
2.0
1.0
Radiance (p/sec/cm2/sr)
Color scale
Max = 6.0 x 10^6
Brain Tumor
+ Untransduced T cells
+ Engineered T cells
BLI (p/s/cm2/sr)
Days (Post Tumor Injection)
Flank Tumor Subcutaneous
+ Untransduced T cells
+ Engineered T cells
BLI (p/s/cm2/sr)
Days (Post Tumor Injection)

FIG. 15A

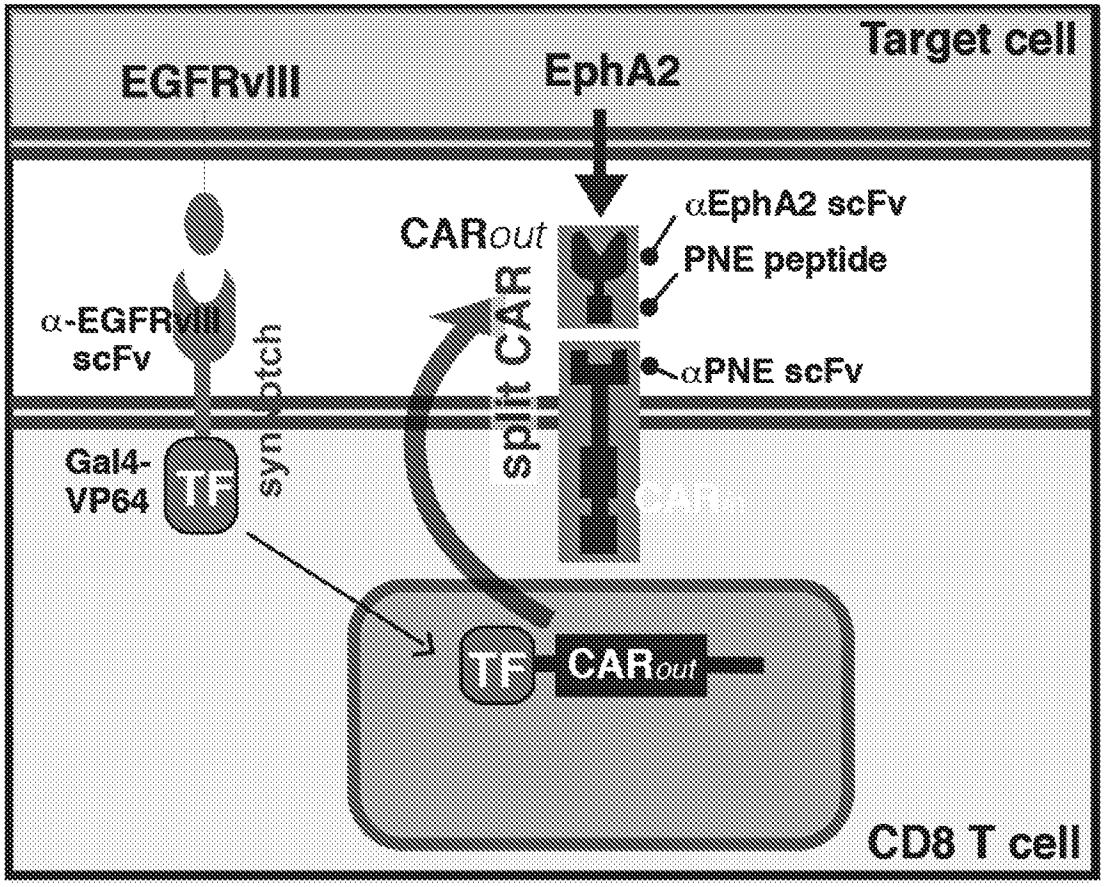
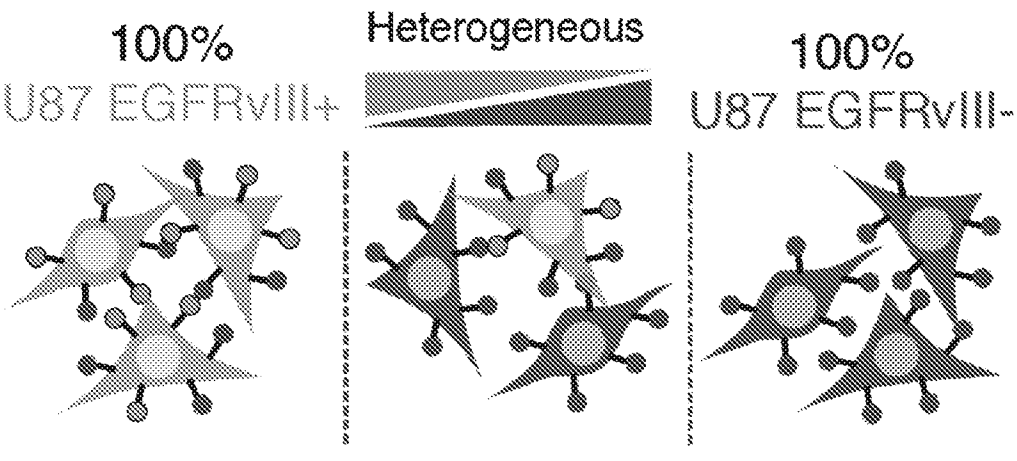


FIG. 15B



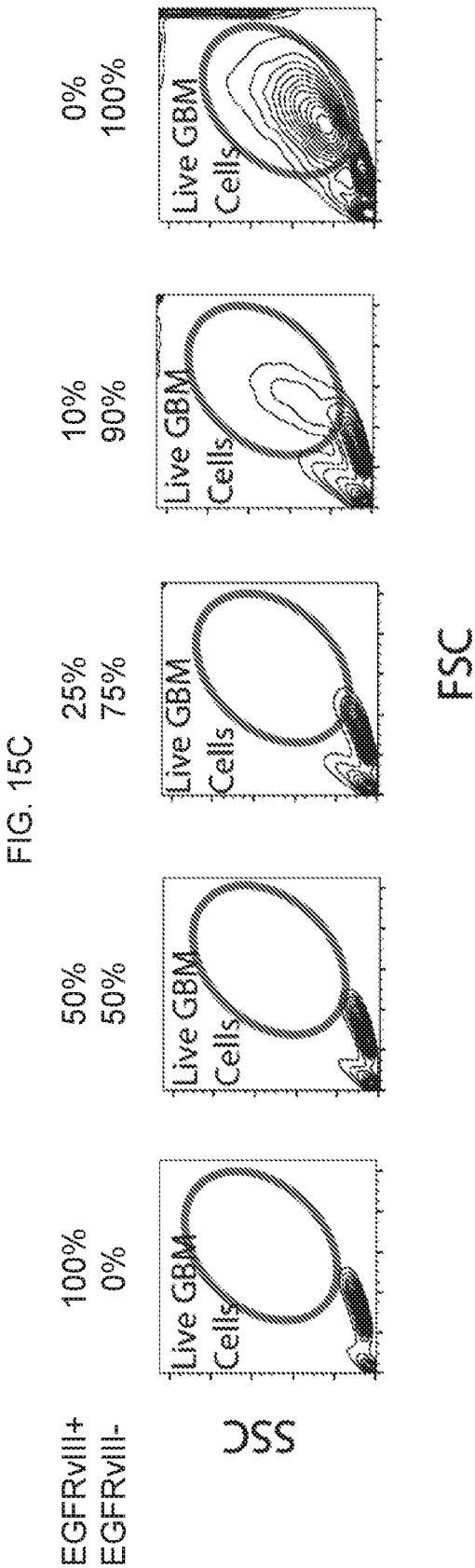


FIG. 15C

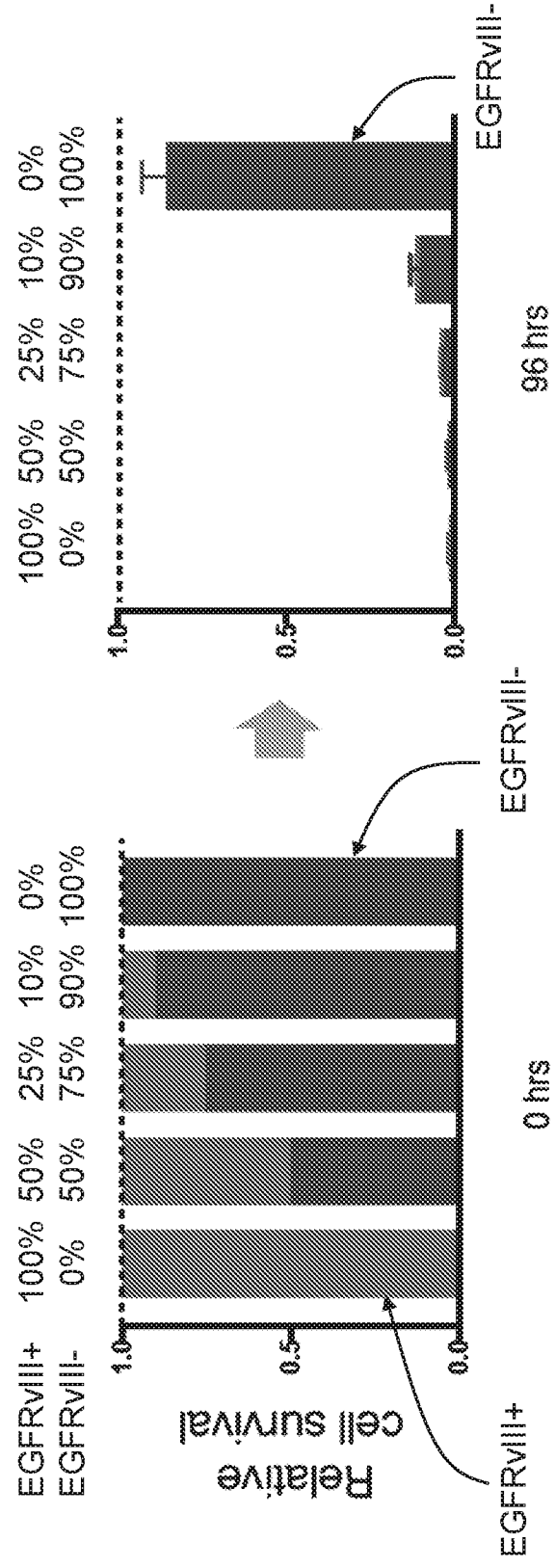
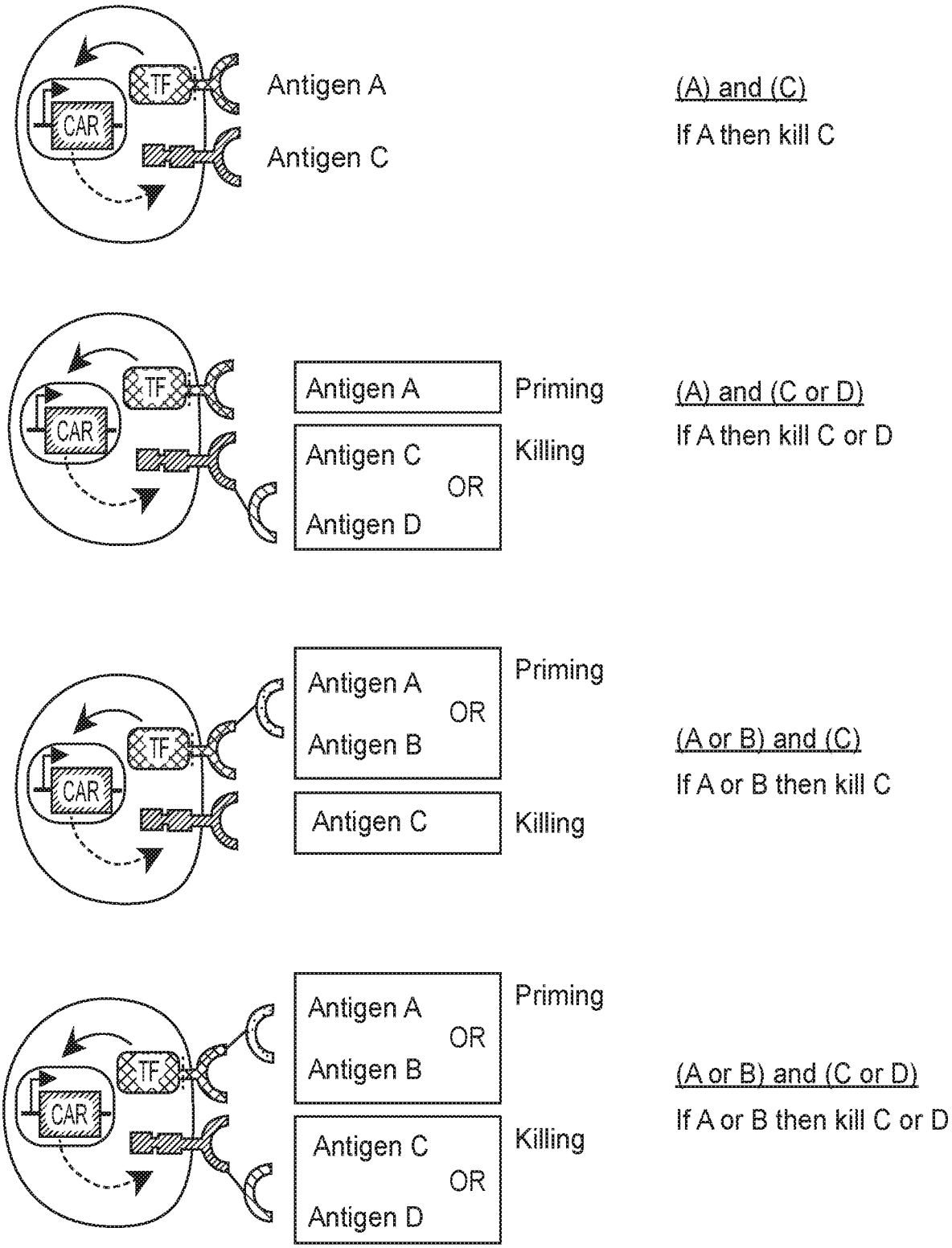


FIG. 15D

FIG. 16


TF
CAR
Antigen A
Antigen C
(A) and (C)
If A then kill C
Antigen A
Priming
Antigen C
OR
Antigen D
Killing
(A) and (C or D)
If A then kill C or D
Antigen A
OR
Antigen B
Priming
Antigen C
Killing
(A or B) and (C)
If A or B then kill C
Antigen A
OR
Antigen B
Priming
Antigen C
OR
Antigen D
Killing
(A or B) and (C or D)
If A or B then kill C or D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/025846

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 35/17; C07K 16/28 (2019.01)
CPC - C07K 16/2863; C07K 2319/33; C07K 2319/74; C12N 2510/00 (2019.05)

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
USPC - 424/93.21; 424/134.1; 424/143.1 (keyword delimited)

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 -- Y	US 2018/0079812 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 22 March 2018 (22.03.2018) entire document	1-3 ----- 4
Y	CHOW et al. "T Cells Redirected to EphA2 for the Immunotherapy of Glioblastoma," Molecular Therapy, 16 October 2012 (16.10.2012), Vol. 21, Iss. 3, Pgs. 629-637. entire document	4
A	WO 2017/025038 A1 (NANJING LEGEND BIOTECH CO., LTD.) 16 February 2017 (16.02.2017) entire document	1-4
A	US 2017/0210811 A1 (PFIZER INC.) 27 July 2017 (27.07.2017) entire document	1-4
A	WO 2014/130657 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA et al) 28 August 2014 (28.08.2014) entire document	1-4

☐ 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
"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	Date of mailing of the international search report
28 May 2019	19 JUL 2019
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 Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/025846

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.: 5-39
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:

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.:

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.