(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
09 November 2017 (09.11.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/192924 A1

(51) International Patent Classification:
A61K 39/00 (2006.01) *A61P 35/00* (2006.01)

(21) International Application Number:
PCT/US2017/031171

(22) International Filing Date:
04 May 2017 (04.05.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/331,906 04 May 2016 (04.05.2016) US

(71) Applicant: **FRED HUTCHINSON CANCER RESEARCH CENTER** [US/US]; 1100 Fairview Avenue North, Seattle, Washington 98109 (US).

(72) Inventors: **RIDDELL, Stanley, R.**; 1763 268th Place SE, Sammamish, Washington 98075 (US). **VEATCH, Joshua**; 505 N 72nd Street, Seattle, Washington 98103 (US).

(74) Agent: **MORGAN, John, A.** et al.; Seed Intellectual Property Law Group LLP, Suite 5400, 701 Fifth Avenue, Seattle, Washington 98104-7064 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) **Title:** CELL-BASED NEOANTIGEN VACCINES AND USES THEREOF

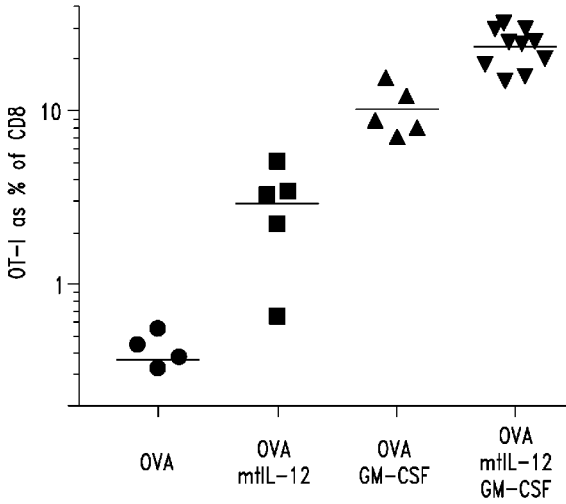

FIG. 5B

(57) **Abstract:** The present disclosure relates to immune cells that express an exogenous neoantigen and an immunogenicity enhancer, or to T cells that express an exogenous neoantigen, and their use in treating a disease or disorder, such as cancer for tumor associated neoantigens. Related expression constructs, kits, host cells, pharmaceutical compositions, and methods are also provided.

WO 2017/192924 A1

[Continued on next page]

Declarations under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

CELL-BASED NEOANTIGEN VACCINES AND USES THEREOF

STATEMENT OF GOVERNMENT INTEREST

This invention was made with government support under CA114536 and CA136551 awarded by the National Institutes of Health. The government has certain rights in the invention.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 360056_440WO_SEQUENCE_LISTING.txt. The text file is 181 KB, was created on May 3, 2017, and is being submitted electronically via EFS-Web.

BACKGROUND

The potential for T cells to recognize and eliminate human cancers of different histologies has been recognized. For example, antibodies that block T cell regulatory molecules (immune checkpoint molecules) have been shown to enhance immune function and have anti-tumor activity (Brahmer *et al.*, *N Engl J Med 366*: 2455, 2012; Topalian *et al.*, *N Engl J Med 366*: 2443, 2012). However, a majority of cancer patients do not achieve durable immune responses when treated with inhibitors of immune checkpoint molecules, perhaps because many patients lack a functional T cell response to tumor antigens that can be induced or rescued (Tumeh *et al.*, *Nature 515*: 568, 2014). Accordingly, methods for eliciting or boosting robust T cell responses to tumor antigens are needed.

Traditional peptide- and dendritic cell-based vaccination platforms have struggled to produce clinically effective T cell responses (Pennock *et al.*, *Trends Immunol 37*: 170, 2016). Autologous T cells may be a useful platform for a cell-based cancer vaccine. T cells modified to express a foreign protein are highly immunogenic in animals (Berger *et al.*, *Blood 103*: 1261, 2004; Russo *et al.*, *J Clin Invest.* 117: 3087, 2007) and humans (Berger *et al.*, *Blood 107*: 2294, 2006; Berger *et al.*, *J Virol 75*: 799,

2001; Riddell *et al.*, *Nat Med 2*: 216, 1996), and elicit high frequency, durable T cell responses to peptide epitopes derived from the foreign protein. Transferred T cells migrate efficiently to secondary lymphoid organs where antigen priming occurs and can be further genetically modified to increase immunogenicity (Russo *et al.*, *J Clin Invest.* 117: 3087, 2007).

One issue that must be addressed during the design of a cancer vaccine is the selection of tumor-associated antigens to target with the vaccine. Many "self" antigens expressed by tumors are generally poor candidates for inclusion in a vaccine due to immune tolerance (*see, e.g.*, Xing and Hogquist, *Cold Spring Harb. Perspect. Biol. 4*:a006957, 2012) and also on-target off-tumor toxicity (*see, e.g.,* Morgan, *Blood* 20:3392 (2013)). But, mutations are a hallmark of cancer, and cancers that are associated with carcinogenic exposures or genomic instability can accumulate large numbers of somatic mutations. While some of these mutations in highly mutated cancers occur in oncogenes and tumor suppressor genes and promote tumor growth, the vast majority are "passenger" mutations and lead to no observable phenotype when re-introduced into normal cells. Passenger mutations are distributed at random throughout the genomes of these cancers and form the bulk of expressed amino acid changes, and can be sampled and recognized by T cells as "foreign." These somatic mutations produce neoantigens and, because the majority are passenger mutations, most neoantigens will be unique to an individual patient's tumor (Schumacher and Schreiber, *Science 348*:69, 2015). Tumor-specific neoantigens may lead to clinically effective T cell responses, and may be better targets for cancer vaccines than some cancer-specific or overexpressed self-antigens that have traditionally been the targets for cancer vaccination (Schumacher and Schreiber, 2015).

Neoantigen vaccine approaches using peptides (Gubin *et al.*, *Nature 515*: 577, 2014) and liposomal RNA preparations (Kreiter *et al.*, *Nature 520*: 692, 2015) have been used in multiple transplantable mouse cancer models, and a peptide-pulsed dendritic cell vaccine has been found to stimulate neoantigen responses in patients with melanoma (Carreno *et al.*, *Science 348*: 803, 2015). Despite evidence supporting a potentially important role for neoantigens in an immune response to highly mutated

solid tumors, a reproducible strategy for exploiting the neoantigen repertoire in an individual's tumor to elicit broadly directed and potent immune responses is lacking.

Therefore, there remains a need for cell-based vaccines that generate immune responses to neoantigens associated with diseases or disorders, such as tumor-specific neoantigens. The embodiments of the present disclosure address such needs, and further provide other related advantages.

BRIEF SUMMARY

In one aspect, the present disclosure provides a T cell, comprising a polynucleotide encoding an exogenous neoantigen associated with a disease or disorder, and a polynucleotide encoding an immunogenicity enhancer.

In another aspect, provided is an immune cell, comprising an exogenous neoantigen associated with a disease or disorder and an immunogenicity enhancer comprising an IL-12 fusion protein that localizes to the cell surface of the T cell, wherein the immune cell is selected from a B cell, a natural killer cell, a dendritic cell, a macrophage, a monocyte, a megakaryocyte, a mast cell, a thrombocyte, an erythrocyte, and a granulocyte.

In a further aspect, a composition comprising a T cell or an immune cell of the present disclosure and a pharmaceutically acceptable carrier, diluent, or excipient is provided.

In another aspect, the present disclosure provides methods of treating a disease or disorder, wherein the methods comprise administering to a human subject in need thereof an effective amount of a T cell, an immune cell, or a composition as described herein.

In yet another aspect, methods are provided for treating a human subject having a disease or disorder associated with expression of a neoantigen, comprising administering to the subject an effective amount of a T cell, of an immune cell, or of a composition as described herein.

In still another aspect, the present disclosure provides a transposon expression construct, comprising a nucleic acid molecule encoding a neoantigen. A kit comprising a transposon expression construct of the present disclosure is also provided.

In another aspect, a host cell, comprising a transposon expression construct of the present disclosure is provided. Compositions comprising a host cell of the present disclosure and a pharmaceutically acceptable carrier, diluent, or excipient are also provided.

In a further aspect, the present disclosure provides a method of preparing a T cell, comprising introducing into the T cell (a) a *piggyBac* transposon plasmid containing a nucleic acid molecule encoding a neoantigen identified in a sample of a subject; and (b) a plasmid comprising a nucleic acid molecule encoding a *piggyBac* transposase, thereby preparing the T cell.

In yet another aspect, a method of preparing an immune cell is provided, comprising introducing into the immune cell (a) a *piggyBac* transposon plasmid containing a nucleic acid molecule encoding a neoantigen identified in a sample of a subject, (b) a plasmid comprising a nucleic acid molecule encoding a *piggyBac* transposase, and (c) a plasmid comprising a nucleic acid molecule encoding an IL-12 fusion protein that localizes to the cell surface of the T cell, thereby preparing the immune cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic of a method of this disclosure for developing a personalized T cell vaccine based on tumor-specific neoantigens. Because the majority of neoantigens are unique to an individual patient's cancer, the "mutanome" of each patient's tumor is determined, allowing for the identification of candidate neoantigens to be targeted by the vaccine. T cells may be obtained from the patient, modified to express the neoantigen, expanded *ex vivo*, and administered to the patient.

Figure 2 shows that treatment with T_{VAX} cells engineered to express ovalbumin (referred to as "T_{OVA}" cells) efficiently induces $CD8^+$ responses to ovalbumin ("OVA") in mice. Six-week-old male C57BL/6 mice were injected with 2×10^4 congenic (CD45.1) naïve OT-1 cells on day -1, and injected with T_{OVA} on days 0 and 14. The frequency of OT-1 cells was determined on days 6, 13, and 20. OVA-specific T cells showed dose-dependent priming after administration of as few as 2×10^5 T_{OVA} cells and

efficient boosting of the response to 5-10% of circulating T cells 7 days following a second 2 x 10^5 T_{OVA} cell dose at day 14.

Figures 3A to 3C show the stable non-viral expression of antigen in primary human T cells by *piggyBac* transposon. Protein expression in T cells was assessed using flow cytometry. (A) Primary human peripheral blood mononuclear cells (PBMCs) were nucleofected with plasmids encoding the *piggyBac* transposase and a *piggyBac* transposon encoding truncated CD19 translationally linked to the CMV NLV epitope. T cells were stained for CD19 7 days following anti-CD3/CD28 stimulation. (B) $CD19^+$ T cells were enriched by CD19 microbeads and stained for CD19 on day 13 of a rapid expansion protocol. (C) Interferon production by CMV NLV-specific $CD8^+$ T cells when co-cultured with T cells stably expressing the NLV epitope (dashed line compared to untransfected (grey) or untransfected T cells pulsed with NLV peptide (solid line).

Figures 4A to 4B show that small amounts of RNA efficiently expand $CD8^+$ and $CD4^+$ memory responses. $CD8^+$ or $CD4^+$ T cells from a CMV-positive donor were stimulated with decreasing amounts of mRNA encoding the CMV pp65 gene. Unmodified cells and unmodified cells pulsed with CMV pp65 peptide served as negative controls and positive controls, respectively. (A) $CD8^+$ T cells were stained with tetramer containing the immunodominant NLV peptide complexed with major histocompatibility complex (MHC). (B) $CD4^+$ T cells were incubated with autologous B cells pulsed with peptides encompassing the pp65 gene in the presence of brefeldin A and stained for intracellular IFN-γ.

Figures 5A to 5B show that expression of adjuvant molecules by T_{VAX} enhances priming of $CD8^+$ T cells. (A) Design scheme of an experiment in which 6-week-old male C57BL/6 mice were injected at day -1 with 500 transgenic donor $CD4.5^{+/+}$ $TCR_{OT-1}^{+/-}$ $CD8^+$ T cells (with TCRs specific for the ovalbumin antigen peptide SIINFEKL). At day 0, mice received a T-cell vaccine (2 x 10^5 T cells expressing full-length ovalbumin, referred to as T_{OVA} cells). Three groups of the injected T_{OVA} cells were transduced to further express a membrane-tethered IL-12 (mtIL-12), secreted GM-CSF, or both. Mice were then bled at day +7 and T cells were stained for CD45.1. (B) Percentages of $CD8^+$ T cells with OT-1-specific TCRs from each tested group.

Figures 6A and 6B show that T priming of $CD8^+$ T cells occurs by cross-presentation. (A) Six-week-old male C57BL/6 mice were injected with 500 $CD4.5^{+/+}$ $TCR_{OT\text{-}1}{}^{+/-}$ $CD8^+$ T cells, vaccinated with 2 x 10^5 Class I MHC^+ or $b2m^{-/-}$ T_{OVA} cells, bled, and stained for CD45.1. (B) Percentages of $CD8^+$ T cells with OT-1-specific TCRs from each tested group.

Figures 7A-7C illustrate that T_{VAX} cells expressing two antigens can prime endogenous $CD4^+$ and $CD8^+$ responses. (A) 6-week-old male C57BL/6 mice were vaccinated using T_{OVA} cells that were further transduced with a virus encoding CD4 antigen LLO190 (from *Listeria monocytogenes*). Two groups of $CD8^+$ cells and one group of $CD4^+$ cells further expressed mtIL-12 and GM-CSF. At day +7, cells were stained (OVA/CD8 tetramers; intracellular cytokine staining for LLO190/CD4) and sorted. (B) OVA/CD8-positive tetramer cells as a percentage of $CD8^+$ T cells. (C) Intracellular interferon-positive cells as a percentage of $CD4^+$ T cells.

Figures 8A-8C illustrate that T_{VAX} cells engineered to express mtIL-12 and containing OVA as a model antigen have therapeutic efficacy in a transplantable mouse melanoma model. (A) Six-week-old male C57BL/6 mice were injected with $5x10^5$ cells from the B16F10-ovalbumin mouse melanoma cell line. At day +1, mice received $2x10^5$ $T_{OVA/mt\text{-}IL12}$ cells, with booster administrations (same dosage) at days +15, and +29. Animals were sacrificed at days +10, +14, +17, and +21, and tumor size was measured. (B) Tumor size (mm^3) for each tested group following injection. (C) Percent survival of each tested group.

Figures 9A-9C show that T_{VAX} cells induce responses against murine sarcoma neoantigens. (A) Six-week-old male C57BL/6 mice were vaccinated with T cells transduced with a viral vector encoding linked murine neoantigens Lama4 and Alg8. Subsets of the T_{VAX} cells were transduced to further express *L. monocytogenes* antigen LLO190, mtIL-12+GM-CSF, or both. At day +13, tetramer staining was performed. (B) Tetramer-positive cells as a percentage of $CD8^+$ T cells for each tested group. (C) In a follow-up experiment, vaccinated mice received an initial priming dose of $2x10^5$ syngeneic T cells modified to express Alg8, Lama4, mt-IL12, and GM-CSF, followed by a "boost" injection ($4x10^5$ cells) at day +28. Tetramer staining was performed at the indicated time points. The limit of detection for these tetramers in this experiment as

compared to mock vaccinated animals was 0.1%. N=3 per group. Error bars represent standard error of the mean.

Figure 10 illustrates an exemplary method for identifying candidate neoantigens from patient tumors (top) and data showing T cell response (Elispot cytokine release assay) from peripheral blood from a lung cancer patient following two rounds of stimulation with candidate neoantigens (bottom left and right).

Figure 11 shows an exemplary scheme for modifying T cells to express a neoantigen to form a T cell vaccine according to the present disclosure. At bottom right, flow cytometry data from a proof-of-principle experiment is provided showing activation of T cells from a lung cancer patient by autologous TERF1 T_{VAX}.

Figures 12A-12D show interferon-release data (Elispot assay) from peripheral blood from cancer patients following stimulation with 20-mer candidate neoantigen peptides, and subsequent validation of candidates. (A) Cytokine release by blood from a melanoma patient following stimulation with pooled candidate neoantigen peptides. (B) Cytokine release by blood from a lung cancer patient following stimulation with pooled candidate neoantigen peptides. (C) Verification of specific T cell response against individual peptides in the melanoma patient sample shown in (A). (D) Verification of specific T cell response against individual peptides in the lung cancer patient sample shown in (B).

Figures 13A-C show interferon-release data (Elispot) from blood samples from three additional lung cancer patients.

DETAILED DESCRIPTION

The present disclosure provides immunogenic compositions and cell-based vaccines for eliciting or boosting immune responses to neoantigens. In certain embodiments, immune cells (*e.g.*, T lymphocytes) comprising one or more polynucleotides encoding exogenous neoantigens and encoding one or more , expression constructs that encode the one or more neoantigens, host cells comprising such expression constructs, and methods for using the same, are provided. The immune and host cells provided herein are modified to express an exogenous neoantigen, such as a tumor neoantigen. In certain embodiments, the immune cells and host cells are

further modified to express one or more immunogenicity enhancers (*e.g.*, foreign or helper antigen, IL-12 (such as a membrane-tethered IL-12), GM-CSF) to augment an immune response against the one or more exogenous neoantigens.

The immune cells or host cells may be used in a personalized, cell-based vaccine for inducing an immune response to one or more neoantigens produced by a particular subject, which may provide a functional T cell response to the neoantigen(s) that can be induced or rescued. In certain embodiments, a neoantigen is a tumor neoantigen. Neoantigens have the advantage of being found in only one or a few specific individuals, not being found in normal tissues (and, therefore, having reduced off-target immunogenicity), and not being subject to central tolerance mechanisms.

Prior to setting forth this disclosure in more detail, it may be helpful to an understanding thereof to provide definitions of certain terms used herein. Additional definitions are set forth throughout this disclosure.

In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated. Also, any number range recited herein relating to any physical feature, such as polymer subunits, size or thickness, are to be understood to include any integer within the recited range, unless otherwise indicated.

As used herein, the term "about" means ± 20% of the indicated range, value, or structure, unless otherwise indicated.

It should be understood that the terms "a" and "an" as used herein refer to "one or more" of the enumerated components. The use of the alternative (*e.g.*, "or") should be understood to mean either one, both, or any combination thereof of the alternatives.

In addition, it should be understood that the individual features or groups of features, derived from the various combinations of the compositions and substituents described herein, are disclosed by the present application to the same extent as if each feature or group of features was set forth individually. Thus, selection of particular structures or particular substituents is within the scope of the present disclosure.

As used herein, the terms "include," "have," and "comprise" are used synonymously, which terms and variants thereof are intended to be construed as non-limiting.

The term "consisting essentially of" limits the scope of a claim to the specified materials or steps, or to those that do not materially affect the basic characteristics of the claimed subject matter. For example, a protein domain, region, or module (*e.g.*, a binding domain, hinge region, linker module) or a protein (which may have one or more domains, regions, or modules) "consists essentially of" a particular amino acid sequence when the amino acid sequence of a domain, region, module, or protein includes extensions, deletions, mutations, or a combination thereof (*e.g.*, amino acids at the amino- or carboxy-terminus or between domains) that, in combination, contribute to at most 20% (*e.g.*, at most 15%, 10%, 8%, 6%, 5%, 4%, 3%, 2% or 1%) of the length of a domain, region, module, or protein and do not substantially affect (*i.e.*, do not reduce the activity by more than 50%, such as no more than 40%, 30%, 25%, 20%, 15%, 10%, 5%, or 1%) the activity of the domain(s), region(s), module(s), or protein (*e.g.*, the target binding affinity of a binding protein).

As used herein, an "immune cell" or "immune system cell" means any cell of the immune system that originates from a hematopoietic stem cell in the bone marrow, which gives rise to two major lineages, a myeloid progenitor cell (which gives rise to myeloid cells such as monocytes, macrophages, dendritic cells, megakaryocytes, mast cells, thrombocytes, erythrocytes, and granulocytes) and a lymphoid progenitor cell (which gives rise to lymphoid cells, or "lymphocytes"). As used herein, the term "lymphocyte" refers to a subtype of white blood cell of a vertebrate immune system that is characterized by its predominant presence in lymph and, generally, by a large nucleus. Lymphocytes include, for example, T cells ($CD4^+$ T cells, $CD8^+$ T cells, $CD4^-$ $CD8^-$ double-negative T cells, γδ T cells, regulatory T cells), B cells, and natural killer (NK) cells. Other exemplary immune system cells include macrophages and dendritic cells, as well as other myeloid cells as described herein. Macrophages and dendritic cells may be referred to as "professional antigen presenting cells" (or "professional APCs"), which are specialized cells that can activate T cells when a major histocompatibility complex (MHC) receptor on the surface of the APC interacts with a

TCR on the surface of a T cell. Alternatively, any hematopoietic stem cell or immune system cell can be converted into an APC by introducing a nucleic acid molecule that expresses an antigen recognized by a TCR or by another antigen binding protein (*e.g.*, chimeric antigen receptor or antibody). Immune cells or lymphocytes used in vaccine compositions or methods of treatment of this disclosure may be autologous, allogeneic, or syngeneic to a subject to receive the composition or the method of treatment.

A "T cell" (or "T lymphocyte") is an immune system cell that matures in the thymus and produces T cell receptors (TCRs), which can be obtained (enriched or isolated) from, for example, peripheral blood mononuclear cells (PBMCs) and are referred to herein as "bulk" T cells. After isolation of T cells, both cytotoxic ($CD8^+$) and helper ($CD4^+$) T cells, can be sorted into naïve, memory, and effector T cell subpopulations, either before or after expansion. T cells can be naïve (not exposed to antigen; increased expression of CD62L, CCR7, CD28, CD3, CD127, and CD45RA, and decreased expression of CD45RO as compared to T_{CM}), memory T cells (T_M) (antigen-experienced and long-lived), and effector cells (antigen-experienced, cytotoxic). T_M can be further divided into subsets of central memory T cells (T_{CM}, increased expression of CD62L, CCR7, CD28, CD127, CD45RO, and CD95, and decreased expression of CD54RA as compared to naïve T cells) and effector memory T cells (T_{EM}, decreased expression of CD62L, CCR7, CD28, CD45RA, and increased expression of CD127 as compared to naïve T cells or T_{CM}). Effector T cells (T_E) refers to antigen-experienced CD8+ cytotoxic T lymphocytes that has decreased expression of CD62L, CCR7, CD28, and are positive for granzyme and perforin as compared to T_{CM}. Helper T cells (T_h) are $CD4^+$ cells that influence the activity of other immune cells by releasing cytokines. $CD4^+$ T cells can activate and suppress an adaptive immune response, and which action is induced will depend on presence of other cells and signals. T cells can be collected in accordance with known techniques, and the various subpopulations or combinations thereof can be enriched or depleted by known techniques, such as by affinity binding to antibodies, flow cytometry, or immunomagnetic selection.

"T cell receptor" (TCR) refers to an immunoglobulin superfamily member (having a variable binding domain, a constant domain, a transmembrane region, and a

short cytoplasmic tail; *see*, *e.g.*, Janeway *et al.*, *Immunobiology: The Immune System in Health and Disease*, 3rd Ed., Current Biology Publications, p. 4:33, 1997) capable of specifically binding to an antigen peptide bound to a MHC receptor. A TCR can be found on the surface of a cell or in soluble form and generally is comprised of a heterodimer having α and β chains (also known as TCRα and TCRβ, respectively), or γ and δ chains (also known as TCRγ and TCRδ, respectively). Like immunoglobulins, the extracellular portion of TCR chains (*e.g.*, α-chain, β-chain) contain two immunoglobulin domains, a variable domain (*e.g.*, α-chain variable domain or V_α, β-chain variable domain or V_β; typically amino acids 1 to 116 based on Kabat numbering, Kabat *et al.*, "Sequences of Proteins of Immunological Interest," US Dept. Health and Human Services, Public Health Service National Institutes of Health, 1991, 5th ed.) at the N-terminus, and one constant domain (*e.g.*, α-chain constant domain or C_α, typically amino acids 117 to 259 based on Kabat, β-chain constant domain or C_β, typically amino acids 117 to 295 based on Kabat) adjacent to the cell membrane. Also like immunoglobulins, the variable domains contain complementary determining regions (CDRs) separated by framework regions (FRs) (*see*, *e.g.*, Jores *et al.*, *Proc. Nat'l Acad. Sci. U.S.A. 87*: 9138, 1990; Chothia *et al.*, *EMBO J. 7*: 3745, 1988; *see also* Lefranc *et al.*, *Dev. Comp. Immunol. 27*: 55, 2003). In certain embodiments, a TCR is found on the surface of T cells (or "T lymphocytes") and associates with the CD3 complex. The source of a TCR as used in the present disclosure may be from various animal species, such as a human, mouse, rat, rabbit or other mammal.

"Antigen" or "Ag" as used herein refers to an immunogenic molecule that provokes an immune response and this immune response may involve antibody production, activation of specific immunologically-competent cells (*e.g.*, T cells), or both. An antigen may be, for example, a peptide, glycopeptide, polypeptide, glycopolypeptide, polynucleotide, polysaccharide, lipid or the like. An antigen can be synthesized, produced recombinantly, or derived from a biological sample using methods known in the art. For example, novel antigens can be generated using methods known in the art such as chromosome rearrangement or breakage. Exemplary biological samples that can contain one or more antigens include tissue samples, tumor samples, cells, biological fluids, or combinations thereof. Antigens can be produced by

cells that have been modified or genetically engineered to express an antigen. Exemplary antigens include α-fetoprotein (AFP), B7H4, BTLA, CD3, CD19, CD20, CD25, CD22, CD28, CD30, CD40, CD44v6, CD52, CD56, CD79b, CD80, CD81, CD86, CD134 (OX40), CD137 (4-1BB), CD151, CD276, CA125, CEA, CEACAM6, c-Met, CT-7, CTLA-4, EGFR, EGFRvIII, ErbB2, ErbB3, ErbB4, EphA2, FLT1, FLT4, Frizzled, O-acetyl-GD2, GD2, GHRHR, GHR, GITR, gp130, HVEM, IGF1R, IL6R, KDR, L1CAM, Lewis A, Lewis Y, LTβR, LIFRβ, LRP5, MAGE, mesothelin, MUC1, NY-ESO-1, a cancer-specific neoantigen, OSMRβ, PD1, PD-L1, PD-L2, PSMA, PTCH1, RANK, Robo1, ROR1, TERT, TGFBR2, TGFBR1, TLR7, TLR9, TNFRSF4, TNFR1, TNFR2, tyrosinase, TWEAK-R, or WT-1, including immunogenic portions or fragments thereof.

As used herein, a "tumor antigen" or "tumor-associated antigen" or "TAA" refers to a mutated protein found in an oncogenic or tumor cell that elicits a humoral immune response, a cellular immune response, or both, which may be found only in tumor cells or may be found in tumor cells and other normal cells. A TAA may be a product of a mutated oncogene (*e.g.*, p53, raf, ras, myc, EGFR), a mutated tumor suppressor gene (*e.g.*, pRb, TP53, PTEN, CD95), a mutated gene that overexpresses or aberrantly expresses a cellular protein, or the like.

As used herein, a "neoantigen" refers to a host cellular product containing a structural change, alteration or mutation that creates a new antigen or antigenic epitope that has not previously been observed in the subject's genome (*i.e.*, in a sample of healthy tissue from the subject) or been "seen" or recognized by the host's immune system. Neoantigens may originate, for example, from coding polynucleotides having alterations (substitution, addition, deletion) that result in an altered or mutated product, or from the insertion of an exogenous nucleic acid molecule or protein into a cell, or from exposure to environmental factors (*e.g.*, chemical, radiological) resulting in a genetic change. Neoantigens may arise separately from a tumor antigen, or may arise from or be associated with a tumor antigen. "Tumor neoantigen" (or "tumor-specific neoantigen") refers to a protein comprising a neoantigenic determinant associated with, arising from, or arising within a tumor cell or plurality of cells within a tumor. . Tumor neoantigenic determinants are found on, for example, antigenic tumor proteins or

peptides that contain one or more somatic mutations encoded by the DNA of tumor cells, as well as proteins or peptides from viral open reading frames associated with virus-associated tumors (*e.g.*, cervical cancers, some head and neck cancers). For example, tumor neoantigens may arise within or from any of the exemplary tumor or other antigens, as well as from "driver" cancer antigens (*e.g.*, G12D neoantigen from KRAS described in Tran *et al.*, *N. Eng. J. Med. 375*:2255-2262 (2016)), as well as in mutated B-Raf, SF31, MYD88, DDX3X, MAPK1, GNB1, and others).

As used herein, an "immunogenicity enhancer" comprises a molecule encoded by a polynucleotide contained in a host cell, such as a T cell, that enhances immunogenicity of an exogenous neoantigen encoded by a polynucleotide contained in the cell. An immunogenicity enhancer encoded by a host cell can provide localized and concentrated adjuvant activity that improves an immune response against a neoantigen. Exemplary immunogenicity enhancers include an IL-12 (such as a membrane-tethered IL-12), a GM-CSF, an inducible cell death factor, a bacterial flagellin, a CD80, a CD137L, a CD40L, a secreted IL-2, a secreted IL-2 that binds T cells independent of CD25, a secreted IL-15, a secreted IL-15-IL-15Rα complex, a secreted IFNβ, a secreted IFN-α1, a secreted IL-7, or any combination thereof. An immunogenicity enhancer may be endogenously expressed by the host cell (*e.g.*, the host cell may endogenously express, for example, GM-CSF), in which case the host cell may be engineered to increase the expression of the immunogenicity enhancer, or the immunogenicity enhancer may be exogenous to the host cell.

As used herein, a "T_{VAX} cell" refers to a T cell comprising a heterologous polynucleotide that encodes one or more neoantigens, wherein the T cell delivers an antigen systemically when administered to a host, such as a human. Such as T_{VAX} cell may further comprise a polynucleotide that encodes an immunogenicity enhancer, wherein the immunogenicity of the neoantigen expressed in the T cell is statistically significantly improved as compared to a T cell encoding one or more neoantigens without the immunogenicity enhancer. Systemic administration of T_{VAX} cells of the present disclosure may result in systemic neoantigen presentation or localized neoantigen presentation, such as for example, localized or concentrated neoantigen presentation at tumor sites or in secondary lymphoid tissues.

A "binding domain" or "binding region," as used herein, refers to a protein, polypeptide, oligopeptide, or peptide (*e.g.*, antibody, receptor) or portion thereof that possesses the ability to specifically recognize and bind to a target (*e.g.*, antigen, ligand). A binding domain includes any naturally occurring, synthetic, semi-synthetic, or recombinantly produced (*i.e.*, engineered by a human) binding partner for a biological molecule or another target of interest. Exemplary binding domains include immunoglobulin light and heavy chain variable regions (*e.g.*, domain antibodies, single chain Fv fragment (scFv or sFv), Fab, $F(ab')_2$), receptor ectodomains, or ligands (*e.g.*, cytokines, such as IL-12). Immunoglobulin variable domains (*e.g.*, scFv, Fab) are referred to herein as "immunoglobulin-based binding domains." A variety of assays are known for identifying binding domains that specifically bind a particular target, including Western blot, ELISA, and Biacore® analysis. In certain embodiments, a binding domain is part of a larger polypeptide or protein and is referred to as a "binding protein."

Sources of binding domains include antibody variable regions from various species (which can be formatted as antibodies, sFvs or scFvs, Fabs, or soluble V_H domain or domain antibodies), including human, rodent, avian, leporine, and ovine. Additional sources of binding domains include variable regions of antibodies from other species, such as camelid (from camels, dromedaries, or llamas; Ghahroudi *et al.*, *FEBS Letters 414*:521, 1997; Vincke *et al.*, *J. Biol. Chem. 284*:3273, 2009; Hamers-Casterman *et al.*, *Nature 363*:446, 1993; Nguyen *et al.*, *J. Mol. Biol. 275*:413, 1998), nurse sharks (Roux *et al.*, *Proc. Nat'l. Acad. Sci. (USA) 95*:11804, 1998), spotted ratfish (Nguyen *et al.*, *Immunogenetics 54*:39, 2002), or lamprey (Herrin *et al.*, *Proc. Nat'l. Acad. Sci. (USA) 105*:2040, 2008; Alder *et al.*, *Nature Immunol. 9*:319, 2008). These antibodies can apparently form antigen-binding regions using only heavy chain variable region, *i.e.*, these functional antibodies are homodimers of heavy chains only (referred to as "heavy chain antibodies") (Jespers *et al.*, *Nature Biotechnol. 22*:1161, 2004; Cortez-Retamozo *et al.*, *Cancer Res. 64*:2853, 2004; Baral *et al.*, *Nature Med. 12*:580, 2006; Barthelemy *et al.*, *J. Biol. Chem. 283*:3639, 2008).

As used herein, "specifically binds" or "specific for" refers to an association or union of a binding protein (*e.g.*, a receptor, an antibody, CAR, or TCR) or a binding

component (or fusion protein thereof) to a target molecule with an affinity or K_a (*i.e.*, an equilibrium association constant of a particular binding interaction with units of 1/M) equal to or greater than 10^5 M^{-1} (which equals the ratio of the on-rate [k_{on}] to the off-rate [k_{off}] for this association reaction), while not significantly associating or uniting with any other molecules or components in a sample. Binding proteins or binding domains (or fusion proteins thereof) may be classified as "high affinity" binding proteins or binding domains (or fusion proteins thereof) or as "low affinity" binding proteins or binding domains (or fusion proteins thereof). "High affinity" binding proteins or binding domains refer to those binding proteins or binding domains having a K_a of at least 10^7 M^{-1}, at least 10^8 M^{-1}, at least 10^9 M^{-1}, at least 10^{10} M^{-1}, at least 10^{11} M^{-1}, at least 10^{12} M^{-1}, or at least 10^{13} M^{-1}. "Low affinity" binding proteins or binding domains refer to those binding proteins or binding domains having a K_a of up to 10^7 M^{-1}, up to 10^6 M^{-1}, up to 10^5 M^{-1}. Alternatively, affinity may be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M (*e.g.*, 10^{-5} M to 10^{-13} M).

A variety of assays are known for identifying binding domains of the present disclosure that specifically bind a particular target, as well as determining binding domain or fusion protein affinities, such as Western blot, ELISA, analytical ultracentrifugation, spectroscopy and surface plasmon resonance (Biacore®) analysis (*see*, *e.g.*, Scatchard *et al.*, *Ann. N.Y. Acad. Sci. 51*:660, 1949; Wilson, *Science 295*:2103, 2002; Wolff *et al.*, *Cancer Res. 53*:2560, 1993; and U.S. Patent Nos. 5,283,173; 5,468,614, or the equivalent).

Terms understood by those in the art of antibody technology are each given the meaning acquired in the art, unless expressly defined differently herein. The term "antibody" refers to an intact antibody comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, as well as any antigen-binding portion or fragment of an intact antibody that has or retains the ability to bind to the antigen target molecule recognized by the intact antibody, such as an scFv, Fab, or Fab′2 fragment. Thus, the term "antibody" herein is used in the broadest sense and includes polyclonal and monoclonal antibodies, including intact antibodies and functional (antigen-binding) antibody fragments thereof, including fragment antigen

binding (Fab) fragments, $F(ab')_2$ fragments, Fab′ fragments, Fv fragments, recombinant IgG (rIgG) fragments, single chain antibody fragments, including single chain variable fragments (scFv), and single domain antibodies (*e.g.*, sdAb, sdFv, nanobody) fragments. The term encompasses genetically engineered and/or otherwise modified forms of immunoglobulins, such as intrabodies, peptibodies, chimeric antibodies, fully human antibodies, humanized antibodies, and heteroconjugate antibodies, multispecific, *e.g.*, bispecific, antibodies, diabodies, triabodies, and tetrabodies, tandem di-scFv, tandem tri-scFv. Unless otherwise stated, the term "antibody" should be understood to encompass functional antibody fragments thereof. The term also encompasses intact or full-length antibodies, including antibodies of any class or sub-class, including IgG and sub-classes thereof, IgM, IgE, IgA, and IgD.

A monoclonal antibody or antigen-binding portion thereof may be non-human, chimeric, humanized, or human. Immunoglobulin structure and function are reviewed, for example, in Harlow *et al.*, Eds., Antibodies: A Laboratory Manual, Chapter 14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, 1988).

For example, the terms "V_L" and "V_H" refer to the variable binding region from an antibody light and heavy chain, respectively. The variable binding regions are made up of discrete, well-defined sub-regions known as "complementarity determining regions" (CDRs) and "framework regions" (FRs). The term "CL" refers to an "immunoglobulin light chain constant region" or a "light chain constant region," *i.e.*, a constant region from an antibody light chain. The term "CH" refers to an "immunoglobulin heavy chain constant region" or a "heavy chain constant region," which is further divisible, depending on the antibody isotype into CH1, CH2, and CH3 (IgA, IgD, IgG), or CH1, CH2, CH3, and CH4 domains (IgE, IgM). A "Fab" (fragment antigen binding) is the part of an antibody that binds to antigens and includes the variable region and CH1 of the heavy chain linked to the light chain via an inter-chain disulfide bond.

The terms "complementarity determining region," and "CDR," synonymous with "hypervariable region" or "HVR," are known in the art to refer to non-contiguous sequences of amino acids within antibody variable regions, which confer antigen specificity and/or binding affinity. In general, there are three CDRs in each heavy chain

variable region (CDR-H1, CDR-H2, CDR-H3) and three CDRs in each light chain variable region (CDR-L1, CDR-L2, CDR-L3). "Framework regions" and "FR" are known in the art to refer to the non-CDR portions of the variable regions of the heavy and light chains. In general, there are four FRs in each full-length heavy chain variable region (FR-H1, FR-H2, FR-H3, and FR-H4), and four FRs in each full-length light chain variable region (FR-L1, FR-L2, FR-L3, and FR-L4).

The precise amino acid sequence boundaries of a given CDR or FR can be readily determined using any of a number of well-known schemes, including those described by Kabat *et al.*, "Sequences of Proteins of Immunological Interest" (5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991) ("Kabat" numbering scheme), Al-Lazikani, *et al.* (*J. Mol. Biol. 273*: 927, 1997) ("Chothia" numbering scheme), MacCallum, *et al.* (*J. Mol. Biol. 262*: 732, 1996) ("Contact" numbering scheme), Lefranc, *et al.* (*Dev. Comp. Immunol. 27*: 55, 2003) ("IMGT" numbering scheme), and Honegger & Plückthun (*J. Mol. Biol. 309*: 657, 2001) ("Aho" numbering scheme). The boundaries of a given CDR or FR may vary depending on the scheme used for identification. For example, the Kabat scheme is based structural alignments, while the Chothia scheme is based on structural information. Numbering for both the Kabat and Chothia schemes is based upon the most common antibody region sequence lengths, with insertions accommodated by insertion letters, for example, "30a," and deletions appearing in some antibodies. The two schemes place certain insertions and deletions ("indels") at different positions, resulting in differential numbering. The Contact scheme is based on analysis of complex crystal structures and is similar in many respects to the Chothia numbering scheme.

Table 1, below, lists exemplary position boundaries of CDR-L1, CDR-L2, CDR-L3 and CDR-H1, CDR-H2, CDR-H3 as identified by Kabat, Chothia, and Contact schemes, respectively. For CDR-H1, residue numbering is listed using both the Kabat and Chothia numbering schemes. FRs are located between CDRs, for example, with FR-L1 located between CDR-L1 and CDR-L2, and so forth. It is noted that because the shown Kabat numbering scheme places insertions at H35A and H35B, the end of the Chothia CDR-H1 loop, when numbered using the shown Kabat numbering convention, varies between H32 and H34, depending on the length of the loop.

Table 1. Exemplary CDR Position Boundaries

CDR	Kabat	Chothia	Contact
CDR-L1	L24--L34	L24--L34	L30--L36
CDR-L2	L50--L56	L50--L56	L46--L55
CDR-L3	L89--L97	L89--L97	L89--L96
CDR-H1 (Kabat Numbering[1])	H31--H35B	H26--H32..34	H30--H35B
CDR-H1 (Chothia Numbering[2])	H31--H35	H26--H32	H30--H35
CDR-H2	H50--H65	H52--H56	H47--H58
CDR-H3	H95--H102	H95--H102	H93--H101

1 – Kabat *et al.*, "Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.
2 – Al-Lazikani *et al.*, *J. Mol. Biol. 273*:927, 1997.

Thus, unless otherwise specified, a "CDR" or "complementary determining region," or individual specified CDRs (*e.g.*, CDR-H1, CDR-H2), of a given antibody or region thereof, such as a variable region thereof, should be understood to encompass a (or the specific) complementary determining region as defined by any of the aforementioned schemes. For example, where it is stated that a particular CDR (*e.g.*, a CDR-H3) contains the amino acid sequence of a corresponding CDR in a given V_H or V_L amino acid sequence, it is understood that such a CDR has a sequence of the corresponding CDR (*e.g.*, CDR-H3) within the variable region, as defined by any of the aforementioned schemes. In some embodiments, specified CDR sequences are specified. Likewise, unless otherwise specified, a FR or individual specified FR(s) (*e.g.*, FR-H1, FR-H2), of a given antibody or region thereof, such as a variable region thereof, should be understood to encompass a (or the specific) framework region as defined by any of the known schemes. In some instances, the scheme for identification of a particular CDR, FR, or FRs or CDRs is specified, such as the CDR as defined by the Kabat, Chothia, or Contact method. In other cases, the particular amino acid sequence of a CDR or FR is given.

Among the provided antibodies are antibody fragments. An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of

an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, $F(ab')_2$; diabodies; linear antibodies; single-chain antibody molecules (*e.g.*, scFv); and multispecific antibodies formed from antibody fragments. In particular embodiments, the antibodies are single-chain antibody fragments comprising a variable heavy chain region and/or a variable light chain region, such as scFvs.

Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody.

Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells. In certain embodiments, the antibodies are recombinantly produced fragments, such as fragments comprising arrangements that do not occur naturally, such as those with two or more antibody regions or chains joined by synthetic linkers, *e.g.*, peptide linkers. In certain embodiments, antibodies are produced by enzyme digestion of a naturally-occurring intact antibody. In some aspects, antibody fragments are scFvs.

As used herein, "Fc region portion" refers to the heavy chain constant region segment of the Fc fragment (the "fragment crystallizable" region or Fc region) from an antibody, which can in include one or more constant domains, such as CH2, CH3, CH4, or any combination thereof. In certain embodiments, an Fc region portion includes the CH2 and CH3 domains of an IgG, IgA, or IgD antibody or any combination thereof, or the CH3 and CH4 domains of an IgM or IgE antibody and any combination thereof. In other embodiments, a CH2CH3 or a CH3CH4 structure has sub-region domains from the same antibody isotype and are human, such as human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, or IgM (*e.g.*, CH2CH3 from human IgG1). By way of background, an Fc region is responsible for the effector functions of an immunoglobulin, such as ADCC (antibody-dependent cell-mediated cytotoxicity), CDC (complement-dependent cytotoxicity) and complement fixation, binding to Fc receptors (*e.g.*, CD16, CD32, FcRn), greater half-life *in vivo* relative to a polypeptide lacking an

Fc region, protein A binding, and perhaps even placental transfer (*see* Capon *et al.*, *Nature 337*:525, 1989). In certain embodiments, an Fc region portion found in immunoglobulin-like binding proteins of the present disclosure will be capable of mediating one or more of these effector functions, or will lack one or more or all of these activities by way of, for example, one or more mutations known in the art.

In addition, antibodies have a hinge sequence that is typically situated between the Fab and Fc region (but a lower section of the hinge may include an amino-terminal portion of the Fc region). By way of background, an immunoglobulin hinge acts as a flexible spacer to allow the Fab portion to move freely in space. In contrast to the constant regions, hinges are structurally diverse, varying in both sequence and length between immunoglobulin classes and even among subclasses. For example, a human IgG1 hinge region is freely flexible, which allows the Fab fragments to rotate about their axes of symmetry and move within a sphere centered at the first of two inter-heavy chain disulfide bridges. By comparison, a human IgG2 hinge is relatively short and contains a rigid poly-proline double helix stabilized by four inter-heavy chain disulfide bridges, which restricts the flexibility. A human IgG3 hinge differs from the other subclasses by its unique extended hinge region (about four times as long as the IgG1 hinge), containing 62 amino acids (including 21 prolines and 11 cysteines), forming an inflexible poly-proline double helix and providing greater flexibility because the Fab fragments are relatively far away from the Fc fragment. A human IgG4 hinge is shorter than IgG1 but has the same length as IgG2, and its flexibility is intermediate between that of IgG1 and IgG2.

As used herein, unless otherwise provided, a position of an amino acid residue in the constant region of human IgG1 heavy chain is numbered assuming that the variable region of human IgG1 is composed of 128 amino acid residues according to the Kabat numbering convention. The numbered constant region of human IgG1 heavy chain is then used as a reference for numbering amino acid residues in constant regions of other immunoglobulin heavy chains. A position of an amino acid residue of interest in a constant region of an immunoglobulin heavy chain other than human IgG1 heavy chain is the position of the amino acid residue in human IgG1 heavy chain with which the amino acid residue of interest aligns. Alignments between constant regions of

human IgG1 heavy chain and other immunoglobulin heavy chains may be performed using software programs known in the art, such as the Megalign program (DNASTAR Inc.) using the Clustal W method with default parameters. According to the numbering system described herein, for example, although human IgG2 C_{H2} region may have an amino acid deletion near its amino-terminus compared with other C_{H2} regions, the position of the "N" located at 296 in human IgG2 C_{H2} is still considered position 297 because this residue aligns with "N" at position 297 in human IgG1 C_{H2}.

The term "epitope" includes any amino acid sequence or protein determinant capable of specific binding to an immunoglobulin, receptor or other binding domain or binding protein. Epitopic determinants generally contain chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three dimensional structural characteristics, as well as specific charge characteristics.

The terms "polypeptide" and "peptide" as used herein refer to a compound made up of amino acid residues that are linked by peptide bonds. The term "protein" may be synonymous with the term "polypeptide" or may refer, in addition, to a complex of two or more polypeptides. A polypeptide may further contain other components (*e.g.*, covalently bound), such as a tag, a label, a bioactive molecule, or any combination thereof. In certain embodiments, a polypeptide may be a fragment. As used herein, a "fragment" means a polypeptide that is lacking one or more amino acids that are found in a parent polypeptide. A fragment can comprise a binding domain, antigen, or epitope found in a parent polypeptide. In certain embodiments, a fragment of a polypeptide can have at least about 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more of amino acids of the amino acid sequence of the parent polypeptide.

As described herein, a "variant" polypeptide species has one or more non-natural amino acids, one or more amino acid substitutions, one or more amino acid insertions, one or more amino acid deletions, or any combination thereof at one or more sites relative to a reference polypeptide as presented herein. In certain embodiments, "variant" means a polypeptide having a substantially similar activity (*e.g.*, enzymatic function, immunogenicity) or structure relative to a reference polypeptide. A variant of a reference polypeptide can have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%,

75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to the amino acid sequence for the reference polypeptide as determined by sequence alignment programs and parameters known in the art. The variant can result from, for example, a genetic polymorphism or human manipulation. Conservative substitutions of amino acids are well known and may occur naturally or may be introduced when a protein is recombinantly produced. Amino acid substitutions, deletions, and additions may be introduced into a protein using mutagenesis methods known in the art (*see, e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 3d ed., Cold Spring Harbor Laboratory Press, NY, 2001). Oligonucleotide-directed site-specific (or segment specific) mutagenesis procedures may be employed to provide an altered polynucleotide that has particular codons altered according to the substitution, deletion, or insertion desired. Alternatively, random or saturation mutagenesis techniques, such as alanine scanning mutagenesis, error prone polymerase chain reaction mutagenesis, and oligonucleotide-directed mutagenesis may be used to prepare polypeptide variants (*see, e.g.*, Sambrook *et al.*, *supra*).

The terms "identical" or "percent identity," in the context of two or more polypeptide or nucleic acid molecule sequences, means two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same over a specified region (*e.g.*, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity), when compared and aligned for maximum correspondence over a comparison window, or designated region, as measured using methods known in the art, such as a sequence comparison algorithm, by manual alignment, or by visual inspection. For example, preferred algorithms suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (*Nucleic Acids Res. 25*:3389, 1977) and Altschul *et al.* (*J. Mol. Biol. 215*:403, 1990), respectively.

As used herein, a "fusion protein" comprises a single chain polypeptide having at least two distinct domains, wherein the domains are not naturally found together in a protein. A nucleic acid molecule encoding a fusion protein may be constructed using PCR, recombinantly engineered, or the like, or such fusion proteins can be made

synthetically. A fusion protein may further contain other components (*e.g.*, covalently bound), such as a tag or bioactive molecule.

A "nucleic acid molecule" or "polynucleotide" refers to a single- or double-stranded linear or circular polynucleotide containing either deoxyribonucleotides or ribonucleotides that are linked by 3′-5′-phosphodiester bonds. A nucleic acid molecule includes RNA, DNA, genomic DNA, mitochondrial DNA, cDNA, or vector DNA. In certain embodiments, the nucleic acids of the present disclosure are produced by PCR. Nucleic acids may be composed of monomers that are naturally occurring nucleotides (such as deoxyribonucleotides and ribonucleotides), analogs of naturally occurring nucleotides (*e.g.*, α-enantiomeric forms of naturally-occurring nucleotides, morpholinos), or a combination of both. Modified nucleotides can have modifications in, or replacement of, sugar moieties, or pyrimidine or purine base moieties. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like.

In certain embodiments, polynucleotides encoding peptides or proteins of the disclosure may be codon optimized to enhance or maximize expression in certain types of cells (*e.g.*, Scholten *et al.*, *Clin. Immunol. 119*:135-145, 2006). As used herein a "codon optimized" polynucleotide is a heterologous polypeptide having codons modified with silent mutations corresponding to the abundances of host cell tRNA levels.

Variants of the polynucleotides of this disclosure are also contemplated. Variant polynucleotides are at least 80%, 85%, 90%, 95%, 99%, or 99.9% identical to a reference polynucleotide as described herein, or that hybridizes to a reference polynucleotide of defined sequence under stringent hybridization conditions of 0.015M sodium chloride, 0.0015M sodium citrate at about 65°-68°C or 0.015M sodium chloride, 0.0015M sodium citrate, and 50% formamide at about 42°C. The polynucleotide variants retain the capacity to encode an immunoglobulin binding protein or antigen-binding fragment thereof having the functionality described herein.

The term "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the co-existing materials in the natural system, is isolated. Such polynucleotide may be part of a vector and/or such polynucleotide or polypeptide may be part of a composition (*e.g.*, a cell lysate), and still be isolated in that such vector or composition is not part of the natural environment for the nucleic acid or polypeptide.

The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell wherein the nucleic acid molecule may be incorporated into the genome of a cell (*e.g.*, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, comprised in an episomal expression vector (*see, e.g.*, Van Caenenbroeck *et al.*, *Eur. J. Biochem. 267*:5665(2000)), or transiently expressed (*e.g.*, transfected mRNA).

As used herein, "heterologous" or "exogenous" nucleic acid molecule, construct or sequence refers to a nucleic acid molecule or portion of a nucleic acid molecule that is not native to a host cell, but may be homologous to a nucleic acid molecule or portion of a nucleic acid molecule from the host cell. The source of the heterologous or exogenous nucleic acid molecule, construct or sequence may be from a different genus or species. In certain embodiments, a heterologous or exogenous nucleic acid molecule is added (*i.e.*, not endogenous or native) to a host cell or host genome by, for example, conjugation, transformation, transfection, electroporation, or the like, wherein the added molecule may integrate into the host genome or exist as extra-chromosomal genetic material (*e.g.*, as a plasmid or other form of self-replicating vector), and may be present in multiple copies. In addition, "heterologous" or "exogenous" refers to a non-native enzyme, protein, or other activity encoded by an exogenous nucleic acid molecule introduced into the host cell, even if the host cell encodes a homologous protein or activity.

In certain embodiments, more than one heterologous nucleic acid molecules can be introduced into a host cell as separate nucleic acid molecules, as a polycistronic nucleic acid molecule, as a single nucleic acid molecule encoding a fusion protein (*e.g.*, a plurality of neoantigens), or any combination thereof, and still be considered as more than one heterologous nucleic acid. For example, as disclosed herein, an immune cell can be modified to contain two or more heterologous or exogenous nucleic acid molecules that encode one or more desired neoantigens or that encode one or more neoantigens and an immunogenicity enhancer (*e.g.*, flagellin, IL-12, GM-CSF). When two or more exogenous nucleic acid molecules encoding a neoantigen or an immunogenicity enhancer are introduced into a host cell, it is understood that the two or more exogenous nucleic acid molecules can be introduced as a single nucleic acid molecule (*e.g.*, on a single vector), or as more than one nucleic acid molecule (*e.g.*, on separate vectors), and can be comprised in an episomal expression vector, or integrated into the host chromosome at a single site or multiple sites, and still be considered two or more exogenous nucleic acid molecules. Thus, the number of referenced heterologous nucleic acid molecules or encoded biological activities refers to the number of encoding nucleic acid molecules or the number of protein activities, not the number of separate nucleic acid molecules introduced into a host cell.

As used herein, the term "endogenous" or "native" refers to a gene, protein, or activity that is normally present in a host cell. Moreover, a gene, protein, or activity that is mutated, overexpressed, shuffled, duplicated, or otherwise altered as compared to a parent gene, protein, or activity is still considered to be endogenous or native to that particular host cell. For example, an endogenous control sequence from a first gene (*e.g.*, promoter, translational attenuation sequences) may be used to alter or regulate expression of a second native gene or nucleic acid molecule, wherein the expression or regulation of the second native gene or nucleic acid molecule differs from normal expression or regulation in a parent cell.

As used herein, the term "recombinant" refers to a cell, microorganism, nucleic acid molecule, or vector that has been modified by introduction of an exogenous nucleic acid molecule through human intervention, or refers to a cell or microorganism that has been altered such that expression of an endogenous nucleic acid molecule or gene is

controlled, deregulated or constitutive, where such alterations or modifications are introduced or induced through human intervention (*e.g.*, by genetic engineering). Genetic alterations may include, for example, modifications introducing nucleic acid molecules (which may include an expression control element, such as a promoter) encoding one or more proteins or enzymes, or other nucleic acid molecule additions, deletions, substitutions, or other functional disruption of or addition to a cell's genetic material. Exemplary modifications include those in coding regions or functional fragments thereof of heterologous or homologous polypeptides from a reference or parent molecule. A cell, microorganism, nucleic acid molecule, or vector that has been modified by introduction of an exogenous nucleic acid molecule may be referred to as "recombinant" or "non-naturally occurring" or "genetically engineered" or "transformed" or "transgenic".

As used herein, "mutation" refers to a change in the sequence of a nucleic acid molecule or polypeptide molecule as compared to a reference or wild-type nucleic acid molecule or polypeptide molecule, respectively. A mutation can result in several different types of change in sequence, including substitution, insertion or deletion of nucleotide(s) or amino acid(s). In certain embodiments, a mutation is a substitution of one to three codons or amino acids, a deletion of one to about five codons or amino acids, or a combination thereof.

A "conservative substitution" is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are well known in the art (*see, e.g.*, WO 97/09433 at page 10; Lehninger, Biochemistry, 2nd Edition; Worth Publishers, Inc. NY, NY, pp. 71-77, 1975; Lewin, Genes IV, Oxford University Press, NY and Cell Press, Cambridge, MA, p. 8, 1990).

Neoantigens

In some aspects, the present disclosure provides immunogenic compositions or vaccines comprising immune cells, such as T cells, that express one or more exogenous neoantigens.

In certain embodiments, a neoantigen is associated with a hyperproliferative disease or disorder (*e.g.*, cancer), such as a tumor neoantigen. Because tumor neoantigens often arise from somatic "passenger" mutations in the DNA of tumor cells,

many tumor neoantigens are unique to an individual patient's cancer. Therefore, in certain embodiments, developing an immunogenic composition or vaccine disclosed herein comprises determining the "mutanome" of an individual patient's tumor, identifying candidate tumor neoantigens, and expressing the tumor neoantigens in immune cells to be delivered to a subject (Figure 1). In certain embodiments, a neoantigen comprises an antigenic peptide or epitope from a protein encoded by a nucleic acid molecule having a missense mutation, nonstop mutation, splice variant, gene fusion, frameshift mutation (*e.g.*, addition or deletion), or combinations thereof, as compared to the wild-type nucleic acid molecule.

Neoantigens may be identified using any of several well-known techniques (*see, e.g.*, Rajasagi *et al.*, *Blood 124*:453, 2014). By way of background and not wishing to be bound by theory, generally, a relatively small fraction of mutations in a malignant clone are able to elicit a new immune response. To be immunogenic, a mutation must change the amino acid sequence of an expressed protein, and the resulting protein must be proteolytically processed into peptides that contain the amino acid change, bind to MHC molecules, and be detected as foreign by the individual's T cells. The resulting mutation can potentially activate a T cell response if it alters processing and binding of the peptide to MHC, or alters the MHC-peptide-T cell receptor (TCR) interaction in a way that promotes specific binding (Gubin *et al.*, *J. Clin. Invest. 125*:3413, 2015). Computational algorithms have been developed to predict proteasome processing as well as peptide binding to MHC.

Exemplary prediction tools for identifying potential peptide binding to MHC *in silico* include, for example, NetMHC (for predicting MHC class I binding for particular human MHC alleles using artificial neural networks (ANNs)) (Andreatta *et al.*, *Bioinformatics 32*(4):511-517 (2016); Segal *et al.*, *Cancer Res 68*(3):889-892 (2008); Nielsen *et al.*, *Protein Sci. 12*(5): 1007-1017 (2003); NetMHCpan (for predicting MHC class I binding for any allele of known sequence) (Nielsen & Andreatta, *Genome Medicine 8*(1):33 (2016); Hoof *et al.*, *Immunogenetics 61*(1): 1-13 (2009), the Stabilized Matrix Method (SMM) (Peters & Sette, *BMC Bioinformatics 6*:132 (2005), and Average Relative Binding (ARB) matrix methods (Bui *et al.*, *Immunogenetics 57*(5):304-314 (2005) (*see also* Fritsch *et al.*, *Cancer Immunol Res 2*(6):522-529

(2014); van Buuren *et al.*, *Oncoimmunology 3*: e28836 (2014); Trolle *et al.*, *Bioinformatics 39*(5):764-768 (2015)). For example, mutations (*e.g.*, missense and frameshift) can be analyzed for formation of novel peptides predicted to bind autologous MHC alleles using NetMHCpan (Trolle *et al.*, *Bioinformatics 39*(5):764-768 (2015).). In addition, HLA typing may be obtained from next generation or exome sequencing using OptiType (*see*, *e.g.*, Szolek *et al.*, *Bioinformatics 30*(23): 3310-3316 (2014)).

A candidate neoantigen for use in an immunogenic composition or a vaccine of the present disclosure may be identified by isolating DNA from a tumor sample; sequencing expressed genes using exome capture, RNA sequencing, whole genome sequencing, or combinations thereof; and using bioinformatics to identify somatic mutations (*e.g.*, point mutations) that are predicted to produce candidate neoantigens.

Neoantigen and Immunogenicity Enhancer Expressing Cells

In certain aspects, the present disclosure provides immune cells (*e.g.*, T cells) comprising one or more exogenous neoantigens and an immunogenicity enhancer. In some embodiments, an immune cell comprising an exogenous neoantigen and an immunogenicity enhancer as disclosed herein may be used in an immunogenic composition or a vaccine for eliciting or boosting an immune response against the neoantigen. For example, in any of the embodiments described herein, T cells are modified to express an exogenous neoantigen. Neoantigens delivered by such engineered T cells will be highly immunogenic when administered to a subject, which can elicit a high frequency, durable T cell response against one or more epitopes of the neoantigen(s). Transferred T cells, to a subject known to have cells producing the neoantigen, can migrate efficiently to secondary lymphoid organs where neoantigen priming can occur, and may activate other T cells indirectly (mediated by dendritic cells) or directly against cells producing the neoantigen. Additionally, the generation of a durable T cell response may prevent relapse or recurrence of a disease associated with a neoantigen (*e.g.*, tumor neoantigen).

Vaccines based on T cells may offer additional practical advantages compared to other approaches, particularly in the context of personalized neoantigen-based vaccines and immunogenic compositions, such as ease of design, production, and

administration. By way of background, vaccines based on naked peptides or nucleic acid molecules are easy to construct and administer, but have not been highly effective. Moreover, increasing their immunogenicity is limited to adding systemic adjuvants, which can be toxic, be of limited efficacy, or both. Dendritic cells are difficult to isolate, genetically modify, or expand to large numbers *ex vivo*. In contrast, T cells are readily isolated, easily genetically modified, and can be expanded *ex vivo*, and there is clinical precedent for adoptively transferring T cells. As described herein, T cells for use in an immunogenic composition or a vaccine of this disclosure may also be modified to increase the potency of specific anti-cancer immune responses that are elicited.

In certain embodiments, the present disclosure provides an immune cell or lymphocyte comprising a nucleic acid molecule encoding an exogenous neoantigen and an immunogenicity enhancer, wherein the nucleic acid molecule encodes one or more exogenous neoantigens. For example, a nucleic acid molecule may encode one, two, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 neoantigens. In certain embodiments, an immune cell comprises a nucleic acid molecule encoding two to about five exogenous neoantigens, or two to about ten exogenous neoantigens, or two to about 20 exogenous neoantigens. In certain other embodiments, an immune cell or lymphocyte comprises separate nucleic acid molecules that each independently encode one or more exogenous neoantigens, such as two to about five exogenous neoantigens or two to about ten exogenous neoantigens or two to about 20 exogenous neoantigens (*e.g.*, each nucleic acid molecule may encode a different number of neoantigens).

In certain embodiments, an immune cell or lymphocyte of the present disclosure comprises a polynucleotide encoding an exogenous neoantigen comprising an epitope that is specifically bound by $CD8^+$ T cell, a $CD4^+$ T cell, or both. In certain embodiments, an immune cell or lymphocyte of the present disclosure comprises a polynucleotide encoding an exogenous neoantigen that is specifically bound by a $CD8^+$ T cell, and comprises a polynucleotide encoding an antigen or neoantigen that is specifically bound by a $CD4^+$ T cell. In further embodiments, the encoded $CD4^+$ antigen or neoantigen is exogenous to the immune cell or lymphocyte. In other

embodiments, an immune cell or lymphocyte comprises a polynucleotide encoding an exogenous neoantigen that is specifically bound by a $CD4^+$ T cell, and comprises a polynucleotide encoding an antigen or neoantigen that is specifically bound by a $CD8^+$ T cell. In further embodiments, the encoded $CD8^+$ antigen or neoantigen is exogenous to the immune cell or lymphocyte. Without wishing to be bound by theory, co-stimulation of CD8 and CD4 activity of a T cell can enhance the immune response against a neoantigen.

In any of the aforementioned embodiments, an immune cell or lymphocyte (*e.g.*, T cell) of the present disclosure comprises an exogenous neoantigen and an immunogenicity enhancer. In any of the aforementioned embodiments, an immune cell or lymphocyte comprises a polynucleotide encoding an exogenous neoantigen and a polynucleotide encoding an immunogenicity enhancer. Exemplary immunogenicity enhancers include an IL-12 (including a membrane-tethered IL-12), a GM-CSF, an inducible cell death factor, an antigen (*e.g.*, bacterial flagellin), or any combination thereof.

In any of the aforementioned embodiments, an exogenous neoantigen-containing immune cell or lymphocyte of the present disclosure may contain an immunogenicity enhancer that comprises a pathogen-associated molecule, such as those recognized by toll-like receptors (TLRs). For example, DNA-encoded TLR agonists may function as immunogenicity enhancers (*i.e.*, molecular adjuvants) for use with an immunogenic composition or a vaccine of this disclosure (*see* Applequist *et al.*, *J. Immunol. 175*:3882, 2005). An exemplary immunogenicity enhancer comprises a bacterial flagellin protein. Expression of a bacterial flagellin protein at the cell surface has been shown to enhance immune responses (Applequist *et al.*, 2005). In some embodiments, an encoded immunogenicity enhancer comprises a bacterial flagellin or immunogenicity enhancing fragment thereof. In certain embodiments, an immune cell or lymphocyte comprising a nucleic acid molecule encoding an exogenous neoantigen and a nucleic acid molecule encoding a bacterial flagellin or immunogenicity enhancing fragment thereof is provided. In particular embodiments, a bacterial flagellin comprises a *Salmonella* phase 1 flagellin. In other embodiments, a bacterial flagellin comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in GenBank

Accession No. NC_003197.1 (which sequence is incorporated herein by reference in its entirety). In further embodiments, a bacterial flagellin comprises a bacterial flagellin fusion protein that localizes to the cell surface of a lymphocyte (*i.e.*, a membrane-tethered bacterial flagellin fusion protein). For example, in certain embodiments, a bacterial flagellin fusion protein comprises (a) a bacterial flagellin domain; and (b) a transmembrane domain. In certain embodiments, a bacterial flagellin fusion protein further comprises (c) a signal domain that directs the fusion protein to a secretory pathway. In particular embodiments, a transmembrane domain comprises a PDGF transmembrane domain. In certain embodiments, a transmembrane domain comprises an amino acid sequence as set forth in SEQ ID NO.:5, or a fragment thereof. In further embodiments, a signal domain comprises an Igκ-chain leader sequence. In certain embodiments, a signal domain comprises an amino acid sequence as set forth in SEQ ID NO.:1. In still further embodiments, a bacterial flagellin domain may be modified to remove eukaryotic glycosylation sites. In certain embodiments, a bacterial flagellin domain comprises an amino acid sequence as set forth in SEQ ID NO.:4. In other embodiments, a bacterial flagellin fusion protein further comprises a tag. In some embodiments, a tag included in a bacterial flagellin fusion protein comprises an amino acid sequence as set forth in SEQ ID NO.:2.

In certain embodiments, a bacterial flagellin fusion protein comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:3.

In any of the aforementioned embodiments, an exogenous neoantigen-containing immune cell or lymphocyte of the present disclosure contains an immunogenicity enhancer that is an IL-12, such as human IL-12. By way of background, IL-12 has been shown to stimulate the priming of Th1 pro-inflammatory responses at the site of antigen delivery (Hsieh *et al.*, *Science 260*:547, 1993), while limiting systemic toxicity. For example, membrane-bound IL-12 can stimulate immune responses to transplanted tumor cell lines (Pan *et al.*, *Mol. Ther. 20*:927, 2012). In certain embodiments disclosed herein, an immune cell or lymphocyte comprising an exogenous neoantigen and an exogenous IL-12 or functional fragment thereof is provided. A nucleic acid sequence encoding human IL-12 is set forth in SEQ ID NO.: 7, and an amino acid sequence of human IL-12 is set forth in SEQ ID NO.: 8. In certain

embodiments, an immune cell or lymphocyte comprising a nucleic acid molecule encoding an exogenous neoantigen and a nucleic acid molecule encoding an IL-12 is provided. In certain embodiments, an IL-12 comprises a single-chain (sc) IL-12. Construction of a single-chain IL-12 is described in, for example, Pan *et al.*, *Mol. Ther.* *20*(5):927-937 (2012). In further embodiments, an IL-12 comprises an IL-12 fusion protein that localizes to the cell surface of the immune cell or lymphocyte (*i.e.*, a membrane-tethered IL-12). In certain embodiments, an IL-12 fusion protein comprises (a) an IL-12 domain; and (b) a transmembrane domain. In certain embodiments, an IL-12 transmembrane domain of B7, CD2, CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD40, CD47, CD79A, CD79B, CD80, CD86, CD95 (Fas), CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD200R, CD223 (LAG3), CD270 (HVEM), CD272 (BTLA), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), CD279 (PD-1), TIM3, CD300, CD357 (GITR), A2aR, DAP10, FcRα, FcRβ, FcRγ, Fyn, GAL9, KIR, Lck, LAT, LPA5, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PTCH2, ROR2, Ryk, Slp76, SIRPα, pTα, TCRα, TCRβ, TIM3, TRIM, or Zap70. In certain embodiments, an IL-12 fusion protein further comprises (c) a signal peptide that directs the fusion protein to a secretory pathway (*i.e.*, to localize to the cell membrane).

In any of the aforementioned embodiments, an exogenous neoantigen-containing immune cell or lymphocyte of the present disclosure contains an immunogenicity enhancer that is a Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF). By way of background, GM-CSF is secreted by multiple cell types in response to cytokine signaling and, among other functions, stimulates production of granulocytes and monocytes, which subsequently mature into macrophages and DCs. GM-CSF plays a role in immune modulation and has been used as an immunogenicity enhancer in cancer immunotherapies. *See, e.g.,* Hong, *Exp. Mol. Med. 48*(7):e242 (2016).

In any of the aforementioned embodiments, the present disclosure provides an immune cell or lymphocyte comprising an exogenous neoantigen and an immunogenicity enhancer, or a polynucleotide encoding an exogenous neoantigen and a polynucleotide encoding an immunogenicity enhancer, wherein the immune cell or lymphocyte comprises a T cell.

Phagocytosis of dying cells by dendritic cells will activate $CD8^+$ T cells by cross-priming (Yatim *et al.*, *Science 350*:328, 2015). Thus, the ability to selectively induce apoptosis or necroptosis in a neoantigen-containing immune cell or lymphocyte of this disclosure may allow for enhanced immunogenicity of the cell, by activating an inflammatory cell death pathway and increasing neoantigen cross-presentation. In certain embodiments, an immune cell or lymphocyte comprises an exogenous neoantigen and an exogenous inducible cell death factor. In certain embodiments, an immune cell or lymphocyte comprises a nucleic acid molecule encoding an exogenous neoantigen and a nucleic acid molecule encoding an exogenous inducible cell death factor.

An "inducible cell death factor" refers to a molecule that is capable of triggering or promoting cell death, and which activity can be selectively induced. In some embodiments, an inducible cell death factor is a fusion protein comprising a cell death signaling domain and a multimerization domain.

A "multimerization domain," as used herein, refers to a polypeptide molecule or region that preferentially interacts or associates with another polypeptide molecule or region, directly or indirectly, wherein the interaction of multimerization domains substantially contribute to or efficiently promote multimerization (*i.e.*, the formation of a dimer, trimer, tetramer, or higher order multimers, which may be a homodimer, heterodimer, homotrimer, heterotrimer, homomultimer, heteromultimer, or the like). For example, multimerization may be due to one or more types of molecular forces, including covalent bonds (*e.g.*, disulfide bonds or bridges), ionic bonds, metallic bonds, electrostatic interactions, salt bridges, dipole-dipole forces, hydrogen bonding, Van der Waals forces, hydrophobic interactions, or any combination thereof. A multimer is stable under appropriate conditions (*e.g.*, physiological conditions, in an aqueous solution suitable for expressing, purifying, or storing recombinant or engineered proteins, or under conditions for non-denaturing or non-reducing electrophoresis). Exemplary multimerization domains associate via a multimerization promoting molecule, such as chemically induced multimerization, wherein multimerization is minimal or does not occur in the absence of the multimerization promoting molecule.

In certain embodiments, an inducible cell death factor is a receptor interacting serine/threonine kinase 3 (RIPK3) (*see*, *e.g.*, Yatim *et al.*, *Science 350*:328, 2015). In particular embodiments, RIPK3 comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in GenBank Accession No. NP_006862 (which sequence is incorporated herein by reference in its entirety). When activated, RIPK3 can trigger necroptosis through the TNF receptor-driven cell death pathway, and chemically enforced oligomerization of RIPK3 can induce necroptosis (Orozco *et al.*, *Cell Death Differ. 21*:1511, 2014). Accordingly, in certain embodiments, an inducible cell death factor is a fusion protein comprising, for example, a RIPK3 cell death signaling domain and a multimerization domain. In certain embodiments, a RIPK3 cell death signaling domain comprises a RIPK3 kinase domain and a multimerization domain comprises an FK506-binding protein or multimerizing portion thereof. The FK506-binding protein binds with high affinity to a synthetic bivalent homologue of rapamycin, resulting in rapid dimerization (Yatim *et al.*, *supra*; Orozco *et al.*, *supra*). In further embodiments, a multimerization promoting molecule comprises rapamycin or analog or derivative thereof. Other molecules that are associated with a protein interaction that leads to cell death include mixed lineage kinase domain-like (MLKL) (*see* Murphy *et al.*, *Immunity 39*:443, 2013) and caspase and GSDMD (*see* Shi *et al.*, *Nature 526*:660, 2015). Immune cells, lymphocytes or T cells comprising an exogenous neoantigen and an inducible MLKL, caspase, or GSMD are also within the scope of this disclosure. Immune cells, lymphocytes or T cells comprising a nucleic acid molecule encoding an exogenous neoantigen and a nucleic acid molecule encoding an inducible MLKL, caspase, or GSMD are also provided.

Other immunogenicity enhancers for use in the cells and methods disclosed herein include, for example, a CD80, a CD137L, a CD40L, a secreted IL-2, a secreted IL-2 that binds T cells independent of CD25, a secreted IL-15, a secreted IL-15-IL-15Rα complex, a secreted IFNβ, a secreted IFN-α1, a secreted IL-7, or any combination thereof.

In any of the aforementioned embodiments, the present disclosure provides an immune cell or lymphocyte comprising an exogenous neoantigen and an exogenous inducible cell death factor, or a polynucleotide encoding an exogenous neoantigen and a

polynucleotide encoding an exogenous inducible cell death factor, wherein the immune cell or lymphocyte comprises a T cell.

In any of the aforementioned embodiments, an exogenous neoantigen-containing immune cell or lymphocyte of the present disclosure may further comprise a costimulatory molecule. In certain embodiments, an immune cell or lymphocyte comprising a nucleic acid molecule encoding an exogenous neoantigen and a nucleic acid molecule encoding a costimulatory molecule is provided. A "costimulatory molecule" as used herein refers to a receptor, ligand, or cell-surface molecule that can deliver or transduce signals into T cells to positively modulate T cell activation (Chen and Flies, *Nat. Rev. Immunol. 13*:227, 2013). By way of background, T cell activation and proliferation requires two signals mediated through engagement of the T cell antigen-specific receptor (TCR) and a costimulatory signal, most typically binding of CD28 by CD80 and CD86 (Ledbetter *et al.*, *Blood 75*:1531, 1990). Costimulatory ligands and counter-receptors are frequently expressed on APCs or other cells that interact with T cells, and provide costimulatory signaling by interacting with cell-surface receptors found on T cells. In some embodiments, an immune cell or lymphocyte comprising an exogenous neoantigen and an exogenous costimulatory molecule is provided, wherein the costimulatory molecule is selected from a CD80, a CD86, a B7RP1, a CD137L, an OX40L, a CD70, a CD30L, a CD154, an ICAM-1, a CD2BP2, a LIGHT, a KLRD1, a ligand that specifically binds to a CD83, or any combination thereof. In particular embodiments, the costimulatory molecule comprises a CD80. In further embodiments, the costimulatory molecule comprises a CD137L. In still further embodiments, the costimulatory molecule localizes to the cell surface of the immune cell or lymphocyte.

In any of the aforementioned embodiments, the present disclosure provides an immune cell or lymphocyte comprising an exogenous neoantigen and an exogenous costimulatory molecule, or a polynucleotide encoding an exogenous neoantigen and a polynucleotide encoding an exogenous costimulatory molecule, wherein the immune cell or lymphocyte comprises a T cell.

In any of the aforementioned embodiments, an exogenous neoantigen-containing immune cell or lymphocyte comprises an exogenous neoantigen associated

with a disease or disorder. In some embodiments, a neoantigen is associated with a hyperproliferative disease or disorder, such as cancer. In certain embodiments, a neoantigen comprises a tumor neoantigen. In particular embodiments, a neoantigen comprises a tumor neoantigen from a protein encoded by a nucleic acid molecule having a missense mutation or frameshift mutation, as compared to the protein encoded by a wild-type nucleic acid molecule.

In any of the aforementioned embodiments, an exogenous neoantigen, an exogenous immunogenicity enhancer, an exogenous inducible cell death factor, or an exogenous costimulatory molecule may be introduced into the immune cell by methods known in the art, such as transfection, transduction, or electroporation of a DNA molecule encoding one or more of the neoantigen(s), immunogenicity enhancer, inducible cell death factor, and costimulatory molecule. In some embodiments, an expression construct is used to introduce a polynucleotide encoding the neoantigen, immunogenicity enhancer, inducible cell death factor, or costimulatory molecule into the immune cell. For example, in some embodiments, one or more of the exogenous neoantigen, exogenous immunogenicity enhancer, exogenous inducible cell death factor, or exogenous costimulatory molecule are introduced into an immune cell, lymphocyte or T cell using a transposon vector or expression construct.

Expression Constructs and Host Cells

In further aspects, the present disclosure provides nucleic acid molecules encoding one or more neoantigens and immunogenicity enhancers, and expression constructs for expressing such neoantigens and immunogenicity enhancers in a host cell (*e.g.*, T cell).

As used herein, "expression construct" refers to a DNA construct containing a nucleic acid molecule that is operably-linked to a suitable control sequence capable of effecting the expression of the nucleic acid molecule in a suitable host. An expression construct may be present in a vector (*e.g.*, a bacterial vector, a viral vector) or may be integrated into a genome. The term "operably linked" refers to the association of two or more polynucleotides on a single polynucleotide fragment so that the function of one is affected by the other. For example, a promoter is operably-linked with a coding

sequence when it is capable of affecting the expression of that coding sequence (*i.e.*, the coding sequence is under the transcriptional control of the promoter). The term "expression control sequence" (also called a regulatory sequence) refers to polynucleotide sequences that effect the expression and processing of coding sequences to which they are operably linked. For example, expression control sequences may include transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequences); sequences that enhance protein stability; and possibly sequences that enhance protein secretion.

In certain embodiments, an expression construct is present in a vector. A "vector" is a nucleic acid molecule that is capable of transporting another nucleic acid. A vector may be, for example, a plasmid, cosmid, virus, RNA vector, or linear or circular DNA or RNA molecule that may include chromosomal, non-chromosomal, semi-synthetic, or synthetic nucleic acids. Exemplary vectors are those capable of autonomous replication (episomal vectors) or expression of nucleic acids to which they are linked (expression vectors). Exemplary viral vectors include retrovirus, adenovirus, parvovirus (*e.g.*, adeno-associated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (*e.g.*, influenza virus), rhabdovirus (*e.g.*, rabies and vesicular stomatitis virus), paramyxovirus (*e.g.*, measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double-stranded DNA viruses including adenovirus, herpesvirus (*e.g.*, Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (*e.g.*, vaccinia, fowlpox and canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example. Examples of retroviruses include avian leukosis-sarcoma, mammalian C-type, B-type viruses, D type viruses, HTLV-BLV group, lentivirus, spumavirus (Coffin, Retroviridae: The viruses and their replication, in Fundamental Virology, Third Edition, B. N. Fields *et al.*, Eds., Lippincott-Raven Publishers, Philadelphia, 1996). In some embodiments, the vector is a plasmid vector (such as sleeping beauty, *piggyBac*, or other transposon vectors). In some embodiments, the vector is a viral vector. In some such embodiments, the viral vector

is a lentiviral vector or a γ-retroviral vector. In some embodiments, a viral or plasmid vector further comprises a gene marker transduction (*e.g.*, green fluorescent protein, huEGFRt).

In certain other aspects, the disclosure provides a host cell comprising an expression construct or vector, or a polynucleotide provided by an expression construct as described herein. As used herein, the term "host" refers to a cell (*e.g.*, T cell, hematopoietic progenitor cell) or microorganism targeted for genetic modification with a heterologous or exogenous nucleic acid molecule to produce a peptide of interest (*e.g.*, a neoantigen). In certain embodiments, a host cell may optionally already possess or be modified to include other genetic modifications that confer desired properties related or unrelated to biosynthesis of the heterologous or exogenous neoantigen peptide or immunogenicity enhancer (*e.g.*, inclusion of a detectable marker). More than one heterologous or exogenous nucleic acid molecule can be introduced into a host cell as separate nucleic acid molecules, as a plurality of individually controlled genes, as a polycistronic nucleic acid molecule, as a single nucleic acid molecule encoding a fusion protein, or any combination thereof. When two or more exogenous nucleic acid molecules are introduced into a host cell, it is understood that the two more exogenous nucleic acid molecules can be introduced as a single nucleic acid molecule (*e.g.*, on a single vector), on separate vectors, integrated into the host chromosome at a single site or multiple sites. The number of referenced heterologous nucleic acid molecules or protein activities refers to the number of encoding nucleic acid molecules or the number of protein activities, not the number of separate nucleic acid molecules introduced into a host cell.

In certain embodiments, an exogenous neoantigen or immunogenicity enhancer is introduced to a host cell using a transposon expression construct. A "transposon" (or "transposable element") refers to a mobile genetic unit that can move positions within a DNA molecule in the presence of a transposase. Transposons mobilize through a cut-and-paste mechanism wherein a transposase enzyme binds to DNA at inverted repeats and catalyzes the excision of the element from a DNA molecule and inserts it in another location. This process of horizontal gene transfer can be used to introduce a gene into a cell. As used herein, a "transposon system" refers to a plasmid-based gene transfer

system comprising a transposon and a transposase. Transposons that may be used to introduce an exogenous gene into a cell include, for example, the sleeping beauty transposon (Ivics *et al.*, *Cell 91*:501, 1997) and the *piggyBac* transposon (Ding *et al.*, *Cell 122*:473, 2005). In the *piggyBac* transposon system, a gene of interest is flanked by transposon inverted terminal repeat (IR) elements, and the construct is typically electroporated into cells along with a plasmid expressing the transposase enzyme. This leads to efficient stable integration into primary human T cells associated with retroviral vectors (Nakazawa *et al.*, *J. Immunother. 32*:826, 2009), and has the advantages of ease of use, low cost of naked DNA, and less theoretical risk for integration related transformation events (Galvan *et al.*, *J. Immunother. 32*:837, 2009).

In certain embodiments, a transposon expression construct, comprising a nucleic acid molecule encoding a neoantigen, is provided. In further embodiments, the transposon expression construct comprises a sleeping beauty transposon or a *piggyBac* transposon. In particular embodiments, a transposon expression construct comprises (a) a promoter; (b) a first *piggyBac* transposon inverted repeat; (b) a nucleic acid molecule encoding a neoantigen; and (c) a second *piggyBac* transposon inverted repeat; wherein the nucleic acid molecule that encodes the neoantigen is positioned between the first *piggyBac* transposon inverted repeat and the second *piggyBac* transposon inverted repeat.

In any of the aforementioned embodiments, a transposon expression construct may be present in a plasmid or delivered to a cell in a plasmid.

In any of the aforementioned embodiments, a transposon expression construct may comprise a minigene, wherein the minigene comprises a nucleic acid molecule encoding a neoantigen. In any of the aforementioned embodiments, a transposon expression construct may comprise tandem minigenes, wherein each minigene comprises a nucleic acid molecule encoding a neoantigen. For example, the tandem minigenes may comprise nucleic acid molecules encoding from two to about 20 neoantigens, or from two to about 10 neoantigens, or from two to about five neoantigens.

In any of the aforementioned embodiments, a transposon expression construct may further comprise a reporter gene. In certain embodiments, the reporter gene is a

green fluorescent protein (GFP) gene. In certain embodiments, a transposon expression construct further comprises a nucleic acid molecule encoding a cell surface marker. For example, in particular embodiments, a cell surface marker comprises a truncated human CD19, a truncated human EGFR, a truncated human CD34, a truncated human NGFR, or any other transduction marker known in the art.

In any of the aforementioned embodiments, a transposon expression construct may further comprise a nucleic acid molecule encoding an immunogenicity enhancer, wherein the immunogenicity enhancer is selected from an IL-12 (such as a membrane-tethered IL-12), and a GM-CSF, an inducible cell death factor, an antigen (*e.g.*, bacterial flagellin), or any combination thereof.

In any of the aforementioned embodiments, a transposase expression construct may further comprise a nucleic acid molecule encoding a *piggyBac* transposase. For example, a transposase may be encoded by the same expression construct as the other exogenous genes and the *piggyBac* transposon inverted repeats. In other embodiments, a transposase is encoded by a nucleic acid molecule contained in a different expression construct. For example, in particular embodiments, a transposase is encoded by a nucleic acid molecule in an expression construct present in a different plasmid.

In another aspect, host cells comprising a transposon expression construct as described herein are provided. In some embodiments, the host cell comprises a transposon expression vector of any of the aforementioned embodiments. In certain embodiments, the host cell further comprises a *piggyBac* transposase enzyme expression construct, wherein the *piggyBac* transposase enzyme construct comprises a nucleic acid molecule encoding a *piggyBac* transposase. In particular embodiments, the first and second constructs are present in different plasmids.

In any of the aforementioned embodiments, a host cell may be an immune system cell. In some embodiments, the host cell comprises a dendritic cell. In other embodiments, the host cell comprises a T cell. In further embodiments, the host cell comprises a T cell selected from a naïve T cell, central memory T cell, naïve and central memory T cells, effector memory T cells, or any combination thereof. In particular embodiments, a host cell is a $CD4^+$ T cell or a $CD8^+$ T cell or both.

In any of the aforementioned embodiments, a host cell may comprise a human cell, such as a human T cell.

In any of the aforementioned embodiments, a polynucleotide encoding an exogenous neoantigen, an exogenous immunogenicity enhancer, an exogenous inducible cell death factor, or an exogenous costimulatory molecule is delivered to a host cell via a viral vector. In some embodiments, the viral vector is a retroviral vector or a lentiviral vector.

In any of the aforementioned embodiments, a viral vector to be used to deliver a polynucleotide of this disclosure, comprises a polynucleotide encoding an immunogenicity enhancer selected from, for example, an IL-12 (such as a membrane-tethered IL-12), a GM-CSF, an inducible cell death factor, an antigen (*e.g.*, bacterial flagellin), or any combination thereof. In any of the aforementioned embodiments, a viral vector to be used to deliver a polynucleotide of this disclosure, comprises a polynucleotide that encodes a CD80, CD137, CD140L, secreted IL-2, IL-2 modified to be CD25 independent in T cell binding (*see* Levin *et al.*, *Nature 484*(7395):529-33, 2012), IL-15, IL-15-IL-15 receptor alpha complex, IFN-B, IFN-A1, IL-7, an inducible death switche such as small molecule dimerizable RIPK3, or any combination thereof.

In any of the aforementioned embodiments, a host cell encoding a neoantigen (*e.g.*, a lymphocyte such as a T cell) further comprises a nucleic acid molecule encoding a costimulatory molecule delivered via a viral vector. In some embodiments, a viral vector comprises a nucleic acid molecule encoding a CD80, a CD86, a B7RP1, a CD137L , an OX40L, a CD70, a CD30L, a CD154, an ICAM-1, a CD2BP2, a LIGHT, a KLRD1, a ligand that specifically binds to a CD83, an agonist of CD137 (4-1BB), an agonist of CD134 (OX-40), an agonist of CD27, an agonist of CD28, an agonist of CD40, an agonist of CD122, an agonist of GITR, an agonist of ICOS, or any combination thereof.. In particular embodiments, a viral vector comprises a nucleic acid molecule encoding a CD80. In other particular embodiments, a viral vector comprises a nucleic acid molecule encoding a CD137L. In certain embodiments, a costimulatory molecule localizes to the cell surface of the host cell, such as a T cell.

Methods and Kits

In further aspects, methods and kits for preparing an immune cell or lymphocyte that expresses an exogenous neoantigen and an immunogenicity enhancer, and optionally any other active molecules, are provided.

In certain embodiments, a method for preparing an immune cell or lymphocyte that comprises an exogenous neoantigen is provided, the method comprising introducing into a lymphocyte (a) a transposon plasmid (*e.g.*, *piggyBac*) containing a nucleic acid molecule encoding a neoantigen (*e.g.*, identified in a sample of a particular subject) and (b) a plasmid comprising a nucleic acid molecule encoding a transposase (*e.g.*, *piggyBac*), thereby preparing the lymphocyte that comprises an exogenous neoantigen.

In further embodiments, a nucleic acid molecule encodes two or more neoantigens or neoantigens are contained in a tandem minigene. For example, a tandem minigene may be used to express multiple neoantigens contained the same plasmid.

In certain embodiments, an immune cell or lymphocyte expressing a neoantigen prepared by the methods described herein is syngeneic, allogeneic, or autologous to the subject to be treated. In particular embodiments, an immune cell or lymphocyte expressing a neoantigen is autologous to the subject to be treated. In certain embodiments, a transposon plasmid (*e.g.*, *piggyBac*) and the plasmid comprising a nucleic acid molecule encoding a transposase (*e.g.*, *piggyBac*) are introduced into an immune cell or lymphocyte (*e.g.*, T cell) *ex vivo*. In some embodiments, a nucleic acid molecule encoding a neoantigen or an immunogenicity enhancer is introduced into the genome of an immune cell or a lymphocyte using known genome editing techniques, such as by use of a CRISPR-Cas9 system or a zinc finger nuclease (ZNF) system. *See, e.g.,* Wang *et al.*, *Nat. Biotech 33*:1256 (2015); *see also* Lombardo *et al.*, *Nat. Biotech. 11*:1298 (2007) and PCT Patent Application US/2016/031366.

In certain embodiments, a kit comprising the above components or a transposon expression construct as disclosed herein is provided.

Pharmaceutical Compositions and Methods of Use

In some aspects, pharmaceutical compositions, immunogenic, compositions, and vaccines are provided. In some embodiments, a therapeutic vaccine for eliciting or boosting a robust immune cell response is provided. In certain embodiments, the vaccine comprises an immune cell modified to express an exogenous neoantigen.

In certain embodiments, the present disclosure provides a composition, comprising a an immune cell or lymphocyte (*e.g.*, T cell) as disclosed herein, and a pharmaceutically acceptable carrier, diluent, or excipient. Pharmaceutically acceptable carriers for diagnostic and therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro (Ed.), 18th Edition, 1990) and in CRC Handbook of Food, Drug, and Cosmetic Excipients, CRC Press LLC (S.C. Smolinski, ed., 1992). Exemplary pharmaceutically acceptable carriers include any adjuvant, carrier, excipient, glidant, diluent, preservative, dye/colorant, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, emulsifier, or any combination thereof. For example, sterile saline and phosphate buffered saline at physiological pH can be suitable pharmaceutically acceptable carriers. Preservatives, stabilizers, dyes or the like may also be provided in the pharmaceutical composition. In addition, antioxidants and suspending agents may also be used. Pharmaceutical compositions may also contain diluents such as water, buffers, antioxidants such as ascorbic acid, low molecular weight polypeptides (less than about 10 residues), proteins, amino acids, carbohydrates (*e.g.*, glucose, sucrose, dextrins), chelating agents (*e.g.*, EDTA), glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary diluents.

In another aspect, methods for treating a disease or disorder comprising administering compositions disclosed herein are provided. In certain embodiments, the present disclosure provides a method for treating a disease or disorder, comprising administering to a human subject in need thereof an effective amount of a lymphocyte as disclosed herein or a composition as disclosed herein.

In another aspect, methods for treating a human subject having a disease or disorder associated with expression of a neoantigen are provided, comprising

administering to a human subject in need thereof an effective amount of an immune cell or lymphocyte as disclosed herein or a composition as disclosed herein. In any of the methods disclosed herein, an immune cell or lymphocyte maybe autologous to the subject, syngeneic to the subject, or allogeneic to the subject. In any of the methods disclosed herein, a lymphocyte comprises a T cell.

In another aspect, methods for preventing relapse or recurrence of a disease or disorder (*e.g.*, cancer, infection) in a human subject having the disease or disorder are provided, comprising administering to a human subject in need thereof an effective amount of a lymphocyte, such as a T cell, containing or expressing a neoantigen and an immunogenicity enhancer as disclosed herein or a composition as disclosed herein. In any of the aforementioned embodiments, a disease or disorder may be a viral infection, bacterial infection, hyperproliferative disorder, or inflammatory or autoimmune disease.

Infectious diseases include those associated with infectious agents and include any of a variety of bacteria (*e.g.*, pathogenic *E. coli*, S. *typhimurium, P. aeruginosa, B. anthracis, C. botulinum, C. difficile, C. perfringens, H. pylori, V. cholerae, Listeria spp., Rickettsia spp., Chlamydia spp.*, and the like), mycobacteria, and parasites (including any known parasitic member of the Protozoa). Infectious viruses include eukaryotic viruses, such as adenovirus, bunyavirus, herpesvirus, papovavirus, papillomavirus (*e.g.*, HPV), paramyxovirus, picornavirus, rhabdovirus (*e.g.*, Rabies), orthomyxovirus (*e.g.*, influenza), poxvirus (*e.g.*, Vaccinia), reovirus, retrovirus, lentivirus (*e.g.*, HIV), flavivirus (*e.g.*, HCV, HBV) or the like. In certain embodiments, infection with cytosolic pathogens whose antigens are processed and displayed with HLA (MHC) Class I molecules, are treated with fusion proteins of this disclosure.

A wide variety of cancers, including solid tumors and leukemias, are amenable to the compositions and methods disclosed herein. Exemplary types of cancer that may be treated include adenocarcinoma of the breast, prostate, and colon; all forms of bronchogenic carcinoma of the lung; myeloid leukemia; melanoma; hepatoma; neuroblastoma; papilloma; apudoma; choristoma; branchioma; malignant carcinoid syndrome; carcinoid heart disease; and carcinoma (*e.g.*, Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous

cell, and transitional cell). Additional types of cancers that may be treated include histiocytic disorders; malignant histiocytosis; leukemia; Hodgkin's disease; immunoproliferative small; non-Hodgkin's lymphoma; plasmacytoma; reticuloendotheliosis; melanoma; chondroblastoma; chondroma; chondrosarcoma; fibroma; fibrosarcoma; giant cell tumors; histiocytoma; lipoma; liposarcoma; mesothelioma; myxoma; myxosarcoma; osteoma; osteosarcoma; chordoma; craniopharyngioma; dysgerminoma; hamartoma; mesenchymoma; mesonephroma; myosarcoma; ameloblastoma; cementoma; odontoma; teratoma; thymoma; trophoblastic tumor. Further, the following types of cancers are also contemplated as amenable to treatment: adenoma; cholangioma; cholesteatoma; cyclindroma; cystadenocarcinoma; cystadenoma; granulosa cell tumor; gynandroblastoma; hepatoma; hidradenoma; islet cell tumor; Leydig cell tumor; papilloma; sertoli cell tumor; theca cell tumor; leiomyoma; leiomyosarcoma; myoblastoma; myomma; myosarcoma; rhabdomyoma; rhabdomyosarcoma; ependymoma; ganglioneuroma; glioma; medulloblastoma; meningioma; neurilemmoma; neuroblastoma; neuroepithelioma; neurofibroma; neuroma; paraganglioma; paraganglioma nonchromaffin. The types of cancers that may be treated also include angiokeratoma; angiolymphoid hyperplasia with eosinophilia; angioma sclerosing; angiomatosis; glomangioma; hemangioendothelioma; hemangioma; hemangiopericytoma; hemangiosarcoma; lymphangioma; lymphangiomyoma; lymphangiosarcoma; pinealoma; carcinosarcoma; chondrosarcoma; cystosarcoma phyllodes; fibrosarcoma; hemangiosarcoma; leiomyosarcoma; leukosarcoma; liposarcoma; lymphangiosarcoma; myosarcoma; myxosarcoma; ovarian carcinoma; rhabdomyosarcoma; sarcoma; neoplasms; nerofibromatosis; and cervical dysplasia.

Exemplifying the variety of hyperproliferative disorders amenable to treatment are B-cell lymphomas (such as various forms of Hodgkin's disease, non-Hodgkin's lymphoma (NHL) or central nervous system lymphomas), leukemias (such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), Hairy cell leukemia, B cell blast transformation of chronic myeloid leukemia) and myelomas (such as multiple myeloma). Additional B cell cancers include small lymphocytic lymphoma, B-cell prolymphocytic leukemia, lymphoplasmacytic lymphoma, splenic

marginal zone lymphoma, plasma cell myeloma, solitary plasmacytoma of bone, extraosseous plasmacytoma, extra-nodal marginal zone B-cell lymphoma of mucosa-associated (MALT) lymphoid tissue, nodal marginal zone B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, diffuse large B-cell lymphoma, mediastinal (thymic) large B-cell lymphoma, intravascular large B-cell lymphoma, primary effusion lymphoma, Burkitt's lymphoma/leukemia, B-cell proliferations of uncertain malignant potential, lymphomatoid granulomatosis, and post-transplant lymphoproliferative disorder.

Inflammatory and autoimmune diseases include arthritis, rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, polychondritis, psoriatic arthritis, psoriasis, dermatitis, polymyositis/dermatomyositis, inclusion body myositis, inflammatory myositis, toxic epidermal necrolysis, systemic scleroderma and sclerosis, CREST syndrome, inflammatory bowel disease, Crohn's disease, ulcerative colitis, respiratory distress syndrome, adult respiratory distress syndrome (ARDS), meningitis, encephalitis, uveitis, colitis, glomerulonephritis, allergic conditions, eczema, asthma, conditions involving infiltration of T cells and chronic inflammatory responses, atherosclerosis, autoimmune myocarditis, leukocyte adhesion deficiency, systemic lupus erythematosus (SLE), subacute cutaneous lupus erythematosus, discoid lupus, lupus myelitis, lupus cerebritis, juvenile onset diabetes, multiple sclerosis, allergic encephalomyelitis, neuromyelitis optica, rheumatic fever, Sydenham's chorea, immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes, tuberculosis, sarcoidosis, granulomatosis including Wegener's granulomatosis and Churg-Strauss disease, agranulocytosis, vasculitis (including hypersensitivity vasculitis/angiitis, ANCA and rheumatoid vasculitis), aplastic anemia, Diamond Blackfan anemia, immune hemolytic anemia including autoimmune hemolytic anemia (AIHA), pernicious anemia, pure red cell aplasia (PRCA), Factor VIII deficiency, hemophilia A, autoimmune neutropenia, pancytopenia, leukopenia, diseases involving leukocyte diapedesis, central nervous system (CNS) inflammatory disorders, multiple organ injury syndrome, myasthenia gravis, antigen-antibody complex mediated diseases, anti-glomerular basement membrane disease, anti-phospholipid antibody syndrome, allergic neuritis, Behcet disease, Castleman's

syndrome, Goodpasture's syndrome, Lambert-Eaton Myasthenic Syndrome, Reynaud's syndrome, Sjorgen's syndrome, Stevens-Johnson syndrome, solid organ transplant rejection, graft versus host disease (GVHD), bullous pemphigoid, pemphigus, autoimmune polyendocrinopathies, seronegative spondyloarthropathies, Reiter's disease, stiff-man syndrome, giant cell arteritis, immune complex nephritis, IgA nephropathy, IgM polyneuropathies or IgM mediated neuropathy, idiopathic thrombocytopenic purpura (ITP), thrombotic throbocytopenic purpura (TTP), Henoch-Schonlein purpura, autoimmune thrombocytopenia, autoimmune disease of the testis and ovary including autoimmune orchitis and oophoritis, primary hypothyroidism; autoimmune endocrine diseases including autoimmune thyroiditis, chronic thyroiditis (Hashimoto's Thyroiditis), subacute thyroiditis, idiopathic hypothyroidism, Addison's disease, Grave's disease, autoimmune polyglandular syndromes (or polyglandular endocrinopathy syndromes), Type I diabetes also referred to as insulin-dependent diabetes mellitus (IDDM) and Sheehan's syndrome; autoimmune hepatitis, lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant), non-specific interstitial pneumonia (NSIP), Guillain-Barré Syndrome, large vessel vasculitis (including polymyalgia rheumatica and giant cell (Takayasu's) arteritis), medium vessel vasculitis (including Kawasaki's disease and polyarteritis nodosa), polyarteritis nodosa (PAN) ankylosing spondylitis, Berger's disease (IgA nephropathy), rapidly progressive glomerulonephritis, primary biliary cirrhosis, Celiac sprue (gluten enteropathy), cryoglobulinemia, cryoglobulinemia associated with hepatitis, amyotrophic lateral sclerosis (ALS), coronary artery disease, familial Mediterranean fever, microscopic polyangiitis, Cogan's syndrome, Whiskott-Aldrich syndrome and thromboangiitis obliterans.

In particular embodiments, the disease or disorder is a hyperproliferative disease or disorder selected from a hematological malignancy or a solid cancer. In certain embodiments, the hyperproliferative disease or disorder is a hematological malignancy selected from acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), chronic eosinophilic leukemia (CEL), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), and multiple myeloma (MM). In some embodiments, the hyperproliferative disease or disorder is a

solid cancer selected from biliary cancer, bladder cancer, bone and soft tissue carcinoma, brain tumor, breast cancer, cervical cancer, colon cancer, colorectal adenocarcinoma, colorectal cancer, desmoid tumor, embryonal cancer, endometrial cancer, esophageal cancer, gastric cancer, gastric adenocarcinoma, glioblastoma multiforme, gynecological tumor, head and neck squamous cell carcinoma, hepatic cancer, lung cancer, mesothelioma, malignant melanoma, neuroblastoma, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, primary astrocytic tumor, primary thyroid cancer, prostate cancer, renal cancer, renal cell carcinoma, rhabdomyosarcoma, skin cancer, soft tissue sarcoma, testicular germ-cell tumor, urothelial cancer, uterine sarcoma, and uterine cancer. In certain embodiments, the disorder or disease being treated is lung cancer. In certain embodiments, the disorder or disease being treated is malignant melanoma.

In certain embodiments, a method for treating a hyperproliferative disease or disorder is provided, wherein the hyperproliferative disease or disorder is a hematological malignancy selected from acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), chronic eosinophilic leukemia (CEL), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), and multiple myeloma (MM).

In further embodiments, a method for treating a hyperproliferative disease or disorder is provided, wherein the hyperproliferative disease or disorder is a solid cancer selected from biliary cancer, bladder cancer, bone and soft tissue carcinoma, brain tumor, breast cancer, cervical cancer, colon cancer, colorectal adenocarcinoma, colorectal cancer, desmoid tumor, embryonal cancer, endometrial cancer, esophageal cancer, gastric cancer, gastric adenocarcinoma, glioblastoma multiforme, gynecological tumor, head and neck squamous cell carcinoma, hepatic cancer, lung cancer, mesothelioma, malignant melanoma, neuroblastoma, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, primary astrocytic tumor, primary thyroid cancer, prostate cancer, renal cancer, renal cell carcinoma, rhabdomyosarcoma, skin cancer, soft tissue sarcoma, testicular germ-cell tumor, urothelial cancer, uterine sarcoma, and uterine cancer.

In any of the aforementioned embodiments, the composition may comprise a lymphocyte or host cell, and the lymphocyte or host cell is syngeneic, allogeneic, or autologous to the human subject. In some embodiments, the composition may comprise a lymphocyte or host cell, and the lymphocyte or host cell is autologous to the human subject.

Compositions of this disclosure may be administered in a manner appropriate to the disease or condition to be treated (or prevented) as determined by persons skilled in the medical art. An appropriate dose, suitable duration, and frequency of administration of the compositions will be determined by such factors as the condition of the patient, size, type and severity of the disease, particular form of the active ingredient, and the method of administration. In certain embodiments, cells or compositions of the present disclosure may be administered in an initial "priming" dose, followed by one or more "boost" dose in an amount and at a frequency, and for a duration, appropriate to the circumstances. In certain embodiments, a one or both of a priming dose and a boost dose comprises a lymphocyte comprising an exogenous neoantigen and one or more exogenous immunogenicity enhancer (*e.g.*, an mtIL-12 and secreted GM-CSF).

The present disclosure provides pharmaceutical compositions comprising cells expressing a fusion protein as disclosed herein and a pharmaceutically acceptable carrier, diluents, or excipient. Suitable excipients include water, saline, dextrose, glycerol, or the like and combinations thereof.

In any of the aforementioned embodiments, the method may further comprise administering an additional therapeutic agent or adjunctive agent. In certain embodiments, a modified lymphocyte (*e.g.*, T cell) comprising a neoantigen and an adjuvant or a composition thereof is administered simultaneously or sequentially with the additional therapeutic or adjunctive agent.

In certain embodiments, an additional therapeutic agent comprises a chemotherapy; an inhibitor of an immune checkpoint molecule; a costimulatory molecule; a molecule that enhances immunogenicity; a cellular therapy; or a vaccine.

In certain embodiments, the additional therapeutic agent comprises a chemotherapy. Exemplary chemotherapeutic agents include, for example, alkylating agents such as thiotepa and cyclosphosphamide; alkyl sulfonates such as busulfan,

improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, caminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSKTM; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2′,2″-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, *e.g.* paclitaxel (Taxol™, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (Taxotere™, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin;

vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins, capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

In certain embodiments, the additional therapeutic agent comprises an inhibitor of an immune checkpoint molecule. In certain embodiments, the inhibitor of an immune checkpoint molecule comprises an agent that blocks the activity or expression of the immune checkpoint molecule. In some embodiments, the immune checkpoint molecule comprises a PD-1, PD-L1, PD-L2, LAG3, CTLA4, B7-H3, B7-H4, CD244/2B4, HVEM, BTLA, CD160, TIM3, GAL9, KIR, PVR1G (CD112R), PVRL2, adenosine, A2aR, immunosuppressive cytokines (*e.g.*, IL-10, IL-4, IL-1RA, IL-35), IDO, arginase, VISTA, TIGIT, LAIR1, CEACAM-1, CEACAM-3, CEACAM-5, Treg cells, or any combination thereof. . In some embodiments, the agent blocks the activity or expression of a PD-1, PD-L1, or PD-L2. In some embodiments In some embodiments, the agent blocks the activity of the immune checkpoint molecule, and the agent comprises an antagonistic antibody or binding fragment thereof, an aptamer, ribozyme, a dominant-negative inhibitor, or a small molecule inhibitor. In some embodiments, the agent blocks the expression of the immune checkpoint molecule, and the agent is a siRNA, a shRNA, an RNAi, an antisense, a CRISPR/Cas system, a zinc finger nuclease, or a TALEN. In some embodiments, the agent is an antibody that blocks the activity of a PD-1, PD-L1, or PD-L2, comprising an antibody that binds to a PD-1.

In some embodiments, an additional therapeutic agent comprises a costimulatory molecule. In some embodiments, the costimulatory molecule comprises a CD80, a CD86, a B7RP1, a CD137L, an OX40L, a CD27, a CD28, a CD122, a GITR, an ICOS, a CD40, a CD70, a CD30L, a CD154, an ICAM-1, a CD2BP2, a LIGHT, KLRD1, or a ligand that specifically binds to a CD83. In particular embodiments, the costimulatory molecule comprises a CD80. In still further embodiments, the costimulatory molecule comprises a CD137L. In some embodiments, an additional therapeutic agent comprises an agonist of a costimulatory molecule. For example, an

additional therapeutic agent may comprise a CD137 (4-1BB) agonist (such as, for example, urelumab), a CD134 (OX-40) agonist (such as, for example, MEDI6469, MEDI6383, or MEDI0562), lenalidomide, pomalidomide, a CD27 agonist (such as, for example, CDX-1127), a CD28 agonist (such as, for example, TGN1412, CD80, or CD86), a CD40 agonist (such as, for example, CP-870,893, rhuCD40L, or SGN-40), a CD122 agonist (such as, for example, IL-2), an agonist of GITR (such as, for example, humanized monoclonal antibodies described in PCT Patent Publication No. WO 2016/054638), an agonist of ICOS, or any combination thereof.

In certain embodiments, an additional therapeutic agent comprises a molecule that enhances immunogenicity, such as a bacterial flagellin, an IL-12, a GM-CSF, an helper antigen, or any combination thereof. In some embodiments, the bacterial flagellin comprises a *Salmonella* phase 1 flagellin. In other embodiments, a bacterial flagellin, IL-12, GM-CSF, helper antigen, or any combination thereof is combined with an adjuvant.

In certain embodiments, an additional therapeutic agent comprises a cellular therapy, such as an immune cell comprising an engineered TCR, a chimeric antigen receptor (CAR), or both. In further embodiments, an additional therapeutic agent comprises a vaccine, such as a peptide vaccine, DNA vaccine, RNA vaccine, cellular vaccine, or any combination thereof. In still further embodiments, a vaccine is administered with an additional therapeutic agent or an adjunctive agent. Exemplary additional therapeutic agents or adjunctive agents may be comprised of a B-Raf inhibitor, a MEK inhibitor, a tyrosine kinase inhibitor, a cytotoxic agent, alum or an aluminum salt, GM-CSF, gamma inulin, ISCOMs, liposomes, MF59, monophosphoryl lipid A, virosomes and other virus-like particles, or Aquila's QS-21 stimulon, CD80, CD137, CD140L, or secretion of IL-2, IL-2 modified to be CD25 independent in T cell binding (*see* Levin *et al.*, 2012), IL-15, IL-15-IL-15 receptor alpha complex, IFN-B, IFN-A1, IL-7, inducible death switches such as small molecule dimerizable RIPK3, or any combination thereof.

Administration of immune cells, lymphocytes, T cells, and compositions thereof of this disclosure, as well as cell-based additional therapies (*e.g.*, an immune cell comprising an engineered TCR or a chimeric antigen receptor (CAR)), will generally be

performed via injection (*e.g.*, parenteral). The term "parenteral" as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection, or infusion techniques. Administration of other additional therapies or their pharmaceutically acceptable salts, in pure form or in an appropriate pharmaceutical composition, can be carried out using any mode of administration for agents serving similar utilities. For example, immune-enhancing agents of this disclosure can be delivered via injection or using a appropriate pharmaceutically acceptable carrier, diluent or excipient, and may be formulated into preparations in solid, semi solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, gels, microspheres, and aerosols. Exemplary routes of administering such pharmaceutical compositions include oral, topical, transdermal, inhalation, parenteral, sublingual, buccal, rectal, vaginal, and intranasal.

Compositions of this disclosure are formulated to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that will be administered to a subject or patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of a compound of this disclosure in aerosol form may hold a plurality of dosage units. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art (*see*, *e.g.*, Remington: The Science and Practice of Pharmacy, 22nd Edition (Pharmaceutical Press, 2012). The composition to be administered will, in any event, contain a therapeutically effective amount of an immune cell or lymphocyte of this disclosure.

EXAMPLES

EXAMPLE 1

T CELLS EXPRESSING AN EXOGENOUS ANTIGEN CAN PRIME AND BOOST RESPONSES TO THE ANTIGEN *IN VIVO*

A mouse model for investigating vaccination with autologous T cells genetically engineered to express candidate neoantigens ("T_{VAX}") was developed. The model can

be used to study the mechanisms of T_{VAX} action and to evaluate strategies for augmenting vaccine immunity. Ovalbumin ("OVA") was used as a model antigen because reagents are readily available to quantify OVA-specific T cells, and OVA-expressing tumors are widely used to investigate immune mechanisms of tumor eradication (Dranoff, *Nat. Rev. Immunol. 12*: 61, 2012). To determine whether murine T cells transduced to express OVA would be immunogenic, wild-type B6 mice (The Jackson Laboratory, USA) were seeded with a small number of CD45.1 congenically marked naïve OVA-specific OT-1 CD8+ T cells and then vaccinated with one of two dose levels of wild-type T cells expressing full-length OVA (referred to herein as "T_{OVA}" cells), or control T cells expressing GFP. Expansion of antigen specific OT-1 cells *in vivo* was followed with the congenic CD45.1 marker.

Dose-dependent priming of OVA-specific T cells after administration of as few as 200,000 T_{OVA} and efficient boosting of the response to 5-10% of circulating T cells seven days following a second 200,000 T_{OVA} cell dose at day 14 were observed (Figure 2). The T_{OVA} model system allows for T cell responses to be primed and boosted with modest doses of T_{OVA}, and can be used to explore the ability of additional genetic modifications to the T_{OVA} cells to enhance the efficiency of priming, boosting, and memory formation of the T cell vaccine.

EXAMPLE 2

MURINE MODEL FOR DEVELOPING A T CELL-BASED VACCINE

Murine models, such as the T_{OVA} model in Example 1, may be used to elucidate mechanisms underlying the effects of T_{VAX}, develop T cell-based vaccine regimens, and identify additional genetic modifications that potentially enhance the ability of T cells to elicit immune responses to tumor-associated antigens. Examples 3-8 describe a series of experiments using the T_{OVA} mouse model. The following materials and methods were used.

Mice

All mouse experiments were performed in 6-week-old male C57BL/6 mice as described above in Example 1. In some experiments, T cell donors for constructing the

vaccine were obtained from B6 background mice that express chicken ovalbumin under the control of the beta actin promoter (The Jackson Laboratory). OT-I mice transgenic for a TCR specific for the ovalbumin SIINFEKL epitope (used herein as a model neoantigen) were crossed with CD45.1 mice (Both obtained from The Jackson Laboratory) to make mice that were heterozygous for the OT-I transgenic TCR and homozygous for the congenic CD45.1 marker.

DNA Constructs

The coding sequence of an mtIL12 construct (Pan *et al.*, *Mol. Ther. 20*(5):927-937 (2012)) was amplified by PCR and cloned into the NotI-EcoRI sites of the retroviral vector MP71 (*Engels et al.*, *Hum. Gene Ther.* 14(12):1155-1168 (2003)) using the NEBuilder cloning kit (NEB) to make the plasmid pJV1. Synthetic DNA fragments encoding a codon-optimized murine GM-CSF were synthesized (Life Sciences) and cloned into the NotI-EcoRI sites of MP71 to make pJV8. Plasmid pJV99 was made by fusing a linear DNA fragment encoding a truncated form of murine CD19 fused at the N-terminus to a linear sequence encoding the SIINFEKL epitope from chicken ovalbumin and the LLO190 epitope (NEKYAQAYPNVS) of *Listeria monocytogenes* listerolysin O (epitopes connected a glycine-serine linker), and cloning this into the NotI-EcoRI sites of the retroviral vector MP71. Plasmid pJV94 was obtained by replacing the SIINFEKL sequence of pJV72 with sequences containing mutated epitopes in the murine Lama4 and Alg8 genes identified by Robert Schrieber and colleagues (Gubin *et al.*, *Nature 515*(7528):577-581 (2014)). The plasmids, constructs, vectors, and the sequences thereof from the above-noted references and sources are herein incorporated by reference in their entireties.

Gene Modification of T cells

Total T cells from 5-10 week old mouse donors were isolated from spleen using the EasySep™ mouse CD8 purification kit (STEMCELL Technologies, Vancouver, BC, Canada) and stimulated with murine CD3/CD28 dynabeads (Thermo Fisher Scientific (Waltham, MA, USA)) in murine T cell medium supplemented with murine IL-2. Retrovirus was produced by transfecting Plat-E cells (Cell Biolabs, Inc., San Diego, CA, USA) with plasmid retroviral constructs using calcium phosphate transfection, and harvesting viral supernatant on days +2 and +3 from transfection.

Viral supernatant with concentrated by centrifugation onto retronectin, and T cells were transduced with virus on days +1 and +2 of stimulation. Transduction of antigens was monitored by mCD19 expression; membrane tethered IL-12 with anti-IL12 antibody (Biolegend), and GM-CSF by ELISA. Cells were moved to medium supplemented with murine IL-15 on day +3 of stimulation, beads were removed on day +5 of stimulation, and cells were cryopreserved or transferred into mice on day +6 of stimulation. For some experiments, T cells from donors constitutively expressing OVA were co-transduced with viruses containing membrane tethered IL-12 and/or secreted GM-CSF. For other experiments, T cell from wildtype donors were transduced with a combination of viruses containing membrane tethered IL-12 and/or secreted GM-CSF and/or viruses containing antigens.

Vaccination Experiments

In experiments involving OT-I mice, $CD8^+$ cells were isolated using the EasySep kit, and 500 cells were transferred per mouse the day prior to vaccination for vaccination experiments, and 100 cells per mouse for the B16 tumor experiment. 2 x 10^5 vaccine cells are transferred for priming dose, and in tumor experiments this was repeated every 14 days. Boost doses were performed using cryopreserved cells that were rested overnight in medium containing murine IL15 prior to injection.

In experiments with OT-I cells, peripheral blood white blood cells were stained with the congenic marker CD45.1 following erythroid lysis. In some experiments, endogenous T cell responses to antigens were detected by staining with tetramers specific for SIINFEKL, mutated Alg8, mutated Lama4 (obtained from the Fred Hutchinson Cancer Research Center Immune Monitoring Lab) or LLO190 (obtained from the NIH Tetramer Core Facility) following erythroid lysis.

To determine effective prime-boost regimens for vaccination with transgenic T cells, T_{OVA} cells from B6 mice were expanded in culture and administered intravenously to mice in various prime-boost regimens. OVA-specific $CD8^+$ and $CD4^+$ T cell responses are measured using standard assays with congenic markers, and their function was assessed by intracellular cytokine staining. $CD8^+$ T cell responses were examined by transferring 200,000 CD45.1 congenically marked OVA-specific OT-1 T cells into B6 mice prior to vaccination. Expanded OT-1 cells were measured on a

weekly basis in the peripheral blood. To examine CD4+ responses, CD45.1 congenically marked OVA-specific CD4+ OT-II cells were transferred prior to vaccination in a similar way. Groups of mice were vaccinated with different schedules of priming and boosting (every week, every 2 weeks, and monthly) (3 mice per group). The methods were also repeated without OT-1/OT-II T cell transfer in order to assess responses of endogenous T cells.

EXAMPLE 3

EXPRESSION OF IMMUNOGENICITY ENHANCERS IMPROVES TVAX IMMUNE RESPONSE

Without wishing to by bound by theory, T_{VAX} cells of the present disclosure may localize and concentrate neoantigen presentation in, *e.g.*, secondary lymphoid tissues and tumor sites. T cell-based vaccines presenting model TAAs had previously showed some efficacy in a murine melanoma model, but a large number of pre-existing antigen-specific T cells was required for this effect. We investigated whether T_{vax} cells may be modified to increase their immunogenicity and thereby overcome this requirement. In this experiment, mice were injected with 500 transgenic donor $CD45.1^{+/+}$ $TCR^{OT\text{-}1+/-}$ $CD8^+$ T cells. At day +1 following injection, the mice were administered a T-cell vaccine (2×10^5 T_{OVA} cells). Three groups of the T_{OVA} cells further expressed a membrane-tethered IL-12, secreted GM-CSF, or both. Mice were then bled at day +7 and T cells were stained for CD45.1. Figure 5A. Data from this experiment is shown in Figure 5B. Animals injected with T_{OVA} cells expressing either or both immunogenicity enhancers showed significant increases in OT-1 specific $CD8^+$ T cells relative to animals receiving unmodified T_{OVA}. Co-expression of mtIL-12 and secreted GM-CSF produced the strongest optimization effect. These data show that T_{VAX} cells modified to express immunogenicity enhancer molecules such as mtIL-12 and GM-CSF induce robust proliferation and memory formation. Such cells may be useful to achieve therapeutic benefits without the need for high numbers of pre-existing antigen-specific T cells.

Other modifications to enhance T_{VAX} immunogenicity include, for example, expression of an inducible cell death factor. Induction of necroptosis in T cells through,

for example, an inducible RIPK3 can be used to enhance immunogenicity of priming and boosting doses of T_{VAX}. T_{VAX} cells are modified to express an inducible RIPK3, which activates an inflammatory cell death pathway and increases antigen cross-presentation (Yatim *et al.*, *Science 350*(6258):328-334 (2015)). Further, this may be tested in a transgenic mouse that expresses the small molecule-inducible allele of RIPK3 in T cells. Vaccination and expansion is performed as described above to measure the expansion and persistence of OVA-specific $CD8^+$ and $CD4^+$ responses to vaccination with necroptotic cell death induced in the setting of priming, boosting, or both.

Other immunogenicity enhancers, such as a pathogenic membrane protein, which can be a membrane-tethered bacterial flagellin, may also be used in T_{vax} of the present disclosure. Antigen presenting cells can be activated *in vivo* by an innate inflammatory signal to the site of antigen presentation, caused by a membrane-tethered bacterial flagellin. This can be tested in T_{VAX} cells by using a retroviral vector to introduce the membrane-tethered version of bacterial flagellin on the surface of T_{OVA} cells. The resulting T_{VAX} cells are characterized in priming and boosting regimens as above.

Additional approaches for enhancing host immunogenic response against T_{VAX} include, for example, CD80, CD137, CD140L, secreted IL-2, IL-2 modified to be CD25 independent in T cell binding (*see* Levin *et al.*, 2012), IL-15, IL-15-IL-15 receptor alpha complex, IFN-B, IFN-A1, and IL-7.

EXAMPLE 4

CD8 PRIMING BY T_{VAX} CELLS OCCURS BY CROSS-PRESENTATION

T_{VAX} priming or boosting may occur through direct presentation by T_{VAX} or by cross-presentation from host DCs. In order to test whether direct presentation by T_{VAX} occurs, mice were injected with 500 $CD4.5^{+/+}$ $TCROT\text{-}1^{+/-}$ $CD8^+$ T cells, then vaccinated with 2 x 10^5 Class I MHC+ or $b2m^{-/-}$ T_{OVA} cells, bled, and stained for CD45.1 (Figure 6A). As shown in Figure 6B, no significant difference was seen between the groups injected with MHC I^+ and $b2m^{-/-}$ T_{OVA} cells, and percentage of OT-

1-specific $CD8^+$ T cells increased with expression of the immune enhancer molecules. These data show that direct presentation by T_{VAX} is not required for CD8 priming.

EXAMPLE 5

T_{VAX} PRIMES ENDOGENOUS CD8 AND CD4 RESPONSES

Next, we asked whether T_{VAX} could prime endogenous CD8 and CD4 responses. For the CD8 experiment, mice were injected with mock control, T cells expressing mtIL-12 and GM-CSF but no antigen, T cells expressing OVA alone, or T cells expressing the antigen and both immunogenicity enhancer molecules. At day +7 following injection, tetramer staining for OVA-specific $CD8^+$ T cells was performed. For the CD4 experiment, mice were injected with a mock control, T cells expressing the virally transduced CD4 model antigen (LLO190) but no immunogenicity enhancers, or T cells expressing antigen and adjuvant molecules. On day +7, intracellular interferon staining was performed (Figure 7A). As shown in Figure 7B, co-expression of the antigen with immunogenicity enhancer molecules showed significantly higher CD8 tetramer staining than the other tested groups. A similar result was seen in the CD4 experiment, where injection with T cells co-expressing antigen and immunogenicity enhancers resulted in much higher levels of intracellular cytokines (Figure 7C).

EXAMPLE 6

T_{VAX} WITH MTIL12 IS THERAPEUTICALLY EFFECTIVE IN A TRANSPLANTABLE TUMOR MODEL

The effectiveness of Tvax when directed against neoantigens may be tested in a transplantable tumor model. The B16F10 transplantable melanoma model is a heavily mutated tumor. While a transplantable model does not fully address issues of tolerance to chronic antigen exposure, the B16 model is poorly immunogenic and is therefore thought to be a relatively stringent test of immune therapies.

B6F10 cells also expressing ovalbumin ($5x10^5$) were injected into male C57BL/6 mice. The following day, the mice were primed with 2×10^5 T_{OVA} cells expressing mtIL12. Boost injections (same dosage) were administered at days 15 and

29 after tumor injection. Figure 8A. Tumor size (mm^3) was measured at 10, 14, 17, and 29 days following tumor injection, and survival of the animals was followed for 60 days. As shown in Figure 8B, the group receiving T_{OVA}/mtIL12 cells experienced a low level of tumor growth, in sharp contrast to the other tested groups. The T_{OVA}/mtIL12-treated group also had a significantly higher survival rate, with 50% of the animals alive at day 40, while the other groups reached 50% survival before day 30 (Figure 8C). These data show that T_{VAX} including mt12 is therapeutically effective in a transplantable tumor model.

EXAMPLE 7

T_{VAX} INDUCES IMMUNE RESPONSE AGAINST MURINE NEOANTIGENS

T cell responses to model antigens such as OVA are easier to provoke than responses against antigens present in cancer. To evaluate the effectiveness of neoantigen-specific T cells, mtIL12$^+$GM-CSF$^+$ T cells were transduced to express the murine sarcoma neoantigens Lama4 or Alg8 (described in Gubin *et al.*, *Nature 515*(7528): 577-581 (2014)) and, optionally, the CD4 helper antigen LLO190 (Figure 9A). Mice were bled at the indicated days and antigen specific cells were measured as a fraction of the CD8$^+$ T cells in the blood that were tetramer positive. The limit of detection for these tetramers in this experiment from analyzing mock vaccinated animals was 0.1%. N=3 per group. Error bars represent standard error of the mean. At day 13 following injection, tetramer staining was performed. Results are shown in Figure 9B. T cells expressing the adjuvant molecules induced a robust vaccine response against both neoantigens, with the highest level of tetramer staining (as a percentage of CD8$^+$ T cells) seen in the additional presence of the helper antigen.

EXAMPLE 8

TOVA-INDUCED IMMUNE RESPONSE IS ENHANCED BY VACCINE BOOST

Vaccine regimens often consist of an initial priming administration followed by one or more subsequent boost injections. Following on the experiment described in Example 7, mice were primed by intravenous injection of 2 x 10^5 syngeneic T cells transduced with a virus encoding a tandem minigene of alg8 and lama4 neoantigens fused to the LLO190 CD4 antigen, as well as 2 additional viruses encoding membrane tethered IL-12 and secreted GM-CSF. At day +28, mice were boosted by intravenous injection of 4 x 10^5 syngeneic T cells retrovirally transduced with a virus encoding the neoantigens alg8 and lama4. Data are shown in Figure 9C. Mice were bled at the indicated days and antigen-specific cells were measured as a fraction of the $CD8^+$ T cells in the blood that were tetramer positive. The limit of detection for these tetramers in this experiment from analyzing mock vaccinated animals was 0.1%. N=3 per group. Error bars represent standard error of the mean. As shown in Figure 9C, the boost injection significantly increased the immune response.

EXAMPLE 9

TRANSPOSON SYSTEM FOR STABLE INTEGRATION OF EXOGENOUS GENES IN HUMAN T CELLS

To develop a personalized cancer vaccine, a new vaccine construct needs to be created for each patient based on predicted neoantigens in the patient's tumor. As a result, production of a clinical grade retrovirus for T cell genetic modification is not feasible. Therefore, a non-viral gene delivery method for T cell transduction was developed using the *piggyBac* (PB) transposon. In order to adapt the PB system to allow for purification and expansion of antigen-expressing T cells, the construct was designed to also encode a cell surface marker, truncated human CD19 (tCD19), for selection of modified T cells based on CD19 expression.

In preliminary experiments to test feasibility, the tCD19 was translationally linked to a minigene encoding the HLA-A2 restricted CMV pp65 epitope NLV and the resulting construct was flanked by PB transposon repeats. The tCD19-CMV construct

was electroporated into human peripheral blood mononuclear cells (PBMCs), and the cells were stimulated with CD3 and CD28 in the presence of IL-15, according to the methods described in Nakazawa, *et al.* (*J. Immunother.* *32*(8):826-869 (2009)). After 7 days of culture, between 5% and 30% of T cells stably expressed tCD19 (Figure 3A), indicating stable integration of the construct. In order to obtain large numbers of T cells expressing the CMV antigen, modified cells were enriched by CD19 magnetic selection and the enriched cells were expanded using a rapid expansion protocol used to expand therapeutic T cell products, resulting in 100-500-fold expansion over 13 days. Over 80% of the expanded T cells expressed the tCD19 marker (Figure 3B). The resulting cells efficiently presented the NLV antigen, as demonstrated by their ability to activate NLV-specific $CD8^+$ T cells obtained from a CMV positive donor to a level equivalent to that of T cells pulsed with the NLV peptide (Figure 3C). Using this procedure, greater than 10^9 antigen expressing T cells can be obtained in 20 days from less than or equal to 5 x 10^6 PBMCs obtained from a 10cc blood draw, demonstrating the feasibility of this approach for preparing Tvax cells and testing Tvax for the ability to elicit neoantigen-specific T cell responses.

EXAMPLE 10

RNA ELECTROPORATION INTO MONOCYTE-DERIVED DENDRITIC CELLS EFFICIENTLY EXPANDS MEMORY $CD8^+$ AND $CD4^+$ T CELLS FROM PERIPHERAL BLOOD

A successful vaccine trial requires accurate prediction of immunogenic antigens and manufacture of the vaccine, as well as reliable tools for identifying and quantitating antigen-specific T cell responses present prior to vaccination or elicited in response to the vaccine. For a T cell-based neoantigen vaccine, Tvax cells could be used to screen for immunogenic neoantigens; however, their efficiency for direct presentation of class II antigens is lower than that of dendritic cells (DCs) or activated B cells, which directly present antigens on both class I and class II MHC to both $CD4^+$ and $CD8^+$ T cells and express costimulatory molecules to optimally stimulate T cells *in vitro*.

Accordingly, DC expression of electroporated mRNA was used to screen for immunogenic neoantigens. For screening, *in-vitro*-transcribed mRNA encoding

candidate antigens was expressed in autologous monocyte-derived DCs obtained from peripheral blood. Intracellular expression from mRNA has been used to screen large numbers of candidate antigens that have been assembled into minigenes (Tran *et al.*, *Science 344*: 641, 2014), and has the advantage of avoiding experimental artifacts from extracellular peptides that bind MHC but are not processed (Carreno *et al.*, *Science 348*: 803, 2015). As an alternative, primary B cells may be used, and they can be efficiently expanded greater than 100-fold from even small clinical samples through co-culture with CD40L (Liebig *et al.*, *Journal of Visualized Experiments 32*: e1373, 2009).

Viral antigen mRNAs were assembled into minigenes and inserted into DCs, illustrating the feasibility of using large pools of potential antigens to expand rare, antigen-specific cells. An expression construct was created by fusing (a) the T7 promoter, (b) the CMV pp65 gene or a minigene containing CMV epitopes, and (c) sequences targeting the fusion protein to the endocytic pathway to enhance class I and class II MHC presentation (Kreiter, *et al.*, *J Immunol 180*: 309, 2008). Decreasing amounts of mRNA *in-vitro* transcribed from the construct were electroporated into DCs and used to stimulate PBMCs from a CMV-positive donor. Cells responding to the immunodominant CMV pp65 $CD8^+$ epitope were efficiently expanded 10 days after stimulation with as little as 0.01ug of mRNA encoding the antigen in target DCs (Figure 4A). Similarly, CMV pp65 $CD4^+$ responses were efficiently expanded by as little as 0.01ug of an mRNA construct encoding pp65 (Figure 4B). These data show that robust viral memory responses to strong antigens can be expanded, at least in the absence of exhaustion that may characterize cancer-specific T cells. These data demonstrate that expressing small amounts of RNA encoding antigenic epitopes as part of a pool can efficiently expand memory $CD4^+$ and $CD8^+$ responses to antigens in that pool. This system can be used to expand rare neoantigen-reactive T cells that can be found in the peripheral blood in clinical samples of melanoma (Snyder *et al.*, *New Engl. J. Med. Medicine 371*: 2189, 2014) and lung cancer patients (Rizvi *et al.*, *Science 348*: 124, 2015) in order to assess naïve or memory responses to candidate neoantigens predicted by a bioinformatics platform.

EXAMPLE 11

IDENTIFICATION OF CANDIDATE NEOANTIGENS FROM CLINICAL SAMPLES IN NON-SMALL CELL LUNG CANCER (NSCLC) AND MELANOMA

Blood and tumor samples were obtained from non-small cell lung cancer (NSCLC) patients (4) and melanoma patients (2). DNA was isolated from archival fixed tissue, and exome capture was performed using SureSelect (Agilent Technologies) followed by paired-end 100 sequencing. The union of missense mutations from MuTect (Cibulskis *et al.*, *Nature Biotechnology 31*: 213, 2013), VarScan (Koboldt *et al.*, *Bioinformatics 25*: 2283, 2009; Koboldt *et al.*, *Genome Research 22*: 568, 2012), and Strelka (Saunders *et al.*, *Bioinformatics 28*: 1811, 2012) were filtered for expression above 0.5 transcripts per million using the trimmed mean expression of 50 similar tumors from the Cancer Genome Atlas dataset (top and bottom deciles removed). Frame-shift mutations not subject to nonsense-mediated decay were identified using the union of VarScan and Strelka. Using this approach, 265 missense mutations and 8 frame-shift mutations that were not subject to nonsense mediate decay were identified. See Figure 10, top schematic.

The identified mutations were encoded in minigenes such that the amino acid sequences included the 13 flanking amino acids on either side of the mutation to encompass potential $CD8^+$ and $CD4^+$ epitopes. The 27 resulting amino acid minigenes were then assembled into 26 tandem minigene molecules that were synthesized and cloned in parallel into an *in-vitro* transcription construct described above in Example 3, sequence verified, and transcribed into mRNA. To determine whether there were pre-existing neoantigen $CD8^+$ and $CD4^+$ memory T cell responses in the patient, the mRNAs were used to stimulate T cells using transfected autologous DCs as antigen presenting cells. Exemplary data is shown in Figure 10, bottom left and right panels.

In particular, exome capture and cDNA capture were performed on tumor tissue from two melanoma patients and one lung cancer patient who had previously attained complete tumor regression following treatment with adoptively transferred, *in vitro*-expanded tumor infiltrating lymphocytes (TILs). Leukapheresis products were also obtained from the three patients prior to and after TIL infusion, and tumor reactive TIL populations and single cell tumor suspensions were obtained. Exome sequences of the

tumor samples were identified. Candidate-expressed neoantigens were incorporated into *in-vitro* transcription products for DC transfection and analysis of potential neoantigen-reactive T cells.

The results of these experiments are useful in validating candidate neoantigens that can be expressed as minigenes for vaccine construction.

EXAMPLE 12

IDENTIFICATION AND CHARACTERIZATION OF IMMUNOGENIC NEOANTIGENS

Computational analysis of sequence variants was used to predict neoantigen binding to patient-specific MHC alleles and to select candidate neoantigens for incorporation into T_{VAX} cells. Samples were obtained from 4 NSCLC patients and 2 melanoma patients and subjected to genomic analysis. Expressed, protein-coding variants were identified as described above in Example 11. HLA typing was determined from exome data using OptiType (Szolek *et al.*, *Bioinformatics 30*(23): 3310-3316 (2014)) and missense and frame shift mutations were analyzed for formation of novel peptides predicted to bind autologous MHC alleles using NetMHCpan (Trolle *et al.*, *Bioinformatics 31*(13):2174-2181 (2015)).

An assay for detecting rare neoantigen-reactive cells in clinical samples from NSCLC and melanoma patients was developed. Specifically, all expressed protein coding mutations were systematically evaluated in order to determine whether antigens with T cell responses would have been predicted by our bioinformatic workflow. Candidate neoantigens were assembled into tandem minigenes as described above in Example 11. Autologous T cells from the blood were stimulated with autologous DC transfected (mRNA) to express 20-mer peptides from a pool comprising approximately 50 of the top candidate neoantigens in order to expand rare neoantigen-reactive T cell populations. The resulting expanded T cells were then tested for reactivity to specific individual neoantigens by incubation with autologous DC or B cells electroporated with mRNA expressing those neoantigens or pulsed with synthetic peptides, followed by measuring interferon release. In a validation experiment, T_{VAX} presenting an exemplary neoantigen (TERF1) from a lung cancer patient activated cancer-specific T cells from the same patient (Figure 11, bottom right); these data are discussed further in Example

14. TILs are analyzed in the same way. This assay platform can be used for immune monitoring in human studies, as well as for investigating whether T cell responses in patients can be successfully predicted bioinformatically.

A method for determining the number of computationally predicted neoepitopes that can prime autologous naïve T cells *in vitro* is developed. It is likely that only a fraction of potentially immunogenic neoantigens that could respond to vaccination will have pre-existing memory responses in cancer patients. For example, in a published trial, the majority (4 of 7) of neoantigen vaccine responses were not detected prior to vaccination (Carreno *et al.*, *Science 348*(6236):803-808 (2015)). In order to identify antigens without memory responses that are nonetheless capable of being processed, being presented, and eliciting a T cell response, a system for priming naïve T cell responses *in vitro* using antigens expressed in DCs is used (Bleakley *et al.*, *Blood 115*(23):4923-4933 (2010)). Briefly, this system involves expressing pools of mRNA encoding candidate epitopes in autologous DCs, and culturing purified naïve (CD45RA+/CD62L+) T cells in the presence of IL-12 and IL-15, followed by screening of these T cells against antigens expressed in autologous B cells or DCs. By systematically evaluating immunogenic neoantigens in both the memory and naïve T cell repertoire, these experiments will allow for bioinformatic epitope prediction and for rationally determining the number and selection criteria for neoantigens to target with a vaccine.

EXAMPLE 13

METHOD OF TREATING NSCLC

Correlative longitudinal studies of treatment with neoantigen-reactive T cells in NSCLC patients undergoing immune checkpoint molecule inhibitor therapy are conducted. Research core needle biopsies are obtained from NSCLC patients prior to initiation of immune checkpoint blockade therapy. If existing neoantigen-specific T cell responses can be identified in the blood of these patients, the unique T cell receptor beta (TCRB) sequences associated with these antigen specific T cells are identified (Adaptive Biotechnologies). Because global sequencing of TCRB is a highly sensitive and quantitative measure of TCR frequency, this technique is used to enumerate

neoantigen-specific T cells in the tumor and longitudinal blood samples of these patients during immune checkpoint inhibition therapy. Tetramer reagents are made for $CD8^+$ antigen specific T cells, allowing for longitudinal analysis of cell phenotype. The phenotype and localization of neoantigen-specific T cells is then observed during lung cancer immunotherapy. Combined with knowledge of tumor gene expression and the microenvironment, these observations could suggest predictive biomarkers for response, or mechanisms of resistance to treatment.

EXAMPLE 14

ACTIVATION OF NEOANTIGEN-SPECIFIC T CELLS BY AUTOLOGOUS T_{VAX}

In this proof-of-principle experiment, T cells from a human lung cancer patient were modified using the *piggy bac* transposon system to express a neoantigen and used to activate endogenous T cells specific for the neoantigen. The plasmid pJV53 was created by cloning linear fragments encoding an ER targeting signal peptide (SEQ ID NO.: 1) derived from murine Igκ chain with a sequence encoding the TERF1 mutation identified in a human lung cancer patient and the C-terminal domain of human class I MHC linked to a T2A skip sequence and truncated human CD19 into the vector PB713B (System Biosciences, Palo Alto, CA, USA) containing *piggy bac* transposon sequences. The plasmid containing the *piggy bac* transposase pb200pa-1 was obtained from System Biosciences. The method of Nakazawa *et al.* (*J. Immunother.* *32*(8):826 (2009)) was followed to introduce the TERF1 transposon into the cells. Briefly, cryopreserved human PBMC were rested overnight in medium containing 5ng/ml recombinant human IL-15, and then nucleofected with 5ug transposon pJV53 and 5ug transposase pb200pa-1 plasmid DNA in solution V using a 2b nucleofector (Lonza, Basel, SU) according to manufacturer's instructions using program U-014. Cells were rested in CTL with 5ng/ml IL-15 for 24 hours and then stimulated with 3/28 human dynabeads for 7 days in CTL with 5ng/ml IL-15. On day +7 of stimulation, modified cells were enriched using the surface marker truncated CD19 using CD19 microbeads (Miltenyi Bio) according to the manufacturer's instructions, and then expanded by a rapid expansion protocol as described previously. Cells were assayed for antigen presentation at day +21 of rapid expansion.

Next, cryopreserved peripheral blood mononuclear cells were thawed and rested overnight in CTL (RPMI (Gibco) supplemented with 10% human serum, beta-mercaptoethanol, penicillin and streptomycin, and l-glutamine) supplemented with 2ng/ml recombinant human IL-7 (PeproTech). The following morning, cells were washed and stimulated at 10^6 cells in the presence of T_{vax}. Recombinant human IL-2 (PeproTech) was added to a final concentration of 10U/ml on day +3, and half media changes with supplemental IL-2 were performed on days +3, +6, and +9. On day +21, antigen-specific T cells were stained for intracellular cytokines (Miltenyi Biotech) following the manufacturer's instructions, using autologous B cells as antigen presenting cells pulsed with 10ug/ml 21-mer TERF1 mutant peptide. CD4+ IFNγ-secreting cells were sorted on a FACS Aria2. Sorted cells were rested in CTL supplemented with 10ng/ml human IL-15 for 5 days, then expanded using a rapid expansion protocol described previously (Riddell *et al., Science 257*(5067): 238-242 (1993)). Cells were used or cryopreserved at day 13 or 14 of this expansion. Cryopreserved cells were thawed and rested overnight in CTL supplemented with 10ug/ml human IL-2 prior to assays.

As shown in Figure 11 (bottom right panel), vaccination with autologous T_{TERF1} cells produced a robust interferon-γ release by cancer-specific T cells from the patient. These data show that autologous T_{VAX} presenting human neoantigens can induce an immune response in the patient, indicating that personalized T_{VAX} having therapeutic efficacy and reduced risk of immunogenicity can be efficiently developed.

EXAMPLE 15

IDENTIFICATION AND TESTING OF NEOANTIGENS FROM CANCER PATIENTS

Blood and tumor samples were obtained from lung cancer and melanoma cancer patients as described in Example 11. Candidate mutations were identified by whole exome sequencing using the publically available algorithms Mutect and Strelka. Mutations identified by both of these algorithms were filtered by variant allele frequency and ranked by expression from similar tumors in the cancer genome atlas, or

by RNA seq of the tumor when this was available. The top ~46 mutations from each patient were chosen for screening.

PBMC from a given patient were rested overnight in IL-7 and stimulated with 1ug/ml of each candidate (crude) peptide in a mix (~92 peptides encompassing the ~46 candidate mutations per patient sample, corresponding to SEQ ID NOs.: 15-512) with IL-2 being added at day +3. Autologous B cells were isolated using immunomagnetic beads targeting CD19 (Milltenyi) and incubated in a 1:1 ratio with NIH 3T3 cells expressing CD40L for 7 days in B cell medium as described by Tran *et al.* (*Science 344*(6184):641-645 (2014)) supplemented with 200U/ml human IL-4 (PeproTech). B cells were subsequently harvested and re-stimulated with 3T3 cd40L every 4 days. B cells were used as antigen-presenting cells in assays at day +3 of stimulation 2 or 3. On day +14, cells were re-stimulated with pairs of peptides representing individual mutations at 10ug/ml and reactivity was read out by Elispot.

Exemplary data from initial screening of 5 patients is shown in Figures 12A, 12C, and 13A-13C. For patients X198 (melanoma) and MA 511 (lung cancer), 80% pure 27-mer peptides containing the mutant peptide (or wildtype counterpart) were used in the Elispot assay to confirm that the T cell response was to the purified peptide and specific for the mutant. Figures 12B and 12D, respectively. This was the case for 7 mutations identified in patient X198 and 3 mutations identified in patient MA 511.

All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification, and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety. The various embodiments described above can be combined to provide further embodiments. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments. These and other changes can be made to the embodiments in light of the above-detailed description.

In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full

scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

What is claimed is:

1. A T cell, comprising a polynucleotide encoding an exogenous neoantigen associated with a disease or disorder, and a polynucleotide encoding an immunogenicity enhancer.

2. The T cell of claim 1, wherein the neoantigen comprises a tumor neoantigen.

3. The T cell of any one of the preceding claims, wherein the T cell comprises a plurality of neoantigens.

4. The T cell of claim 3, wherein the T cell comprises from two to about 20 neoantigens.

5. The T cell of claim 3 or 4, wherein the T cell comprises from two about 10 neoantigens.

6. The T cell of any one of claims 1-5, wherein the T cell is a naïve, central memory, naïve and central memory, effector memory, or any combination thereof.

7. The T cell of any one of claims 1-6, wherein the T cell is a $CD4^+$T cell, a $CD8^+$ T cell, or both.

8. The T cell of any one of claims 1-7, wherein the T cell is a human T cell.

9. The T cell according to any one of the preceding claims, wherein the immunogenicity enhancer comprises an IL-12, a GM-CSF, an inducible cell death factor, a bacterial flagellin, a CD80, a CD137L, a CD40L, a secreted IL-2, a secreted

IL-2 that binds T cells independent of CD25, a secreted IL-15, a secreted IL-15-IL-15Rα complex, a secreted IFNβ, a secreted IFN-α1, a secreted IL-7, or any combination thereof.

10. The T cell of any one of claims 1-9, wherein the immunogenicity enhancer comprises an IL-12.

11. The T cell of 10, wherein the IL-12 comprises an IL-12 fusion protein that localizes to the cell surface of the T cell.

12. The T cell of claim 11, wherein the IL-12 fusion protein comprises (a) an IL-12 domain; and (b) a transmembrane domain.

13. The T cell of claim 12, wherein the IL-12 fusion protein further comprises (c) a signal domain that directs the fusion protein to a secretory pathway.

14. The T cell of any one of claims 9-13, wherein the immunogenicity enhancer comprises an IL-12 and a GM-CSF.

15. The T cell of any one of claims 9-14, wherein the immunogenicity enhancer comprises an inducible cell death factor.

16. The T cell of claim 15, wherein the inducible cell death factor comprises a receptor interacting serine/threonine kinase 3 (RIPK3).

17. The T cell of claim 15, wherein the inducible cell death factor comprises a fusion protein comprising a cell death signaling domain and a multimerization domain.

18. The T cell of claim 17, wherein the cell death signaling domain comprises a RIPK3 kinase domain and the multimerization domain comprises a FK506-binding protein or multimerizing portion thereof.

19. The T cell of any one of claims 9-18, wherein the immunogenicity enhancer comprises a bacterial flagellin.

20. The T cell of claim 19, wherein the bacterial flagellin comprises a *Salmonella* phase 1 flagellin.

21. The T cell of claim 19 or 20, wherein the bacterial flagellin comprises a bacterial flagellin fusion protein that localizes to the cell surface of the T cell.

22. The T cell of claim 21, wherein the bacterial flagellin fusion protein comprises (a) a bacterial flagellin domain; and (b) a transmembrane domain.

23. The T cell of claim 22, wherein the bacterial flagellin fusion protein further comprises (c) a signal domain that directs the fusion protein to a secretory pathway.

24. The T cell of any one of the preceding claims, further comprising an exogenous costimulatory molecule.

25. The T cell of claim 24, wherein the costimulatory molecule comprises a CD80, a CD86, a B7RP1, a CD137L , an OX40L, a CD70, a CD30L, a CD154, an ICAM-1, a CD2BP2, a LIGHT, a KLRD1, a ligand that specifically binds to a CD83, an agonist of CD137 (4-1BB), an agonist of CD134 (OX-40), an agonist of CD27, an agonist of CD28, an agonist of CD40, an agonist of CD122, an agonist of GITR, an agonist of ICOS, or any combination thereof.

26. The T cell of claim 25, wherein the costimulatory molecule comprises a CD80.

27. The T cell of any one of claims 24-26, wherein the costimulatory molecule comprises a CD137L.

28. The T cell of any one of claims 24-27, wherein the costimulatory molecule localizes to the cell surface of the T cell.

29. An immune cell, comprising an exogenous neoantigen associated with a disease or disorder and an immunogenicity enhancer comprising an IL-12 fusion protein that localizes to the cell surface of the immune cell, wherein the immune cell is selected from a B cell, a natural killer cell, a dendritic cell, a macrophage, a monocyte, a megakaryocyte, a mast cell, a thrombocyte, an erythrocyte, and a granulocyte.

30. The immune cell of claim 29, wherein the IL-12 fusion protein comprises (a) an IL-12 domain; and (b) a transmembrane domain.

31. The immune cell of claim 30, wherein the IL-12 fusion protein further comprises (c) a signal domain that directs the fusion protein to a secretory pathway.

32. The immune cell of any one of claims 30-32, further comprising a second immunogenicity enhancer selected from a GM-CSF, an inducible cell death factor, a bacterial flagellin, a CD80, a CD137L, a CD40L, a secreted IL-2, a secreted IL-2 that binds T cells independent of CD25, a secreted IL-15, a secreted IL-15-IL-15Rα complex, a secreted IFNβ, a secreted IFN-α1, a secreted IL-7, or any combination thereof.

33. A composition, comprising (a) a T cell of any one of claims 1-28 or an immune cell of any one of claims 29-32, or any combination thereof, and (b) a pharmaceutically acceptable carrier, diluent, or excipient.

34. A method for treating a disease or disorder, comprising administering to a human subject in need thereof an effective amount of a T cell of any one of claims 1-28, of an immune cell of any one of claims 29-32, or of a composition of claim 33.

35. A method for treating a human subject having a disease or disorder associated with expression of a neoantigen, comprising administering to the subject an effective amount of a T cell of any one of claims 1-28, of an immune cell of any one of claims 29-32, or of a composition of claim 33.

36. The method of claim 34 or claim 35, wherein relapse or recurrence of the disease or disorder is prevented.

37. The method of any one of claims 34-36, wherein the T cell, the immune cell, or the composition is administered multiple times.

38. The method of claim 37, wherein the time between administrations is selected from 1 week, 2 weeks, 3 weeks, and 1 month.

39. The method of claim any one of claims 34-38, wherein the disease or disorder is a viral infection, bacterial infection, hyperproliferative disorder, or autoimmune disease.

40. The method of claim 39, wherein the disease or disorder is a hyperproliferative disease or disorder.

41. The method of claim 40, wherein the hyperproliferative disease or disorder is a hematological malignancy or a solid cancer.

42. The method of claim 41, wherein the hyperproliferative disease or disorder is a hematological malignancy selected from acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML),

chronic eosinophilic leukemia (CEL), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), and multiple myeloma (MM).

43. The method of claim 41, wherein the hyperproliferative disease or disorder is a solid cancer selected from biliary cancer, bladder cancer, bone and soft tissue carcinoma, brain tumor, breast cancer, cervical cancer, colon cancer, colorectal adenocarcinoma, colorectal cancer, desmoid tumor, embryonal cancer, endometrial cancer, esophageal cancer, gastric cancer, gastric adenocarcinoma, glioblastoma multiforme, gynecological tumor, head and neck squamous cell carcinoma, hepatic cancer, lung cancer, mesothelioma, malignant melanoma, neuroblastoma, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic ductal adenocarcinoma, primary astrocytic tumor, primary thyroid cancer, prostate cancer, renal cancer, renal cell carcinoma, rhabdomyosarcoma, skin cancer, soft tissue sarcoma, testicular germ-cell tumor, urothelial cancer, uterine sarcoma, and uterine cancer.

44. The method of claim 43, wherein the hyperproliferative disease or disorder is lung cancer.

45. The method of claim 43, wherein the hyperproliferative disease or disorder is malignant melanoma.

46. The method of any one of claims 34-45, wherein the T cell or the immune cell is syngeneic, allogeneic, or autologous to the human subject.

47. The method of claim 46, wherein the T cell or the immune cell is autologous to the human subject.

48. The method of any one of claims 34-47, further comprising administering an additional adjunctive therapeutic agent.

49. The method of claim 48, wherein the lymphocyte or composition is administered simultaneously or sequentially with the adjunctive therapeutic agent.

50. The method of claim 48 or 49, wherein the adjunctive therapeutic agent comprises a chemotherapy; an inhibitor of an immune checkpoint molecule; a costimulatory molecule; a molecule that enhances immunogenicity; a cellular therapy; or a vaccine.

51. The method of any one of claims 48-50, wherein the adjunctive therapeutic agent comprises a chemotherapy.

52. The method of any one of claims 48-51, wherein the adjunctive therapeutic agent comprises an inhibitor of an immune checkpoint molecule.

53. The method of claim 52, wherein the inhibitor of an immune checkpoint molecule comprises an agent that blocks activity or expression of a PD-1, a PD-L1, or a PD-L2 a LAG3, a CTLA4, a B7-H3, a B7-H4, a CD244/2B4, a HVEM, a BTLA, a CD160, a TIM3, a GAL9, a KIR, a PVR1G (CD112R), a PVRL2, an adenosine, A2aR, an immunosuppressive cytokine, an IDO, an arginase, a VISTA, a TIGIT, a LAIR1, a CEACAM-1, a CEACAM-3, a CEACAM-5, a Treg cell, or any combination thereof.

54. The method of claim 53, wherein the agent that blocks the activity or expression of a PD-1, a PD-L1, or a PD-L2 comprises an antibody that binds to a PD-1.

55. The method of any one of claims 48-54, wherein the adjunctive therapeutic agent comprises a costimulatory molecule.

56. The method of claim 55, wherein the costimulatory molecule comprises a CD80, a CD86, a B7RP1, a CD137L , an OX40L, a CD70, a CD30L, a CD154, an ICAM-1, a CD2BP2, a LIGHT, a KLRD1, a ligand that specifically binds to a CD83, an agonist of CD137 (4-1BB), an agonist of CD134 (OX-40), an agonist of CD27, an

agonist of CD28, an agonist of CD40, an agonist of CD122, an agonist of GITR, an agonist of ICOS, or any combination thereof.

57. The method of claim 56, wherein the costimulatory molecule comprises a CD80.

58. The method of claim 56 or 57, wherein the costimulatory molecule comprises a CD137L.

59. The method of any one of claims 48-58, wherein the adjunctive therapeutic agent comprises a vaccine.

60. The method of any one of claims 34-59, further comprising administering an adjuvant.

61. The method of claim 60, wherein the adjuvant comprises alum or an aluminum salt, GM-CSF, gamma inulin, ISCOMs, liposomes, MF59, monophosphoryl lipid A, virosomes and other virus-like particles, or Aquila's QS-21 stimulon.

62. A transposon expression construct, comprising a nucleic acid molecule encoding a neoantigen.

63. The transposon expression construct of claim 62, comprising (a) a promoter; (b) a first *piggyBac* transposon inverted repeat; (b) the nucleic acid molecule encoding the neoantigen; and (c) a second *piggyBac* transposon inverted repeat, wherein the nucleic acid molecule that encodes the neoantigen is positioned between the first *piggyBac* transposon inverted repeat and the second *piggyBac* transposon inverted repeat.

64. The transposon expression construct of claim 62 or 63, wherein the transposon expression construct is present in a plasmid.

65. The transposon expression construct of any one of claims 62-64, further comprising a nucleic acid molecule encoding a cell surface marker.

66. The transposon expression construct of claim 65, wherein the cell surface marker comprises a truncated human CD19, a truncated human EGFR, a truncated human NGFR, a truncated human CD34, or any combination thereof.

67. The transposon expression construct of any one of claims 62-66, further comprising a nucleic acid molecule encoding an immunogenicity enhancer selected from an IL-12, a GM-CSF, an inducible cell death factor, a bacterial flagellin, a CD80, a CD137L, a CD40L, a secreted IL-2, a secreted IL-2 that binds T cells independent of CD25, a secreted IL-15, a secreted IL-15-IL-15 receptor alpha complex, a secreted IFNβ, a secreted IFN-α1, a secreted IL-7, or any combination thereof.

68. A kit, comprising the transposon expression construct of any one of claims 62-67.

69. A host cell, comprising a transposon expression construct of any one of claims 62-67.

70. The host cell of claim 69, further comprising a *piggyBac* transposase enzyme expression construct, wherein the *piggyBac* transposase enzyme construct comprises a nucleic acid molecule encoding a *piggyBac* transposase.

71. The host cell of claim 69 or 70, wherein the host cell is an immune system cell.

72. The host cell of claim 71, wherein the immune system cell is a dendritic cell, a T cell, a B cell, a natural killer cell, a macrophage, a monocyte, a megakaryocyte, a mast cell, a thrombocyte, an erythrocyte, or a granulocyte.

73. The host cell of claim 72, wherein the immune system cell is a T cell.

74. The host cell of claim 73, wherein the T cell is naïve, central memory, naïve and central memory, effector memory, or any combination thereof.

75. The host cell of claim 73 or 74, wherein the T cell is a $CD4^+$T cell, a $CD8^+$ T cell, or both.

76. The host cell of any one of claims 69-75, wherein the host cell is a human cell.

77. The host cell of any one of claims 69-76, further comprising a viral vector.

78. The host cell of claim 77, wherein the viral vector is a retroviral vector.

79. The host cell of claim 80, wherein the viral vector is a lentiviral vector.

80. The host cell of any one of claims 77-79, wherein the viral vector comprises a nucleic acid molecule encoding an immunogenicity enhancer selected from an IL-12, a GM-CSF, an inducible cell death factor, a bacterial flagellin, a CD80, a CD137L, a CD40L, a secreted IL-2, a secreted IL-2 that binds T cells independent of CD25, a secreted IL-15, a secreted IL-15-IL-15 receptor alpha complex, a secreted IFNβ, a secreted IFN-α1, a secreted IL-7, or any combination thereof.

81. The host cell of claim 80, wherein the viral vector comprises (a) a nucleic acid molecule encoding an IL-12 comprised in a fusion protein that localizes to the cell surface of the host cell and (b) a nucleic acid molecule that encodes a GM-CSF.

82. The host cell of any one of claims 77-81, wherein the viral vector comprises a nucleic acid molecule encoding a costimulatory molecule.

83. The host cell of claim 82, wherein the nucleic acid molecule encodes a costimulatory molecule comprising CD80, a CD86, a B7RP1, a CD137L , an OX40L, a CD70, a CD30L, a CD154, an ICAM-1, a CD2BP2, a LIGHT, a KLRD1, a ligand that specifically binds to a CD83, an agonist of CD137 (4-1BB), an agonist of CD134 (OX-40), an agonist of CD27, an agonist of CD28, an agonist of CD40, an agonist of CD122, an agonist of GITR, an agonist of ICOS, or any combination thereof.

84. A composition, comprising a host cell of any one of claims 69-83 and a pharmaceutically acceptable carrier, diluent, or excipient.

85. A method of preparing a T cell of any one of claims 1-28, comprising introducing into the T cell (a) a *piggyBac* transposon plasmid containing a nucleic acid molecule encoding a neoantigen identified in a sample of a subject; and (b) a plasmid comprising a nucleic acid molecule encoding a *piggyBac* transposase, thereby preparing the T cell.

86. The method of preparing a T cell of claim 85, further comprising identifying the neoantigen using whole exome or RNA sequencing of the sample; and inserting the nucleic acid molecule encoding the neoantigen into the *piggyBac* transposon plasmid.

87. The method of claim 85 or 86, wherein the nucleic acid molecule encoding a neoantigen comprises a tandem minigene.

88. The method of claim 87, wherein the tandem minigene comprises from two to about 20 neoantigens.

89. The method of claim 87 or 88, wherein the tandem minigene comprises from two to about 10 neoantigens.

90. The method of any one of claims 85-89, wherein the T cell is syngeneic, allogeneic, or autologous to the subject.

91. The method of claim 90, wherein the T cell is autologous to the subject.

92. The method of any one of claims 85-91, wherein the *piggyBac* transposon plasmid and the plasmid comprising a nucleic acid molecule encoding a *piggyBac* transposase are introduced into the T cell *ex vivo*.

93. The method of any one of claims 85-92, further comprising introducing into the T cell a plasmid comprising a nucleic acid molecule encoding an immunogenicity enhancer.

94. The method of claim 93, wherein the nucleic acid molecule encoding an immunogenicity enhancer is comprised in the *piggyBac* transposon plasmid.

95. A method of preparing an immune cell of any one of claims 29-32, comprising introducing into the immune cell (a) a *piggyBac* transposon plasmid containing a nucleic acid molecule encoding a neoantigen identified in a sample of a subject, (b) a plasmid comprising a nucleic acid molecule encoding a *piggyBac* transposase, and (c) a nucleic acid molecule encoding an IL-12 fusion protein that localizes to the cell surface of the T cell, thereby preparing the immune cell.

96. The method of claim 95, wherein the IL-12 fusion protein encoded by the nucleic acid molecule of (c) comprises an IL-12 domain and a transmembrane domain.

97. The method of claim 96, wherein the IL-12 fusion protein encoded by the nucleic acid molecule of (c) further comprises a signal domain that directs the fusion protein to a secretory pathway.

98. The method of any one of claims 95-97, wherein the nucleic acid molecule encoding an IL-12 fusion protein is comprised in a plasmid.

99. The method of any one of claims 95-98, wherein the nucleic acid molecule encoding an IL-12 fusion protein is comprised in the *piggyBac* transposon plasmid of (a).

100. The method of any of claims 95-99, further comprising identifying the neoantigen using whole exome or RNA sequencing of the sample; and inserting the nucleic acid molecule encoding the neoantigen into the *piggyBac* transposon plasmid.

101. The method of any of claims 95-100, wherein the nucleic acid molecule encoding a neoantigen comprises a tandem minigene.

102. The method of claim 101, wherein the tandem minigene comprises from two to about 20 neoantigens.

103. The method of claim 101 or 102, wherein the tandem minigene comprises from two to about 10 neoantigens.

104. The method of any one of claims 95-103, wherein the immune cell is syngeneic, allogeneic, or autologous to the subject.

105. The method of claim 104, wherein the immune cell is autologous to the subject.

106. The method of any one of claims 95-105, wherein the *piggyBac* transposon plasmid and the plasmid comprising a nucleic acid molecule encoding a *piggyBac* transposase are introduced into the immune cell *ex vivo*.

107. The method of any one of claims 95-106, further comprising introducing into the immune cell a plasmid comprising a nucleic acid molecule encoding a second immunogenicity enhancer.

108. The method of claim 107, wherein the second immunogenicity enhancer comprises GM-CSF.

109. The method of claim 107 or 108, wherein the nucleic acid molecule encoding a second immunogenicity enhancer is comprised in the *piggyBac* transposon plasmid containing a nucleic acid molecule encoding a neoantigen.

110. The method of claim 109, wherein the plasmid comprising a nucleic acid molecule encoding a GM-CSF is introduced into the immune cell *ex vivo*.

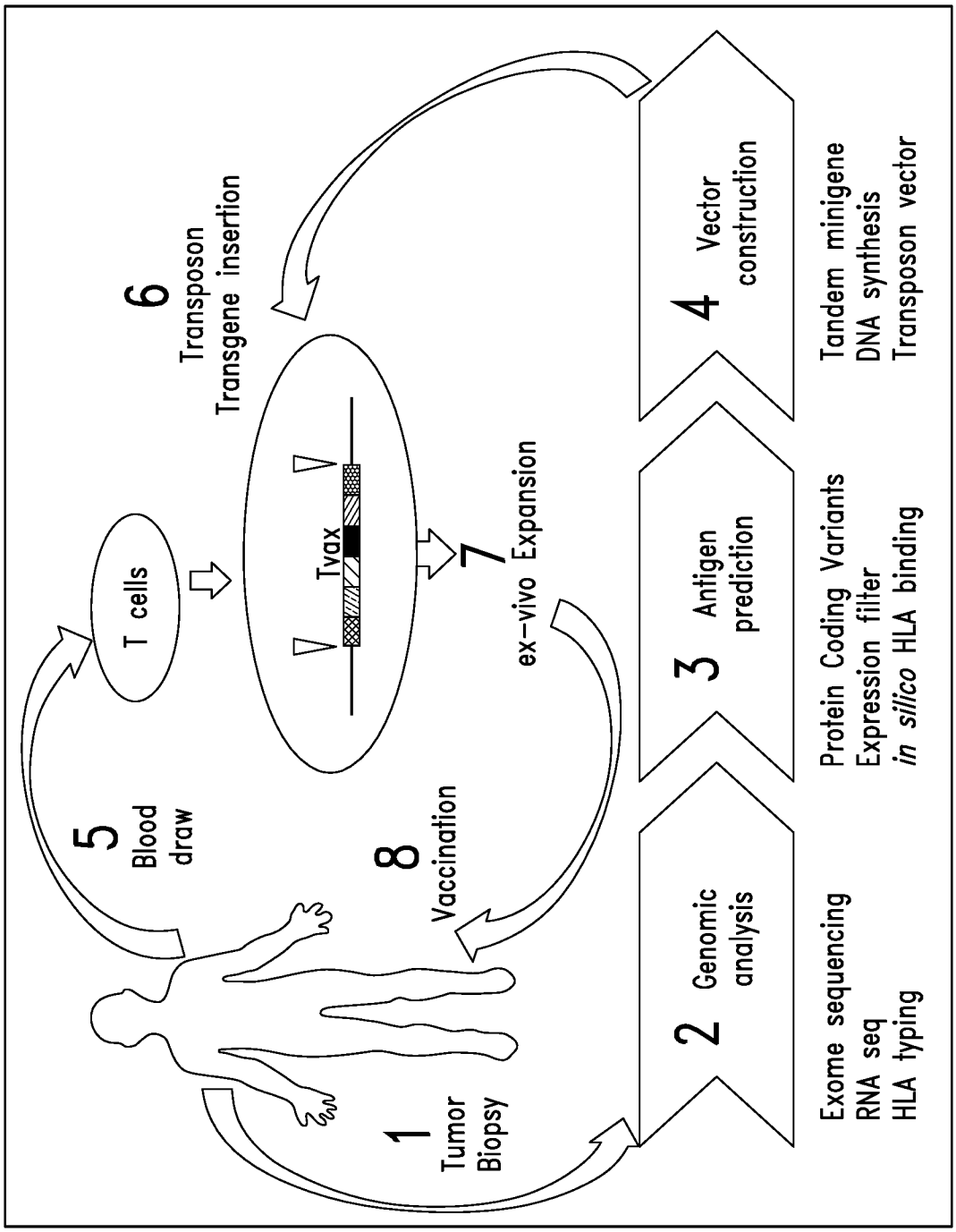
1 Tumor Biopsy
Exome sequencing
RNA seq
HLA typing
2 Genomic analysis
3 Antigen prediction
Protein Coding Variants
Expression filter
in silico HLA binding
4 Vector construction
Tandem minigene
DNA synthesis
Transposon vector
5 Blood draw
T cells
6 Transposon Transgene insertion
Tvax
7 ex-vivo Expansion
8 Vaccination

FIG. 1

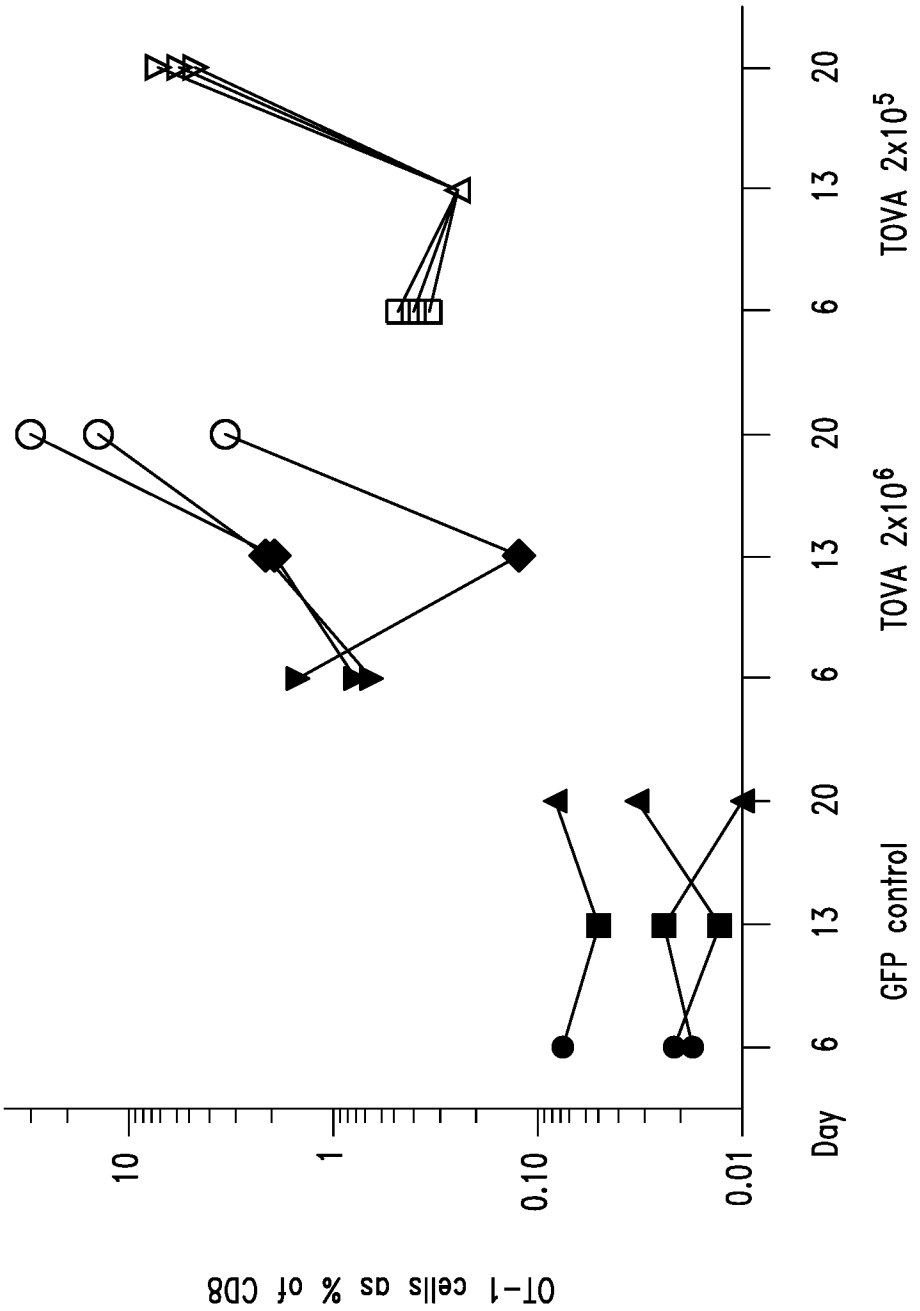
OT-1 cells as % of CD8
10
1
0.10
0.01
Day
6
13
20
6
13
20
6
13
20
GFP control
TOVA 2x10^6
TOVA 2x10^5

FIG. 2

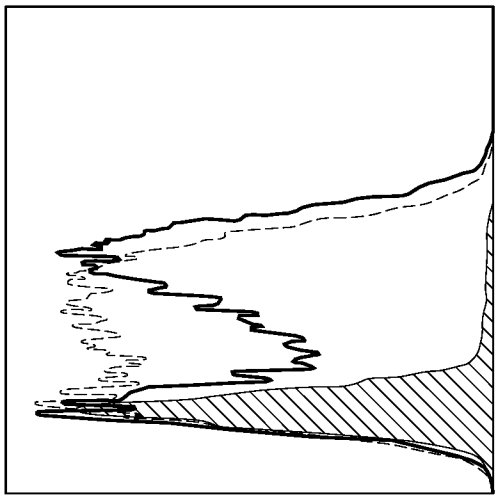

FIG. 3C

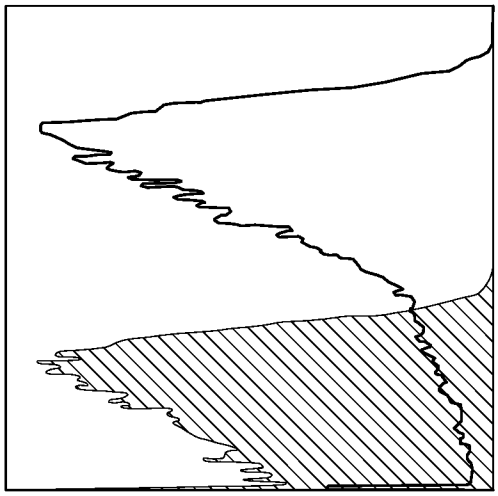

FIG. 3B

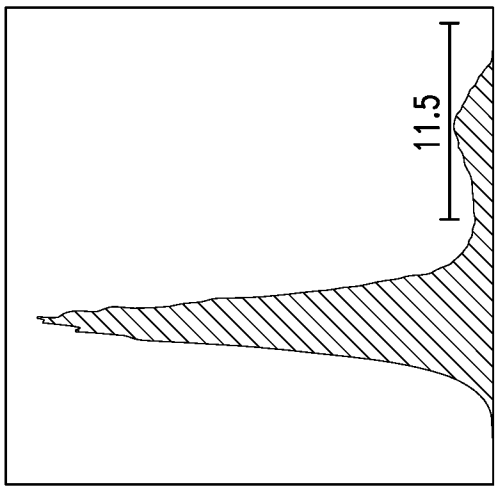

FIG. 3A

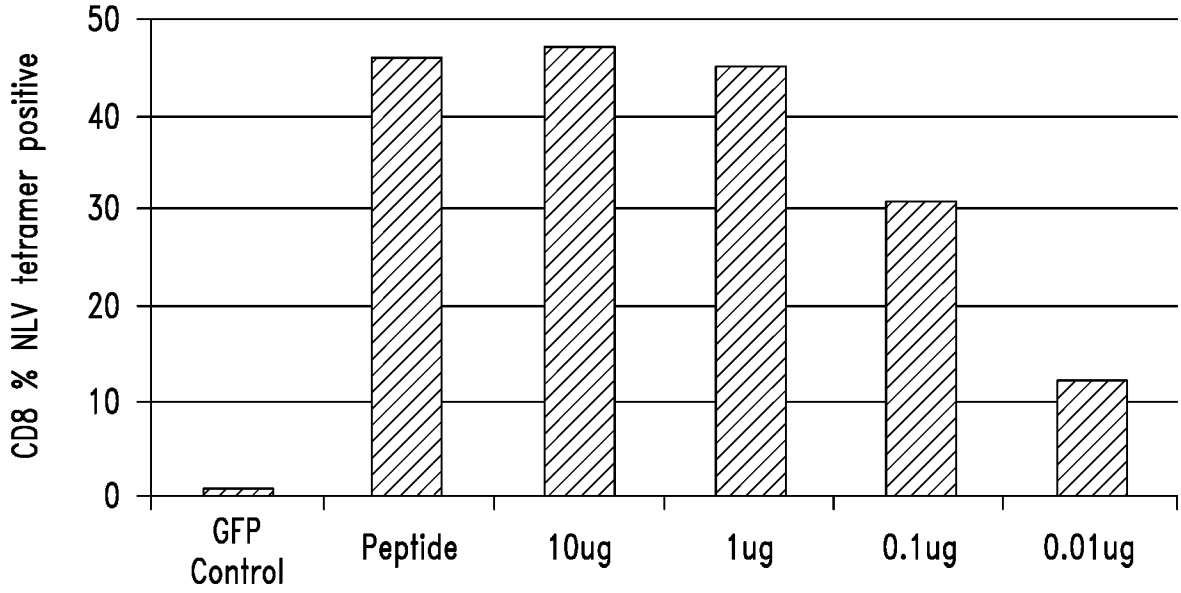
CD8 % NLV tetramer positive
50
40
30
20
10
0
GFP Control
Peptide
10ug
1ug
0.1ug
0.01ug

FIG. 4A

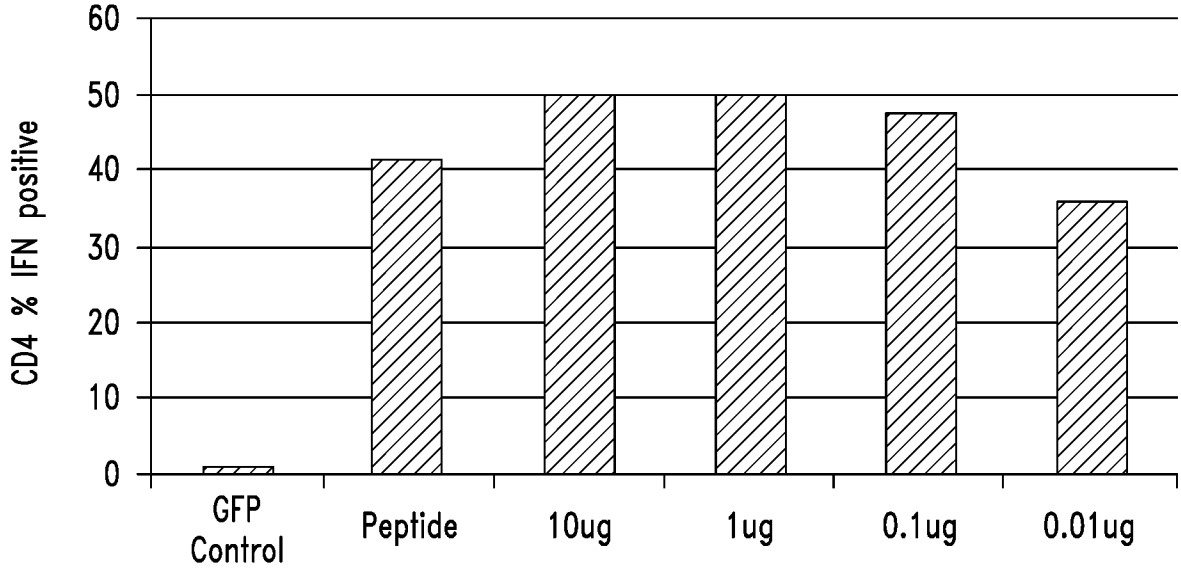
CD4 % IFN positive
60
50
40
30
20
10
0
GFP Control
Peptide
10ug
1ug
0.1ug
0.01ug

FIG. 4B

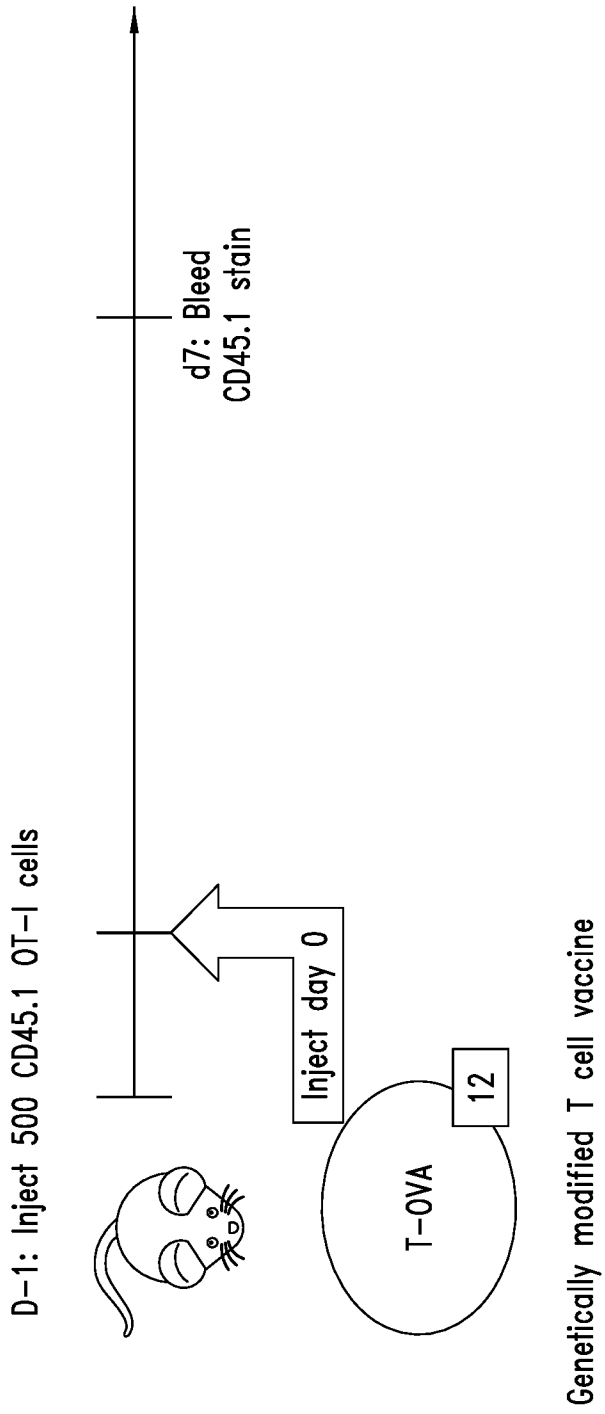
D-1: Inject 500 CD45.1 OT-I cells
Inject day 0
T-OVA
12
d7: Bleed
CD45.1 stain
Genetically modified T cell vaccine
+/- membrane-tethered IL-12
+/- secreted GM-CSF

FIG. 5A

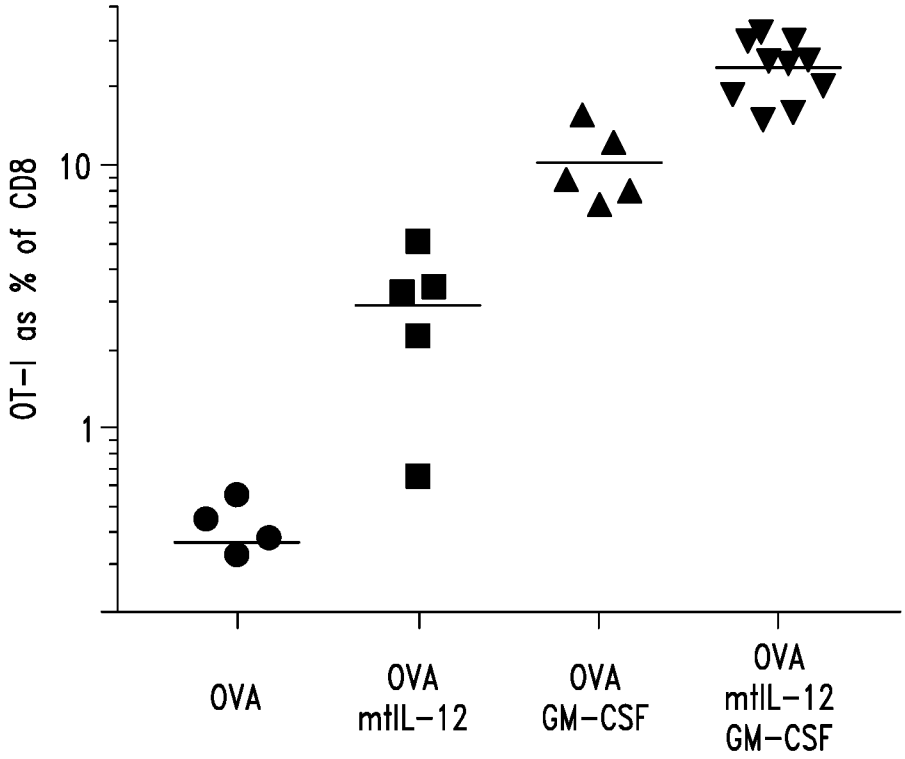

FIG. 5B

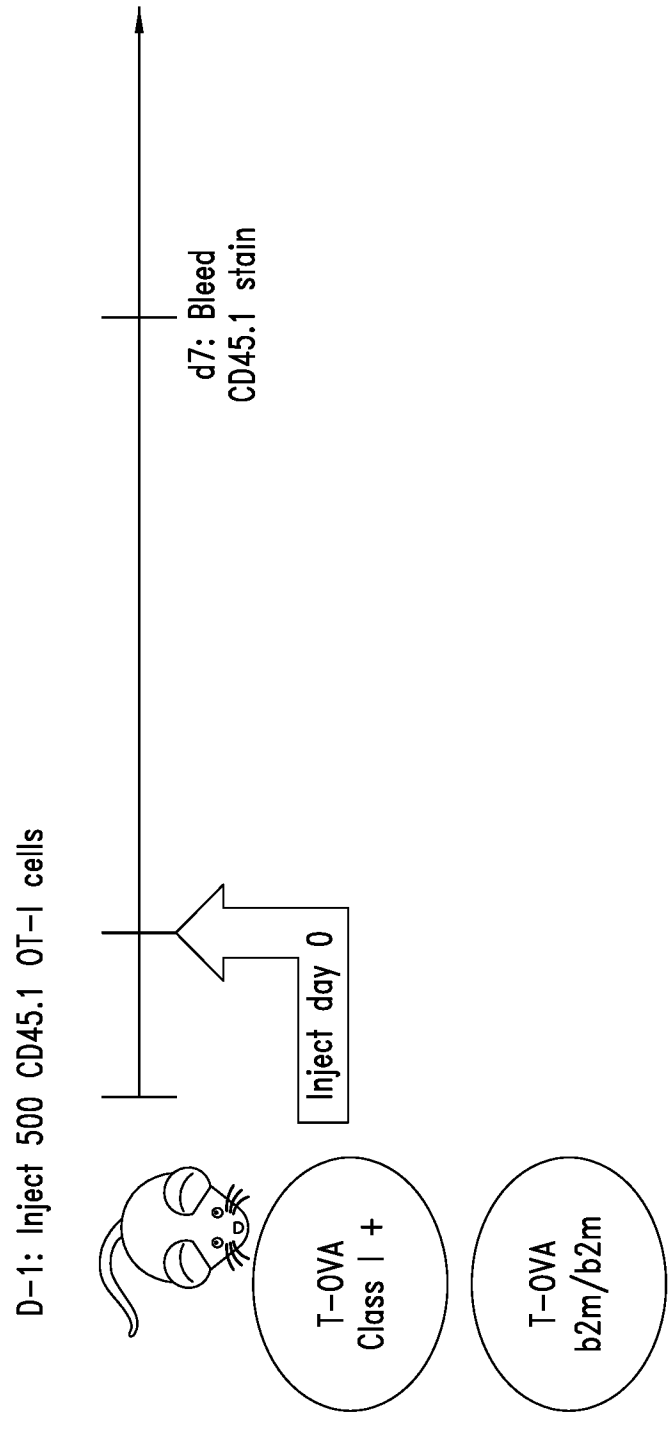
D-1: Inject 500 CD45.1 OT-I cells
Inject day 0
d7: Bleed
CD45.1 stain
T-OVA
Class I +
T-OVA
b2m/b2m
Genetically modified T cell
vaccine with or without
class I MHC expression

FIG. 6A

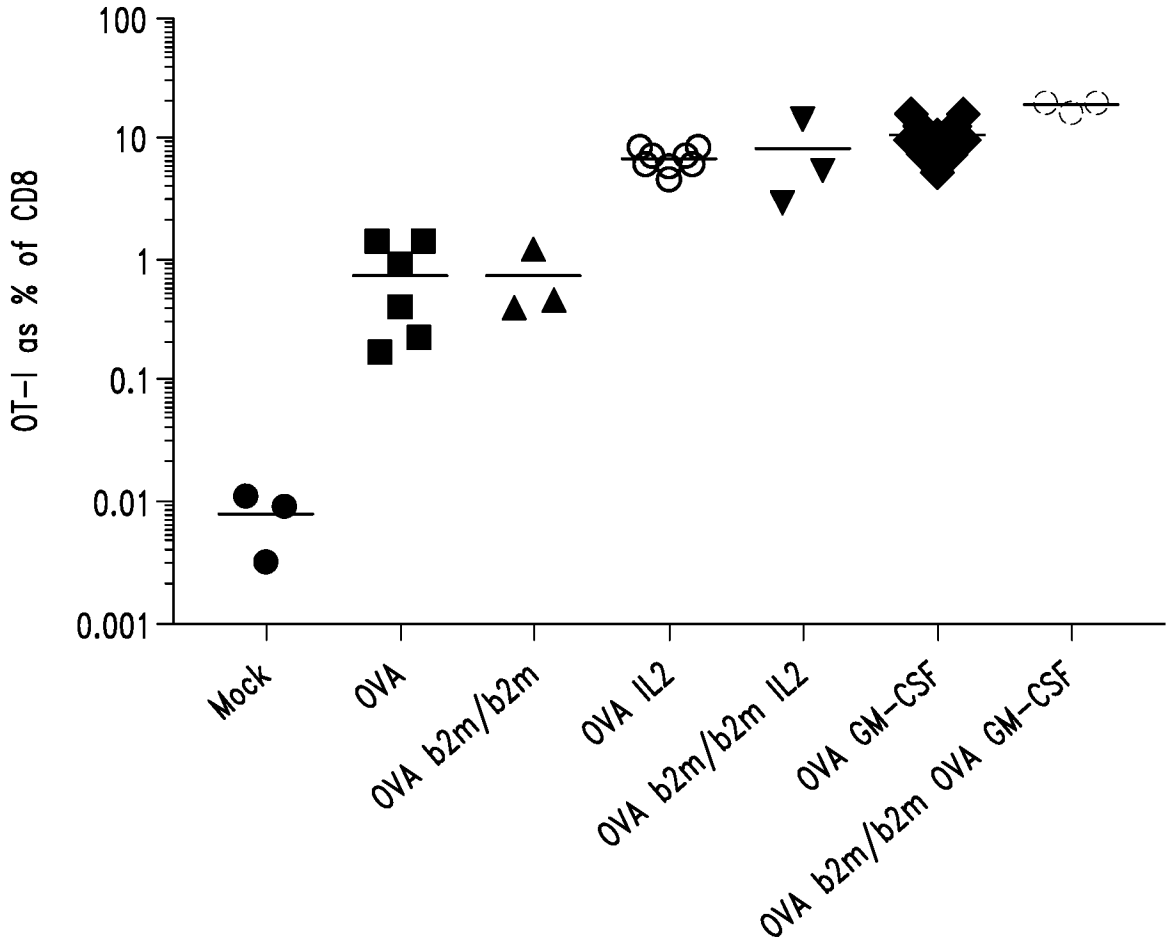
100
10
1
0.1
0.01
0.001
OT-I as % of CD8
Mock
OVA
OVA b2m/b2m
OVA IL2
OVA b2m/b2m IL2
OVA GM-CSF
OVA b2m/b2m OVA GM-CSF

FIG. 6B

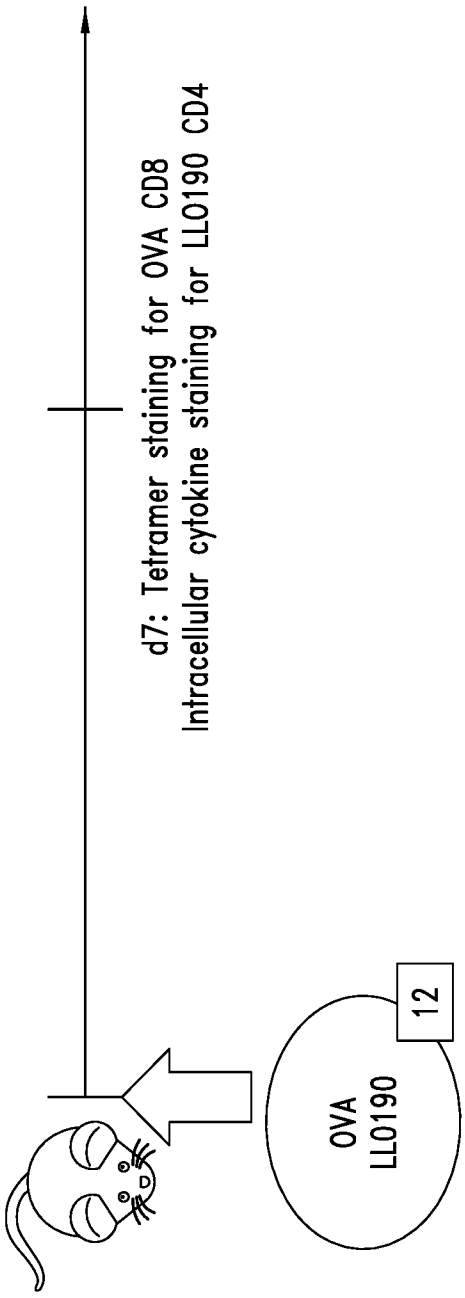
d7: Tetramer staining for OVA CD8
Intracellular cytokine staining for LL0190 CD4
OVA
LL0190
12
Genetically modified T cell vaccine
expressing OVA and LL0190 antigens
+/- localized membrane tethered
IL-12 and secreted GM-CSF

FIG. 7A

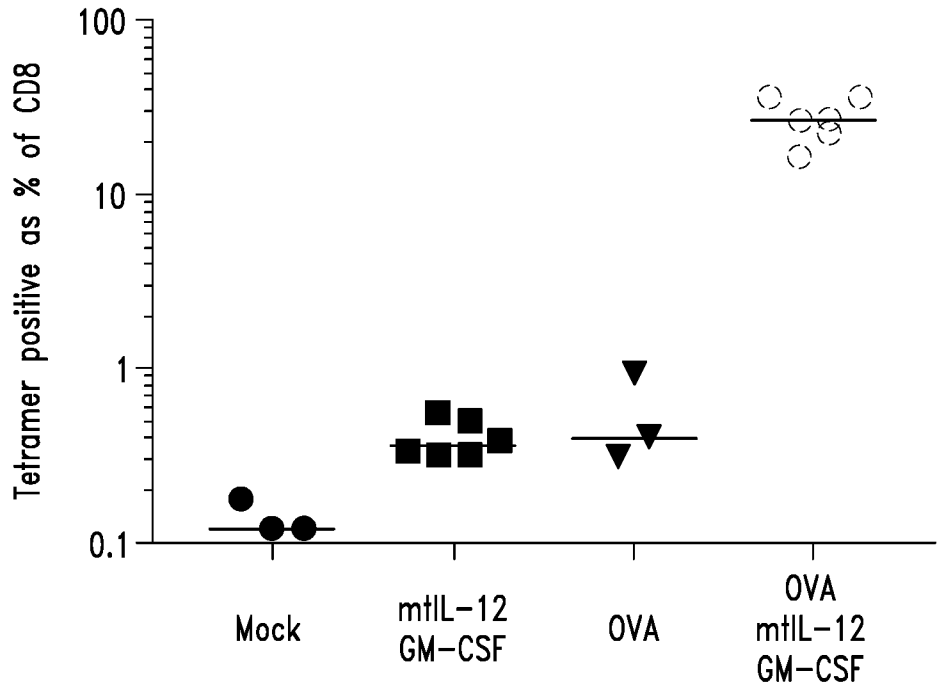
100
10
1
0.1
Tetramer positive as % of CD8
Mock
mtIL-12
GM-CSF
OVA
OVA
mtIL-12
GM-CSF

FIG. 7B

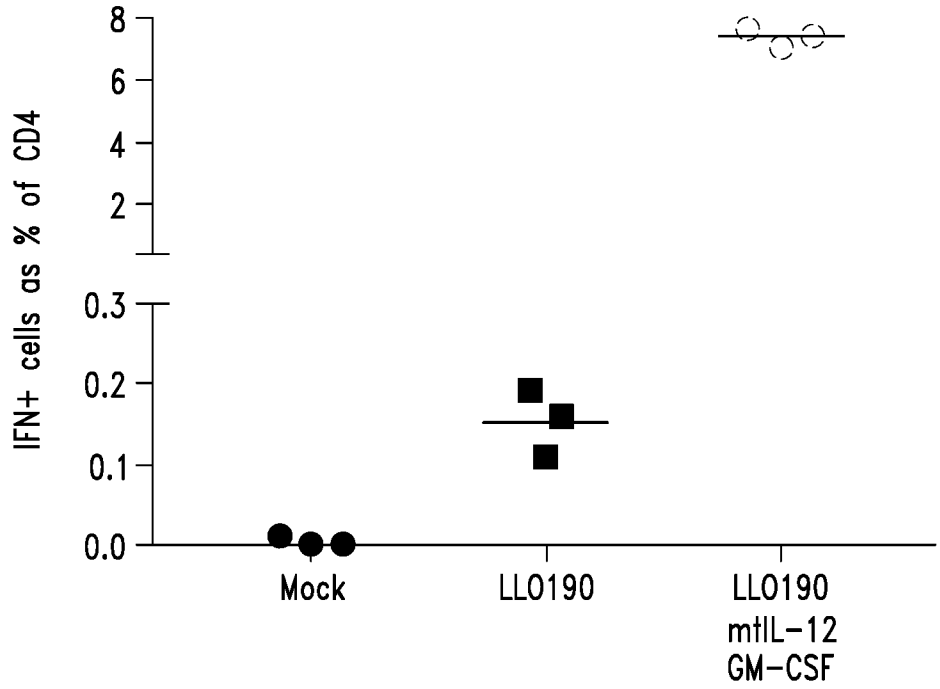
IFN+ cells as % of CD4
8
6
4
2
0.3
0.2
0.1
0.0
Mock
LLO190
LLO190
mtIL-12
GM-CSF

FIG. 7C

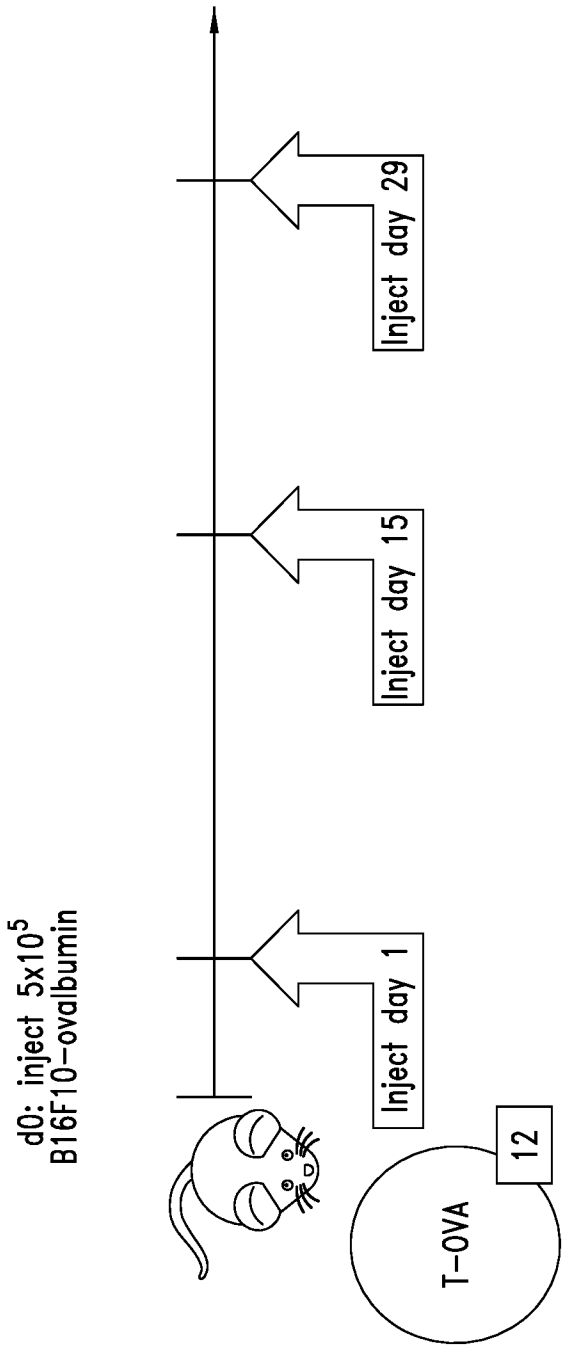
d0: inject 5x10^5
B16F10-ovalbumin
Inject day 1
Inject day 15
Inject day 29
T-OVA
12

FIG. 8A

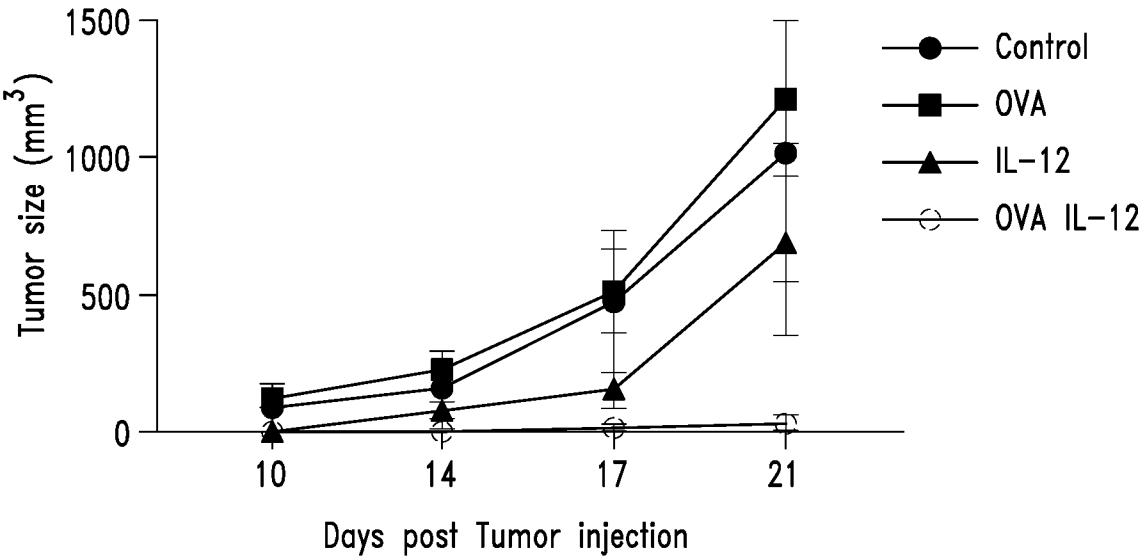
1500
1000
500
0
Tumor size (mm3)
10
14
17
21
Days post Tumor injection
Control
OVA
IL-12
OVA IL-12

FIG. 8B

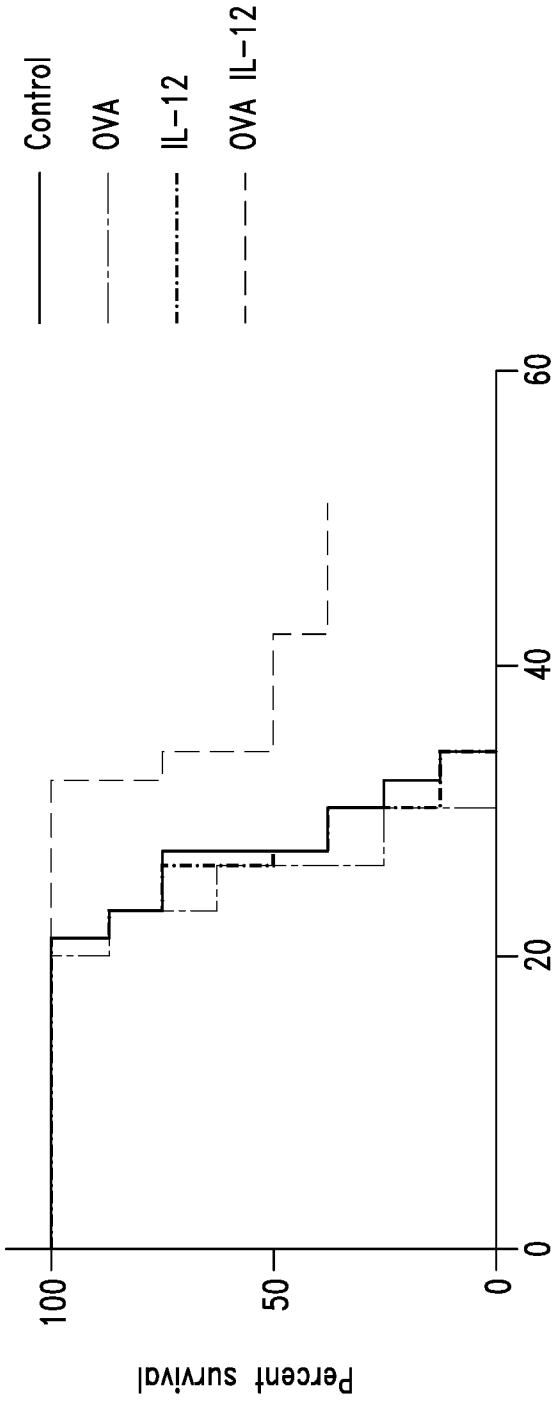
Control
OVA
IL-12
OVA IL-12
Percent survival
100
50
0
0
20
40
60
Days

FIG. 8C

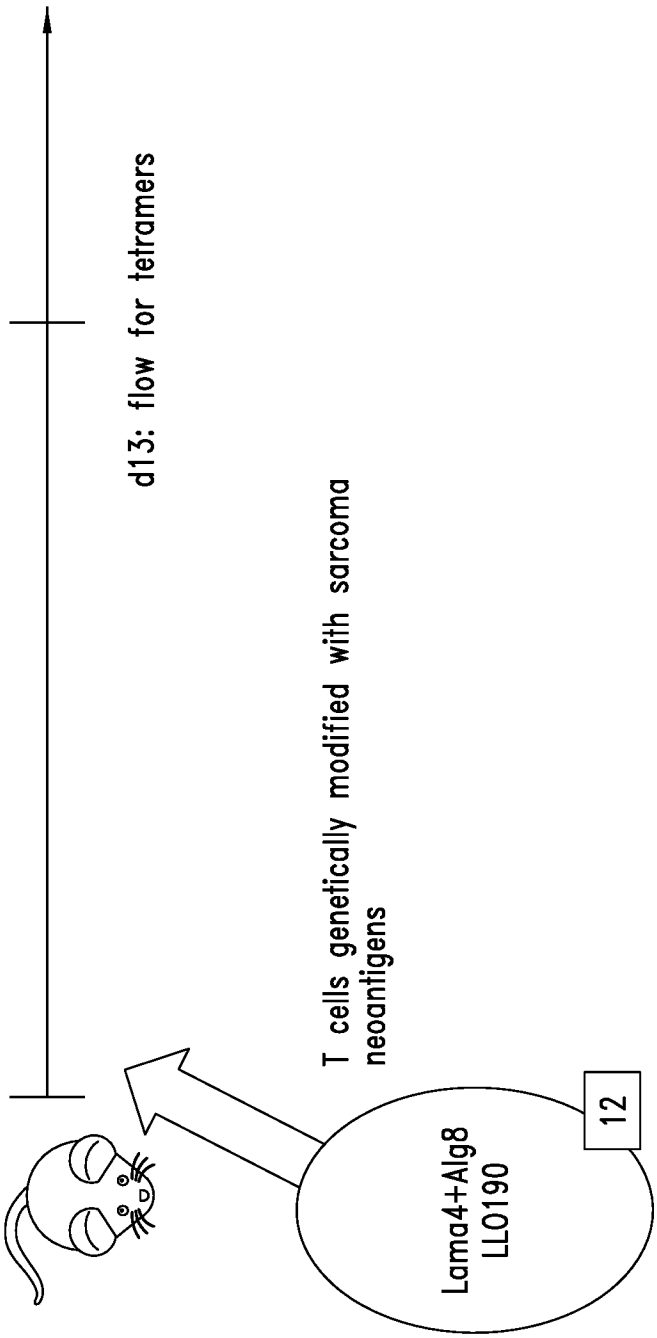
d13: flow for tetramers
T cells genetically modified with sarcoma neoantigens
Lama4+Alg8
LL0190
12

FIG. 9A

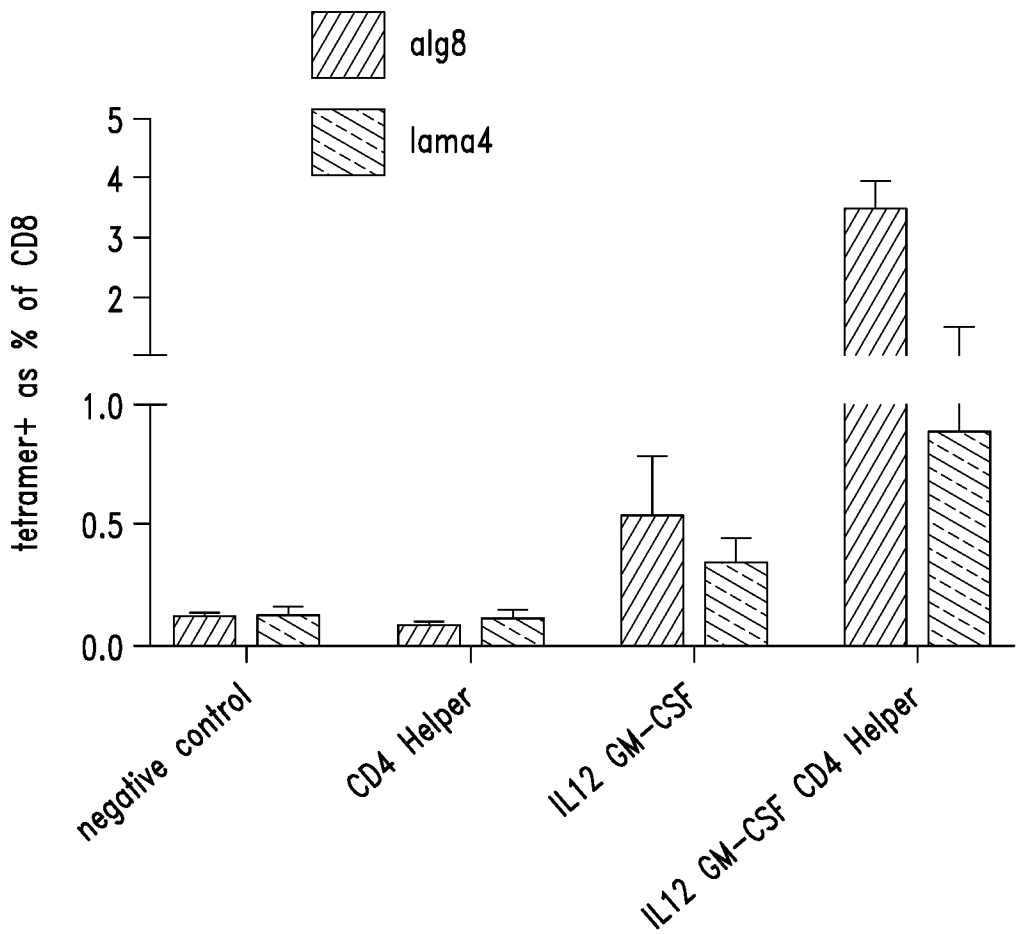
alg8
lama4
tetramer+ as % of CD8
5
4
3
2
1.0
0.5
0.0
negative control
CD4 Helper
IL12 GM-CSF
IL12 GM-CSF CD4 Helper

FIG. 9B

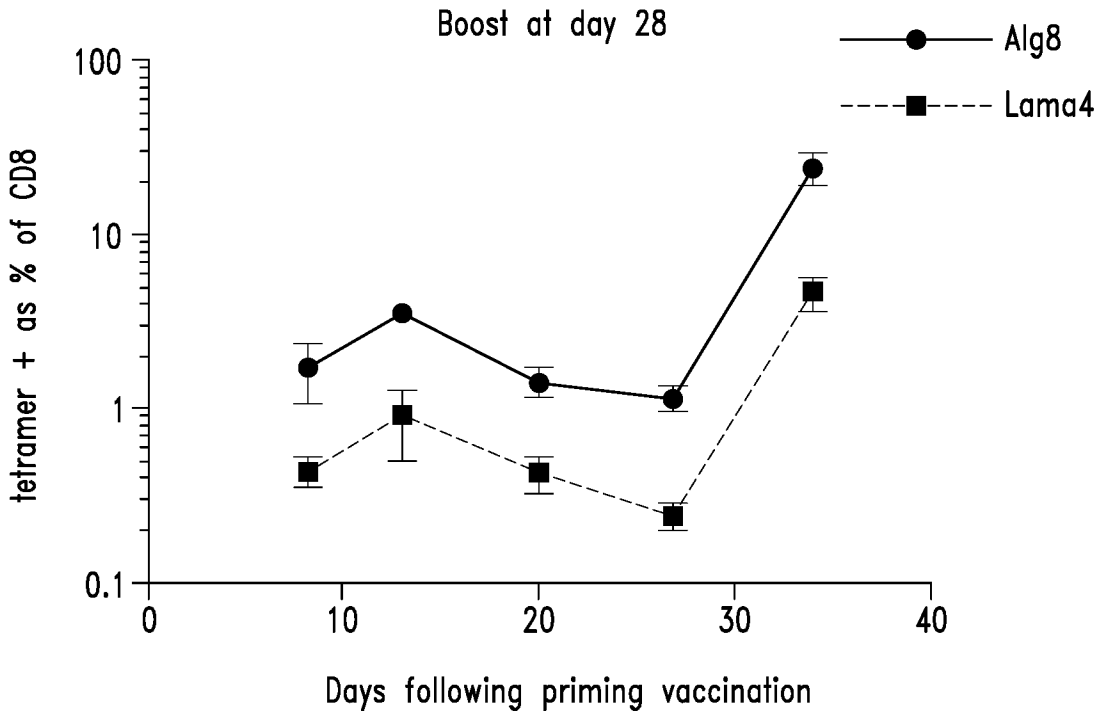
Boost at day 28
Alg8
Lama4
100
10
1
0.1
tetramer + as % of CD8
0
10
20
30
40
Days following priming vaccination

FIG. 9C

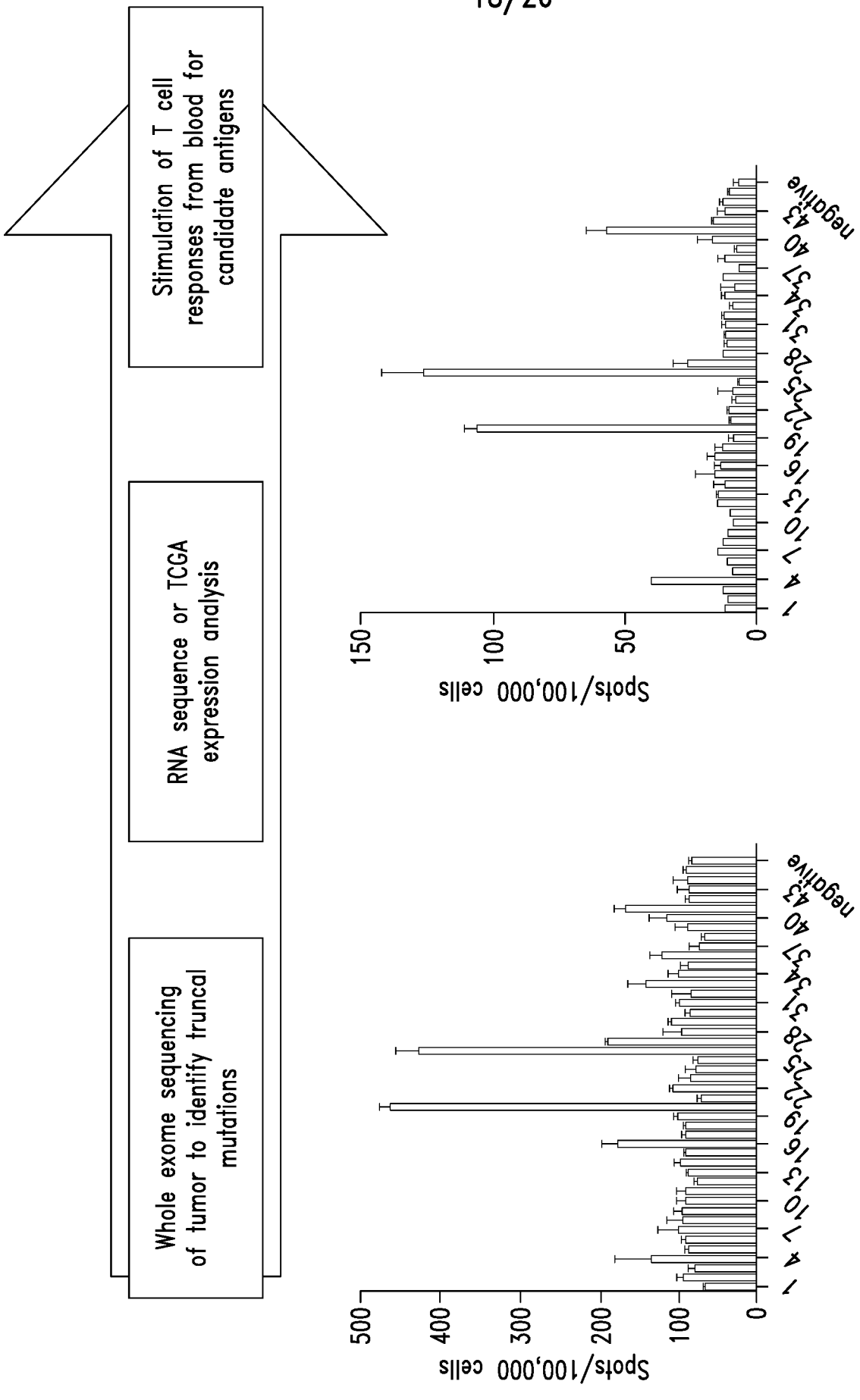
Whole exome sequencing of tumor to identify truncal mutations
RNA sequence or TCGA expression analysis
Stimulation of T cell responses from blood for candidate antigens
Spots/100,000 cells
0
100
200
300
400
500
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
negative
IFN-γ
Spots/100,000 cells
0
50
100
150
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
negative
IL-5

FIG. 10

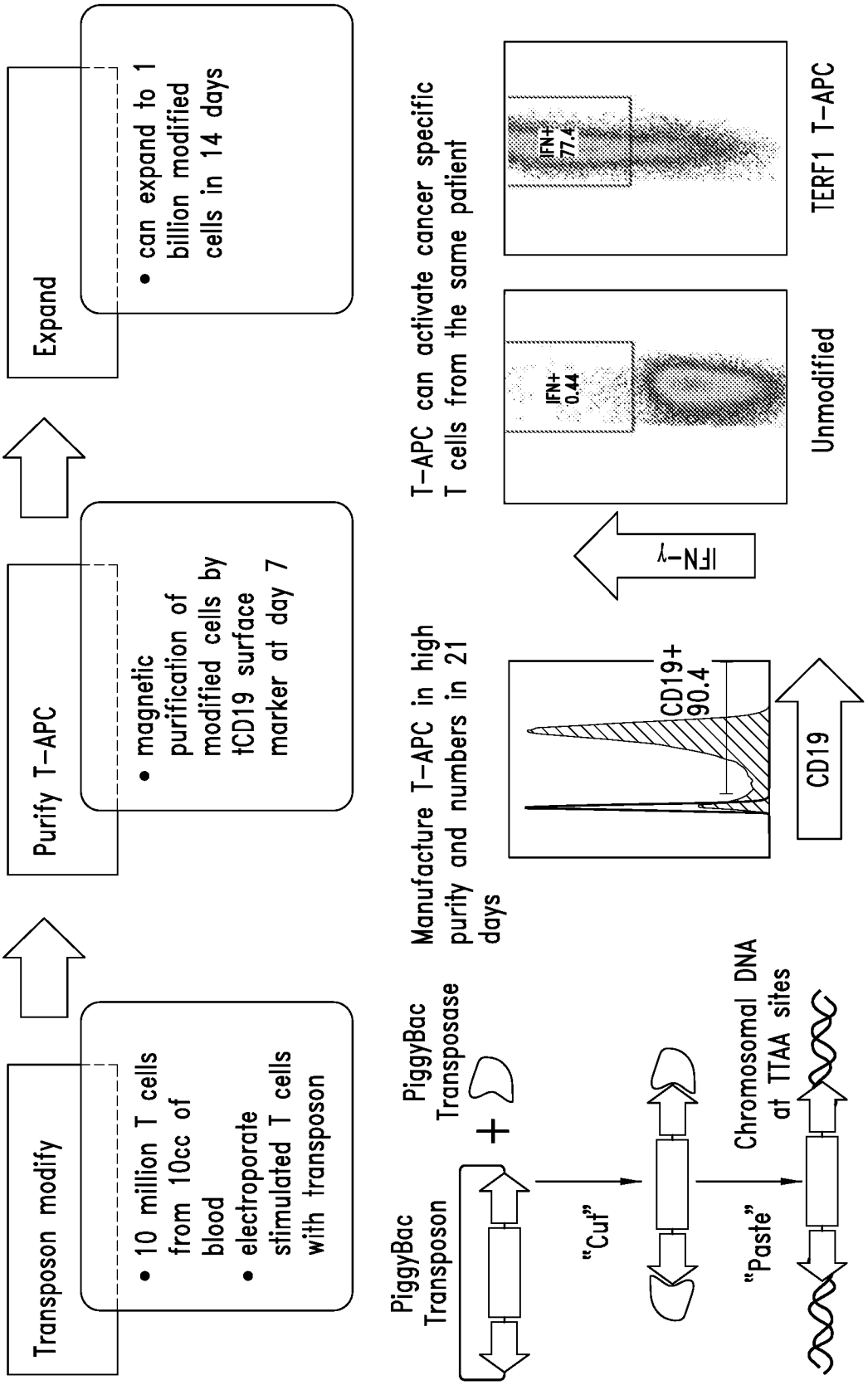
Transposon modify
• 10 million T cells from 10cc of blood
• electroporate stimulated T cells with transposon
Purify T-APC
• magnetic purification of modified cells by tCD19 surface marker at day 7
Expand
• can expand to 1 billion modified cells in 14 days
PiggyBac Transposon
PiggyBac Transposase
"Cut"
"Paste"
Chromosomal DNA at TTAA sites
Manufacture T-APC in high purity and numbers in 21 days
CD19+ 90.4
CD19
IFN-γ
T-APC can activate cancer specific T cells from the same patient
IFN+ 0.44
Unmodified
IFN+ 77.4
TERF1 T-APC

FIG. 11

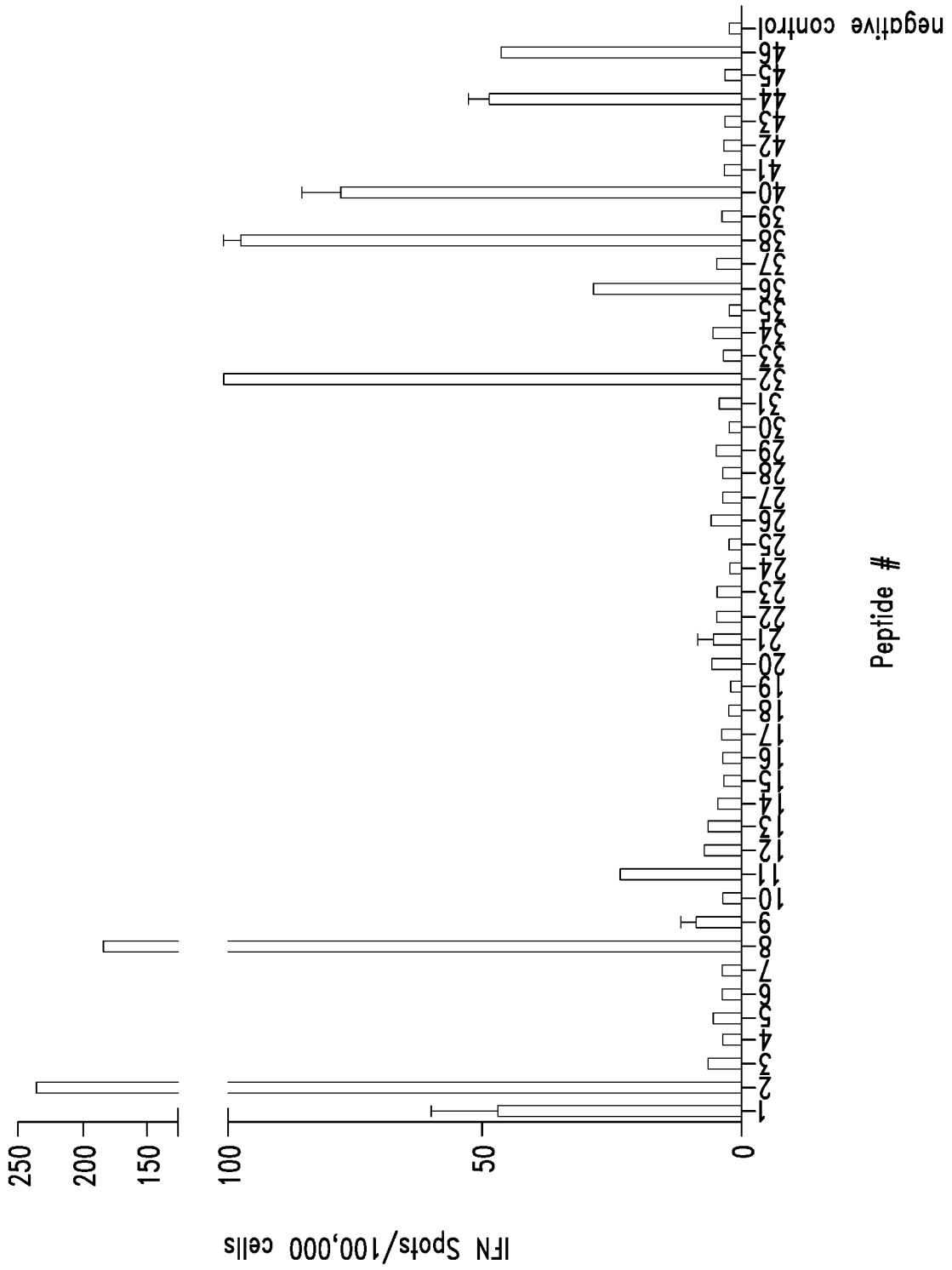
IFN Spots/100,000 cells
250
200
150
100
50
0
Peptide #
negative control
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

FIG. 12A

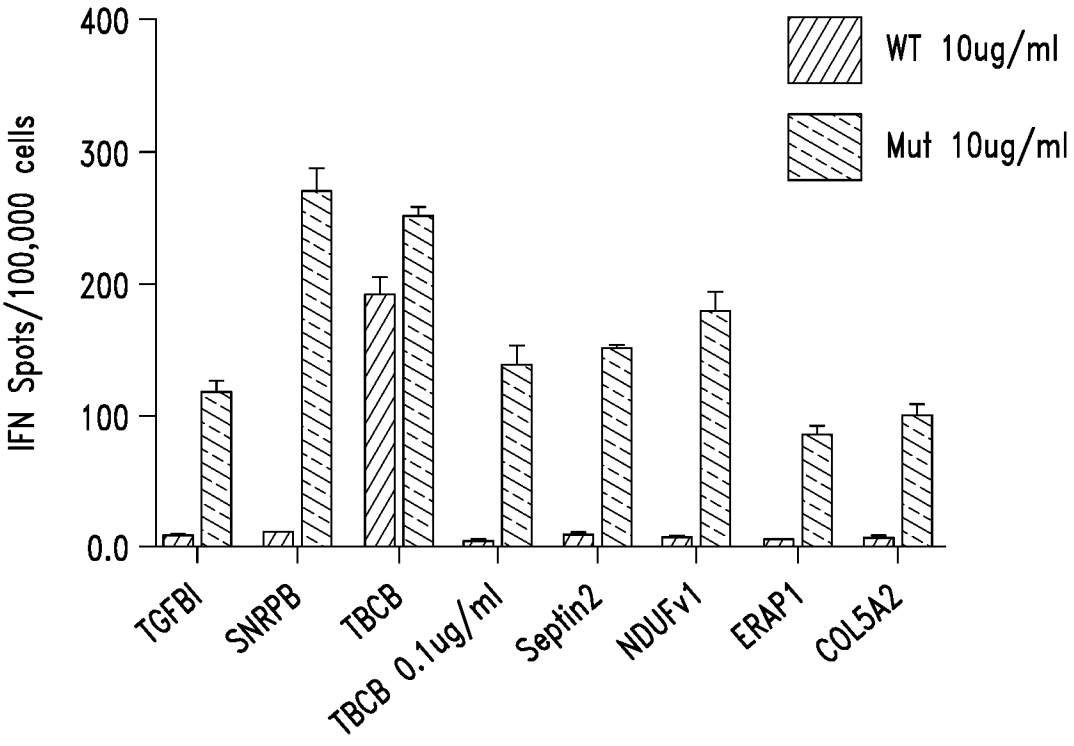
400
300
200
100
0.0
IFN Spots/100,000 cells
WT 10ug/ml
Mut 10ug/ml
TGFBI
SNRPB
TBCB
TBCB 0.1ug/ml
Septin2
NDUFv1
ERAP1
COL5A2
Genes containing neoantigens

FIG. 12B

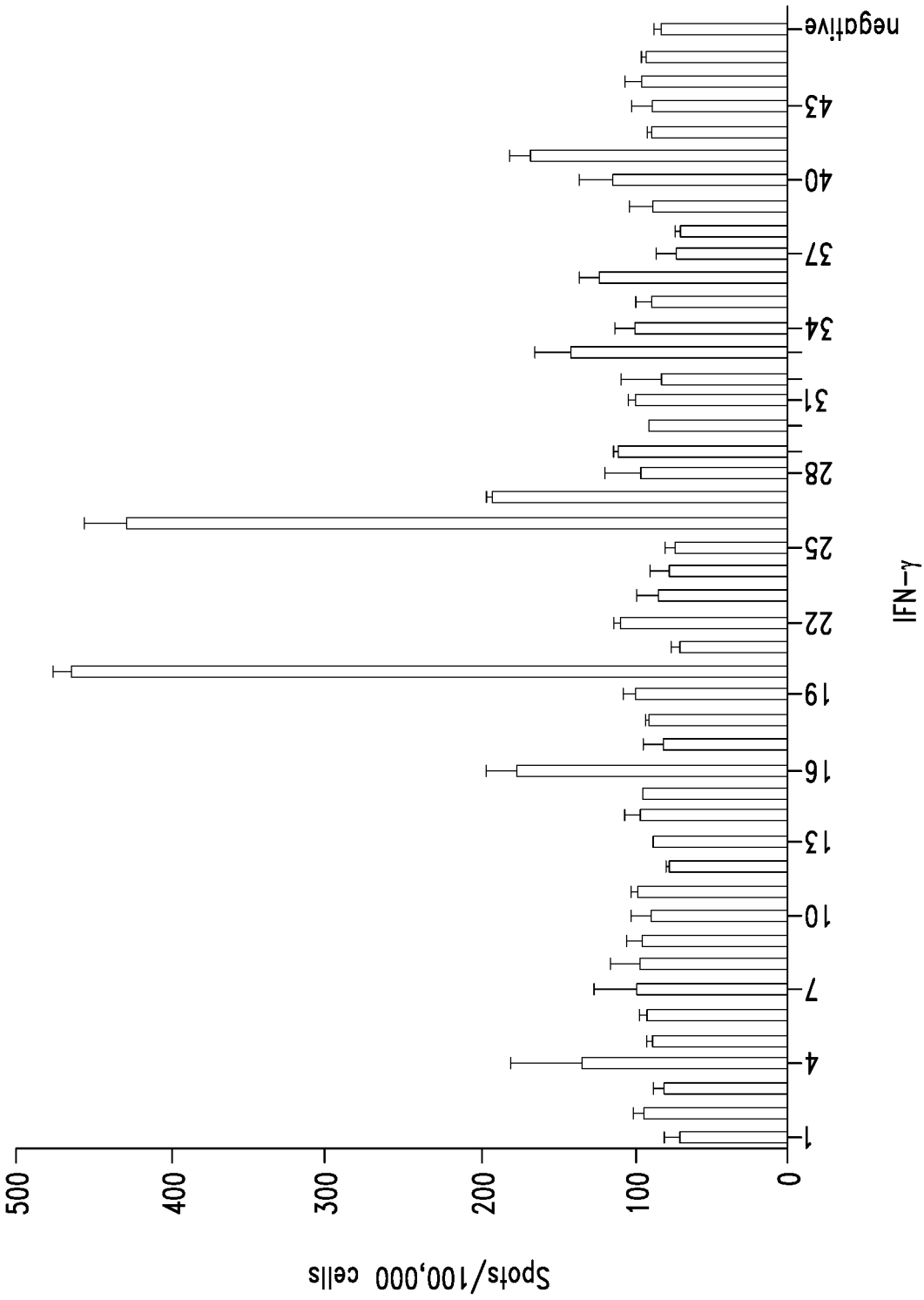
Spots/100,000 cells
500
400
300
200
100
0
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
negative
IFN-γ

FIG. 12C

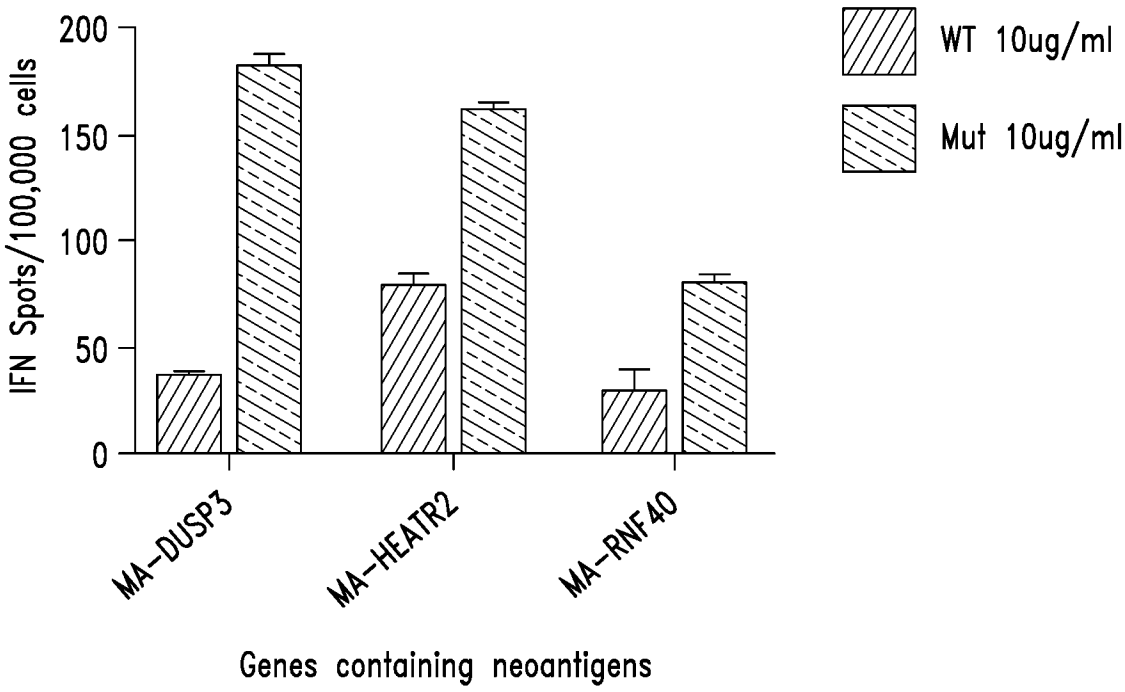
200
150
100
50
0
IFN Spots/100,000 cells
MA-DUSP3
MA-HEATR2
MA-RNF40
Genes containing neoantigens
WT 10ug/ml
Mut 10ug/ml

FIG. 12D

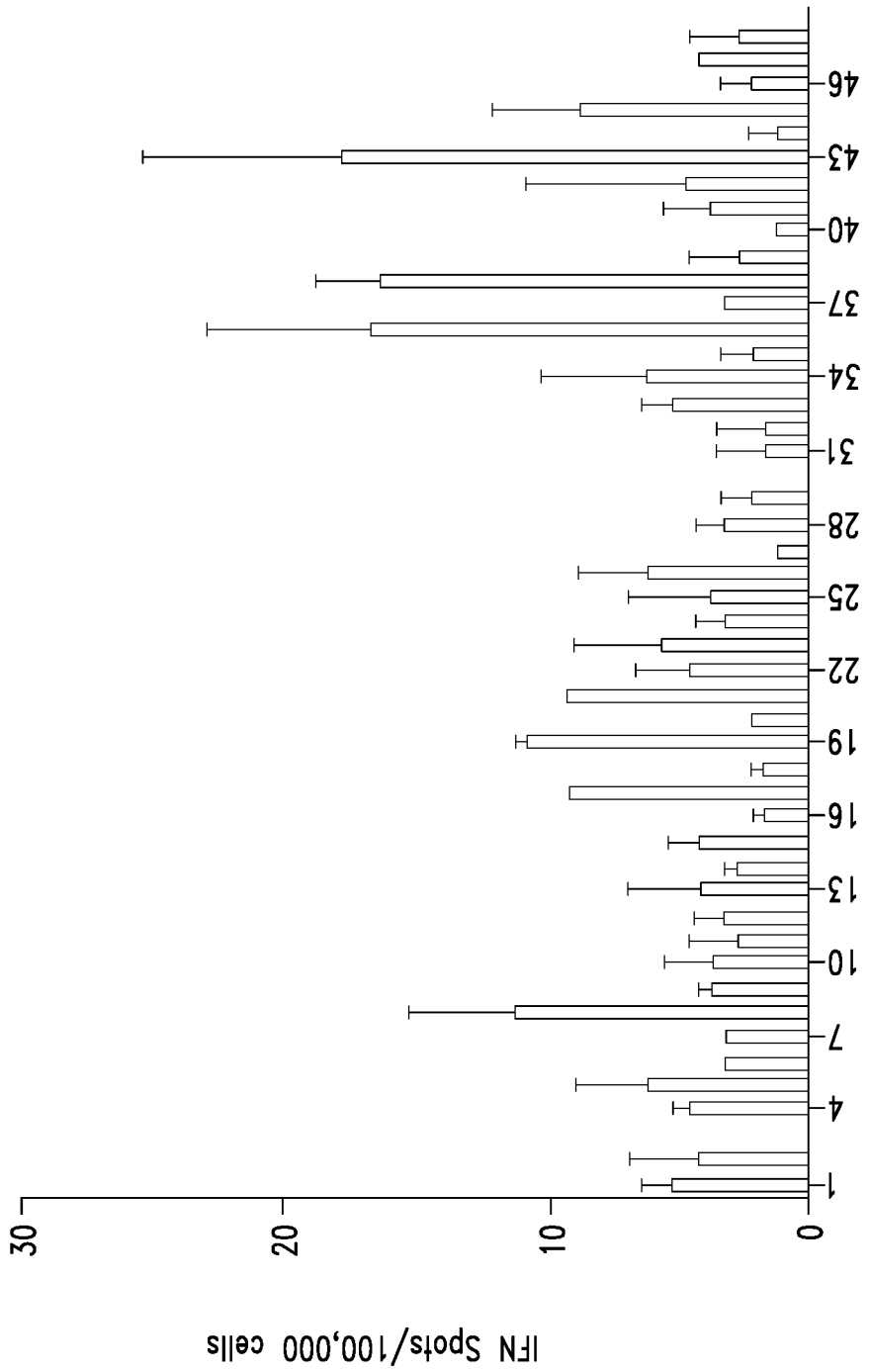
IFN Spots/100,000 cells
0
10
20
30
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46

FIG. 13A

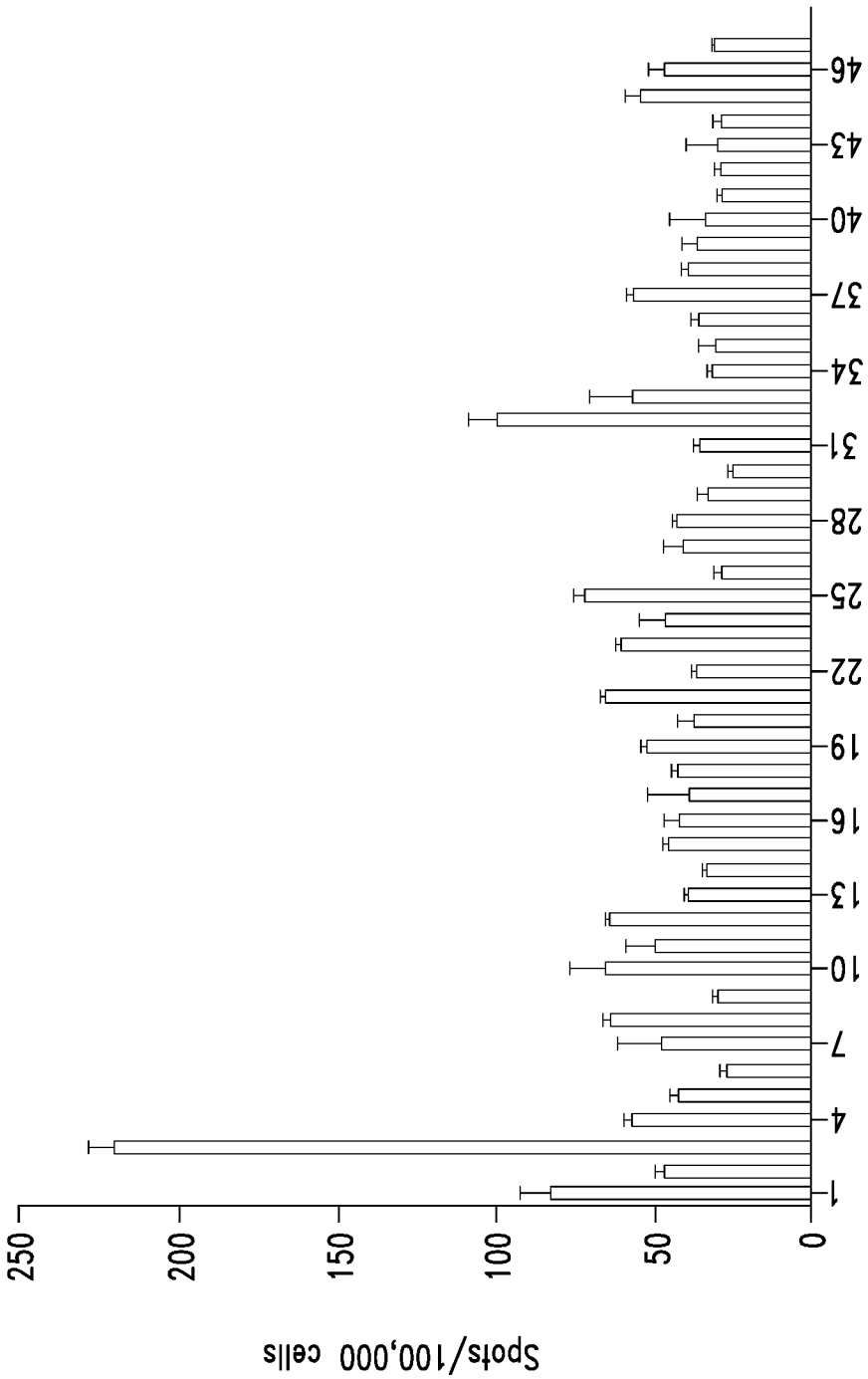
Spots/100,000 cells
250
200
150
100
50
0
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46

FIG. 13B

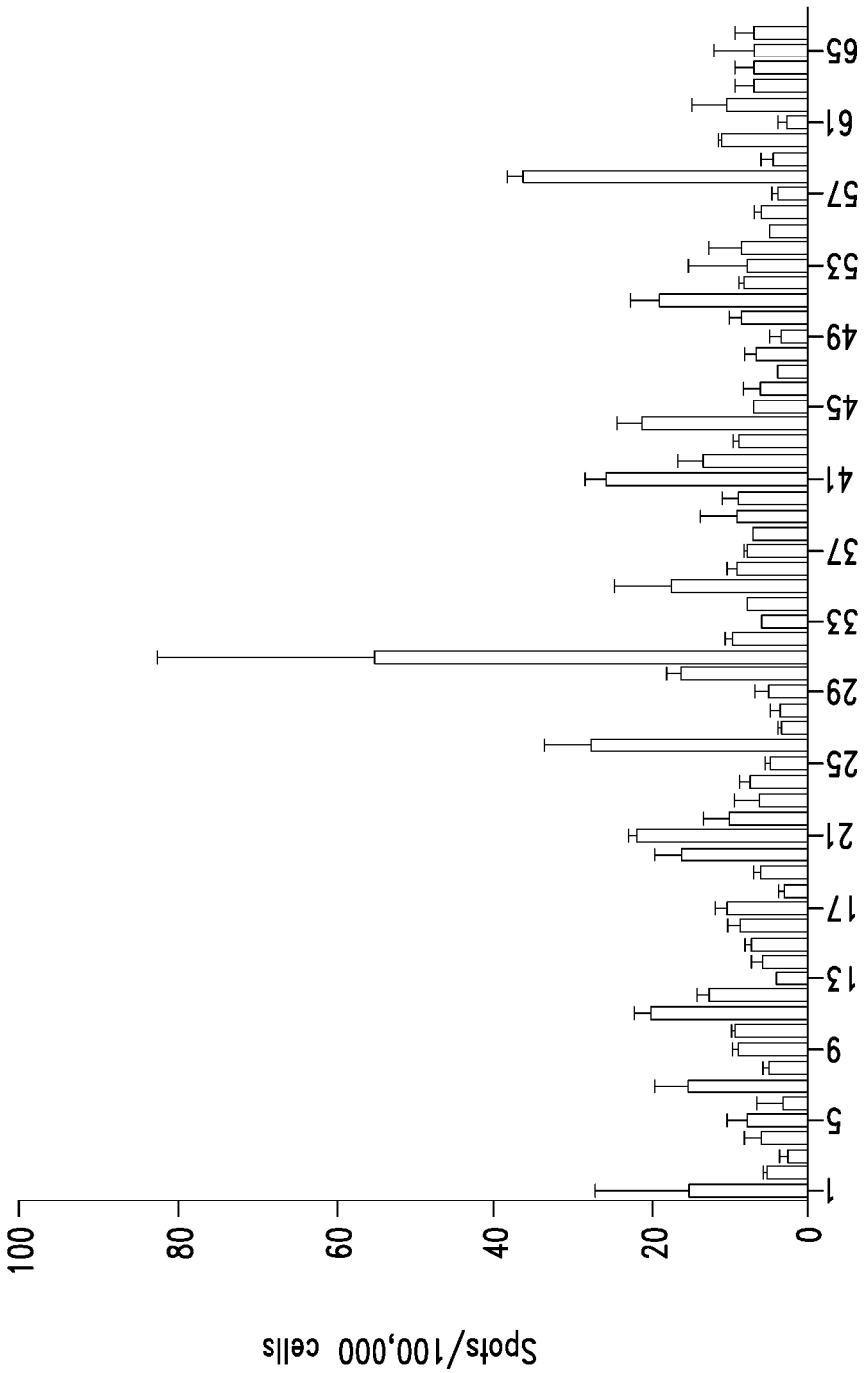
Spots/100,000 cells
100
80
60
40
20
0
1
5
9
13
17
21
25
29
33
37
41
45
49
53
57
61
65

FIG. 13C

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/031171

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 A61P35/00
ADD.

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)
A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ADHAM S. BEAR ET AL: "T Cells as Vehicles for Cancer Vaccination", JOURNAL OF BIOMEDICINE AND BIOTECHNOLOGY, vol. 2011, 1 January 2011 (2011-01-01), pages 1-7, XP055390438, US ISSN: 1110-7243, DOI: 10.1155/2011/417403 page 2, column 2, paragraph 3 - page 3, column 2, paragraph 2 figure 1 page 4, column 2	1-28, 33-61, 80-94

-/--

[X] Further documents are listed in the continuation of Box C.

[X] 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
13 July 2017	21/09/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Noë, Veerle

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/031171

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CARRENO BEATRIZ M ET AL: "Cancer immunotherapy. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells.", SCIENCE (NEW YORK, N.Y.), vol. 348, no. 6236, 15 May 2015 (2015-05-15), pages 803-808, XP002772056, ISSN: 1095-9203 cited in the application abstract page 804 page 807, columns 2-3	1-28, 33-61, 80-94
Y	YOZO NAKAZAWA ET AL: "Optimization of the PiggyBac Transposon System for the Sustained Genetic Modification of Human T Lymphocytes", NIH Public Access Author Manuscript , October 2009 (2009-10), pages 1-18, XP055181605, Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2796278/pdf/nihms-139400.pdf [retrieved on 2017-07-13] abstract page 2, last paragraph - page 3, paragraph 1 Published in final edited form as: J Immunother. 2009 October ; 32(8): 826-836. doi:10.1097/CJI.0b013e3181ad762b	85-94
Y	WILGENHOF SOFIE ET AL: "Therapeutic vaccination with an autologous mRNA electroporated dendritic cell vaccine in patients with advanced melanoma", JOURNAL OF IMMUNOTHERAPY, vol. 34, no. 5, 1 June 2011 (2011-06-01), pages 448-456, XP009174114, , LIPPINCOTT WILLIAMS & WILKINS, USA ISSN: 1537-4513, DOI: 10.1097/CJI.0B013E31821DCB31 abstract page 448, column 2, last paragraph - page 449, column 1, paragraph 1-4	1-28, 33-61, 80-94

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/031171

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J A KYTE ET AL: "Phase I/II trial of melanoma therapy with dendritic cells transfected with autologous tumor-mRNA", CANCER GENE THERAPY, vol. 13, no. 10, 5 May 2006 (2006-05-05), pages 905-918, XP055390452, GB ISSN: 0929-1903, DOI: 10.1038/sj.cgt.7700961 abstract ----- SONG-NAN ZHANG ET AL: "Optimizing DC Vaccination by Combination With Oncolytic Adenovirus Coexpressing IL-12 and GM-CSF", MOLECULAR THERAPY, vol. 19, no. 8, 1 August 2011 (2011-08-01) , pages 1558-1568, XP055070307, ISSN: 1525-0016, DOI: 10.1038/mt.2011.29 abstract ----- LIANG WEN ET AL: "In vitro induction of specific anti-tumoral immunity against laryngeal carcinoma by using human interleukin-12 gene-transfected dendritic cells", CHINESE MEDICAL JOURNAL / ZHONGHUA YIXUE ZAZHI YINGWEN, CHINESE MEDICAL ASSOCIATION, BEIJING, CN, vol. 124, no. 9, 1 January 2011 (2011-01-01), pages 1357-1361, XP008180899, ISSN: 0366-6999 abstract ----- SCHREIBELT GERTY ET AL: "Abstract IA44:Cancer prevention: Dendritic cell enhanced immune responses towards neoantigens in patients with Lynch syndrome", CANCER IMMUNOLOGY RESEARCH , vol. 4, no. 1 January 2016 (2016-01), XP002772057, & CRI-CIMT-EATI-AACR INAUGURAL INTERNATIONAL CANCER IMMUNOTHERAPY CONFERENCE; NEW YORK, NY, USA; SEPTEMBER 16 -19, 2015 DOI: 10.1158/2326-6074.CRICIMTEATIAACR15-IA44 Retrieved from the Internet: URL:http://cancerimmunolres.aacrjournals.org/content/4/1_Supplement/IA44 [retrieved on 2017-07-13] cited in the application the whole document ----- -/--	1-28, 33-61, 80-94 9-28, 80-84 1-28, 33-61, 80-94 1-28, 33-61, 80-94

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/031171

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SEBASTIAN KREITER ET AL: "Mutant MHC class II epitopes drive therapeutic immune responses to cancer", NATURE, vol. 520, no. 7549, 22 April 2015 (2015-04-22), pages 692-696, XP055231810, ISSN: 0028-0836, DOI: 10.1038/nature14426 cited in the application the whole document	1-28, 33-61, 80-94
A	WO 2004/035768 A1 (MOLMED SPA [IT]; TRAVERSARI CATIA [IT]; BORDIGNON CLAUDIO [IT]) 29 April 2004 (2004-04-29) the whole document	1-28, 33-61, 80-94
A	RUSSO VINCENZO ET AL: "Clinical and immunologic responses in melanoma patients vaccinated with MAGE-A3-genetically modified lymphocytes.", INTERNATIONAL JOURNAL OF CANCER, vol. 132, no. 11, 1 June 2013 (2013-06-01) , pages 2557-2566, XP002772062, ISSN: 1097-0215, DOI: 10.1002/ijc.27939 the whole document	1-28, 33-61, 80-94

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/031171

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.:
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:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-28, 85-94(completely); 33-61, 80-84(partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-28, 85-94(completely); 33-61, 80-84(partially)

   A T cell comprising a polynucleotide encoding an exogenous neoantigen associated with a disease and a polynucleotide encoding an immunogenicity enhancer; a composition comprising the T-cell; method of treatment comprising administering the T-cell; method for preparing the T-cell.

   ---

2. claims: 29-32, 95-110(completely); 33-61, 80-84(partially)

   An immune cell which is not a T-cell comprising an exogenous neoantigen associated with a disease and an immunogenicity enhancer comprising an IL-12 fusion protein that localizes to the cell surface of the immune cell; a composition comprising the immune cell; method of treatment comprising administering the immune cell; method for preparing the immune cell.

   ---

3. claims: 62-79(completely); 80-84(partially)

   a transposon expression construct comprising a nucleic acid molecule encoding an neoantigen, a kit comprising the transposon expression construct; a host cell comprising the transposon expression construct.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2017/031171

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004035768 A1	29-04-2004	AU 2003278502 A1	04-05-2004
		CA 2463439 A1	21-04-2004
		CN 1705739 A	07-12-2005
		JP 2006503878 A	02-02-2006
		KR 20050059279 A	17-06-2005
		RU 2330884 C2	10-08-2008
		US 2004235172 A1	25-11-2004
		US 2008286305 A1	20-11-2008
		WO 2004035768 A1	29-04-2004