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(54) Title: IDENTIFICATION OF T-CELL TRAFFICKING GENES AND USES THEREOF FOR INCREASING INFILTRATION
OF T-CELLS INTO SOLID TUMORS

(57) Abstract: Disclosed are compositions, kits, and methods for identifying genes that are involved in T-cell trafficking. In particular, the compositions, kits, and methods may be used to identify genes involved in T-cell trafficking and/or infiltration into tumors such as genes that encode immune checkpoint regulators and/or stimulatory agents. The disclosed compositions, kits, and methods utilize the Sleeping Beauty transposon system in a mouse tumor model to identify genes that are involved in T-cell trafficking and infiltration into tumors. The genes identified in the disclosed methods may provide immunotherapy targets in the tumor microenvironment. The identified genes may be utilized in order to develop therapies that enhance T-cell trafficking and infiltration into tumors and/or T-cell killing of tumors such as in chimeric antigen receptor (CAR) T cell therapies.



IDENTIFICATION OF T-CELL TRAFFICKING GENES AND USES THEREOF FOR INCREASING INFILTRATION OF T-CELLS INTO SOLID TUMORS

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0001] This invention was made with government support under CA097274 awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

[0002] The present application claims the benefit of priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 62/545,758, filed on August 15, 2017 and to U.S. Provisional Application No. 62/486,677, filed on April 18, 2017, the contents of which are incorporated herein by reference in their entireties.

BACKGROUND

[0003] The field of the invention relates to compositions, kits, and methods for identifying and utilizing genes that are involved in T-cell trafficking into solid tumors. In particular, the field of the invention relates to the genes *Aak1* and *Crct3* and modifying the expression of *Aak1* and *Crct3* of gene products encoded by *Aak1* and *Crct3* in T-cells in order to increase infiltration of the T-cells into solid tumors.

[0004] T cells in particular and the immune system in general have amazing potential to eliminate tumor cells throughout the body. This therapeutic potential is evidenced by the clinical success of immunotherapies designed to enhance T cell function, such as immune checkpoint blockade and chimeric antigen receptor T cell (CAR-T) therapies. Despite the remarkable progress to date, expanding success to a broader number of patients is a top priority in the field. T cell infiltration into tumors appears to be an important prerequisite for the success of both immune checkpoint blockade and CAR-T therapies. For example, patients with inflamed tumors have a better response rate to anti-PD-1 than patients whose tumors are immune excluded (T cells present at tumor border, but blocked from entry) or immune deserts (very few, or no,

intratumoral T cells). Preclinical models have also demonstrated that increasing intratumoral T cell number enhances immune checkpoint blockade efficacy. Thus, increasing T cell infiltration into the tumor could have beneficial therapeutic impact.

[0005] To this end, we developed an innovative, systematic method to screen for novel therapeutic targets in T cells that impact T cell infiltration into tumors. We have identified 386 T cell genes using various cancer models, most of which have no previously described role in T cells or immunotherapy. Uses for the disclosed technology include, but are not limited to: 1) Using these genes to develop a diagnostic platform to predict immunotherapy success in patients, 2) Using these genes as the basis for developing novel co-therapies to existing immunotherapies including immune checkpoint blockade and CAR-T technologies, and 3) Using these genes as targets for novel single-agent immunotherapies--all of which would aim to improve our ability to treat human disease.

SUMMARY

[0006] Disclosed are compositions, kits, and methods for identifying genes that are involved in T-cell trafficking into tumors and activation. In particular, the compositions, kits, and methods may be used to identify genes involved in T-cell trafficking and activation in tumors such as genes that encode immune checkpoint regulators and/or stimulatory agents. The disclosed compositions, kits, and methods may utilize the Sleeping Beauty transposon system in a tumor model system to identify genes that are involved in T-cell trafficking into tumors and activation, which may provide immunotherapy targets in the tumor microenvironment. The identified genes may be utilized in order to develop therapies that enhance T-cell trafficking into tumors and activation. Particularly disclosed are the genes *Aak1* and *Crct3* which are shown to be involved in T-cell trafficking and whose expression may be modified in T-cells in order to increase infiltration and/or activation of the T-cells in solid tumors.

[0007] Also disclosed herein are genetically modified T-cells and therapeutic compositions comprising the genetically modified T-cells. As disclosed herein, T-cells may be genetically modified in order to modulate the expression of a gene that is involved in T-cell trafficking into tumors and/or activation, including, but not limited to, the genes *Aak1* and *Crct3*.

The genetically modified T-cells disclosed herein may include T-cells that have been genetically modified to express a chimeric antigen receptor (CAR), and in addition have been further genetically modified to modulate the expression of a gene that is involved in T-cell trafficking into tumors and/or activation, including, but not limited to, the genes *Aak1* and *Crc3*. As such, disclosed herein are CAR T-cells that have been generically modified further to increase in infiltration and/or activation of the CAR T-cells in solid tumors. The further generically modified CAR T-cells and therapeutic compositions comprising the further generically modified CAR T-cells may be administered to treat cancers characterized by solid tumors in a subject in need thereof.

BRIEF DESCRIPTION OF THE FIGURES

[0008] Figure 1. Immunotherapy target discovery screen design A. Mice with T cell-specific SB transposition were injected on both flanks with 8,000 B16F0 cells in growth factor-reduced Matrigel and tumors were allowed to grow for 21 days, at which point bilateral tumors and spleens were harvested. Genetic analysis was performed on either whole tissue or single cells suspensions of sorted CD4+ and CD8+ T cell populations. B and C. Overview of bioinformatics approach to identify candidate T cell genes involved in intratumoral T cell accumulation. Transposon insertion sites are sequenced in spleens and both tumors using Illumina Sequencing of SB amplicon libraries. Within each mouse, insertion sites identified in the spleen are used to represent background, or insertions that do not contribute to intratumoral accumulation. Insertions that are present in both tumors, but absent from spleen, within a single mouse are considered candidate genes (B). Furthermore, candidate genes are compared across animals to identify genes with greater confidence (C).

[0009] Figure 2. Identified genes impact T cell function. A. Flow cytometry analysis of tumors included in the B16F0 melanoma cohort revealed that a minority of tumors displayed very high T cell percentages compared to the nonmutagenized (-SB) control cohort. Yet, the identifiable gene signatures come mostly from these highly infiltrated tumors, suggesting SB insertions are functionally contributing to increased T cell accumulation in tumors. B. One immunotherapy gene candidate, *Crc3*, was specifically shown to be involved in regulating the expression of cytokines by activated T cells. Briefly, murine T cell lymphoma cells (EL4) were

transduced with either a lentiviral CRISPR vector targeting *Crtc3* or a non-targeting control. After selecting an EGFP positive clones and verifying complete knock out of *Crtc3*, T cells were activated for 24 hours with anti-CD3/anti-CD28 beads and Luminex was performed. Loss of *Crtc3* resulted in altered (though largely impaired) cytokine production, which could impact an anti-tumor response. (Additional cytokine data not shown.)

[0010] Figure 3. *Aak1* is a promising immunotherapy target candidate. A. Three SB screens have been performed using two different tumor models. Numbers represent the tumor-enriched genes discovered in each cohort (mutated in tumors, but not spleens, of at least one mouse). As indicated, some genes are shared between two of the three cohort screens. The only gene shared between all three is *Aak1*, which we hypothesize would enhance intratumoral T cell infiltration. B. The *Aak1* gene (to scale). Red triangle indicates where transposon insertions are clustered. Because the protein coding sequence for the kinase domain spans exons 2-3, we hypothesize insertions are introducing a loss-of-function mutation.

[0011] Figure 4. Experimental design for Aim 1A measuring the effect of *Aak1* loss on intratumoral T cell infiltration. A. Donor T cells will be harvested and transduced *ex vivo* with a lentiviral vector containing CRISPR-Cas9 machinery targeting *Aak1* for knock out and containing an mCherry reporter. Control knock out cells are transduced with a nontargeting CRISPR-Cas9 vector containing an EGFP reporter. Fluorophore positive KO cells will be purified using FACS live sorting (Aria, BD), mixed 1:1, and adoptively transferred into tumor-bearing recipient mice. Intratumoral T cell accumulation will be measured as percent mCherry or EGFP positive cells of all live cells 1, 3, and 5 days later by flow cytometry. B. Anticipated results if our hypothesis that *Aak1* KO will result in enhance intratumoral T cell infiltration, where the ratio of *Aak1* KO (mCherry) to control KO (EGFP) cells is larger in tumors than in spleen.

[0012] Figure 5. Experimental design for Aim 1B: measuring effect of *Aak1* loss on tumor regression with and without anti-PD-1 treatment. We hypothesize that knocking out *Aak1* in adoptively transferred T cells will enhance anti-PD-1 therapy. We propose initial assessment of therapeutic response by measuring tumor regression, but other markers of enhanced T cell

response may also be assessed, including expression of CD137 and other anti-tumor activation markers.

[0013] Figure 6. Schematic representation of *Aak1* gene illustrated cluster of insertions in intron 2.

[0014] Figure 7. Western blot of cell lysates using Aak1 monoclonal antibody specific for the long form of Aak1.

[0015] Figure 8. Western blot of cell lysates using Aak1 monoclonal antibody specific for the long form of Aak1 and Aak1 monoclonal antibody for both of the long form of Aak1 and the short form of Aak1.

[0016] Figure 9. In vitro treatment of primary mouse CD8+ T cells with Aak1 inhibitor (iAak1) results in a transient increase in PD-1 expression.

[0017] Figure 10. In vitro treatment of primary mouse CD8+ T cells with Aak1 inhibitor (iAak1) modestly enhances proliferation.

[0018] Figure 11. In vitro treatment of primary mouse splenocytes with Aak1 inhibitor (iAak1) increases PD-1 expression on CD8+ T cells.

[0019] Figure 12. Aak1 inhibitor (iAak1) results in increased EG7 tumor growth in vivo.

[0020] Figure 13. Aak1 inhibitor (iAak1) increased PD-1 expression on tumor-infiltrating but not splenic CD8+ T cells in EG7 tumor model.

[0021] Figure 14. Aak1 inhibitor (iAak1) did not directly increase EG7 cell proliferation in vitro.

[0022] Figure 15. Aak1 inhibitor (iAak1) enhanced A20 tumor growth *in vivo*.

DETAILED DESCRIPTION

[0023] Disclosed are compositions, kits, and methods for identifying genes associated with T-cell trafficking in solid tumors and methods for treating cancer in a subject in need thereof, in particular in a subject having a solid tumor. The compositions, kits, and methods may be further described as follows.

[0024] Unless otherwise specified or indicated by context, the terms “a”, “an”, and “the” mean “one or more.” In addition, singular nouns such as “gene,” “T-cell,” and “CAR T-cell” should be interpreted to mean “genes,” “one or more T-cells,” and “one or more CAR T-cells,” respectively, unless otherwise specified or indicated by context. Similarly, the phrase “at least one”, when employed herein to refer to an entity, refers to, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, or more of that entity, including but not limited to whole number values between 1 and 100 and greater than 100.

[0025] As used herein, the term “and/or” when used in the context of a list of entities, refers to the entities being present singly or in combination. Thus, for example, the phrase “A, B, C, and/or D” includes A, B, C, and D individually, but also includes any and all combinations and subcombinations of A, B, C, and D.

[0026] As used herein, “about”, “approximately”, “substantially,” and “significantly” will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which they are used. If there are uses of the term which are not clear to persons of ordinary skill in the art given the context in which it is used, “about” and “approximately” will mean plus or minus $\leq 10\%$ of the particular term and “substantially” and “significantly” will mean plus or minus $>10\%$ of the particular term.

[0027] As used herein, the terms “include” and “including” have the same meaning as the terms “comprise” and “comprising.” The terms “comprise” and “comprising” should be interpreted as being “open” transitional terms that permit the inclusion of additional components further to those components recited in the claims. The terms “consist” and “consisting of” should be interpreted as being “closed” transitional terms that do not permit the inclusion of additional components other than the components recited in the claims. The term “consisting

essentially of” should be interpreted to be partially closed and allowing the inclusion only of additional components that do not fundamentally alter the nature of the claimed subject matter.

[0028] The terms “subject,” “patient,” or “host” may be used interchangeably herein and may refer to human or non-human animals. Non-human animals may include, but are not limited to non-human primates, dogs, and cats.

[0029] The terms “subject,” “patient,” or “individual” may be used to a human or non-human animal having or at risk for acquiring a cell proliferative disease or disorder. Individuals who are treated with the compositions disclosed herein may be at risk for cancer or may have already acquired cancer including cancers characterized by solid tumors.

[0030] As used herein, the terms “condition”, “disease condition”, “disease”, “disease state”, and “disorder” refer to physiological states in which diseased cells can be targeted with the genetically modified cells disclosed herein. Any solid tumor cell that expresses a tumor-associated epitope can be targeted with the genetically modified cells disclosed herein, and any disease, disorder, or condition associated with solid tumors can be treated, and/or a symptom thereof can be ameliorated, using the genetically modified cells disclosed herein.

[0031] As used herein, the phrase “disease targeted by genetically modified cells” encompasses the targeting of any cell involved in any manner in any disease by the genetically modified cells of the presently disclosed subject matter, irrespective of whether the genetically modified cells target diseased cells or healthy cells to effectuate a therapeutically beneficial result. In some embodiments, the genetically modified cells target diseased cells only in order to effectuate a therapeutically beneficial result. The genetically modified cells include but are not limited to genetically modified T-cells, NK cells, hematopoietic stem cells, pluripotent embryonic stem cells, and embryonic stem cells. The genetically modified cells may express CARs, which CARs can target any of the antigens and/or epitopes expressed on the surface of target cells, including epitopes expressed on the surface of various cancer and tumor cells.

[0032] As used herein, the term “tumor” refers to any neoplastic cell growth and/or proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

The terms “cancer” and “tumor” are used interchangeably herein and can refer to both primary and metastasized solid tumors and carcinomas of any tissue in a subject, including but not limited to breast; colon; rectum; lung; oropharynx; hypopharynx; esophagus; stomach; pancreas; liver; gallbladder; bile ducts; small intestine; urinary tract including kidney, bladder, and urothelium; female genital tract including cervix, uterus, ovaries (e.g., choriocarcinoma and gestational trophoblastic disease); male genital tract including prostate, seminal vesicles, testes and germ cell tumors; endocrine glands including thyroid, adrenal, and pituitary; skin (e.g., hemangiomas and melanomas), bone or soft tissues; blood vessels (e.g., Kaposi's sarcoma); brain, nerves, eyes, and meninges (e.g., astrocytomas, gliomas, glioblastomas, retinoblastomas, neuromas, neuroblastomas, Schwannomas, and meningiomas). As used herein, the terms “cancer” and “tumor” are also intended to refer to multicellular tumors as well as individual neoplastic or pre-neoplastic cells. In some embodiments, a cancer or a tumor comprises a cancer or tumor of an epithelial tissue such as, but not limited to a carcinoma. In some embodiments, a tumor is an adenocarcinoma, which in some embodiments is an adenocarcinoma of the pancreas, breast, ovary, colon, or rectum, and/or a metastatic cell derived therefrom.

[0033] As used herein, the phrase “tumor-associated” refers to a disease, disorder, condition, status, antigen, epitope, or glycosylation state that is primarily or secondarily the result of the presence of a tumor or cancer or a cell's status as being a tumor cell or a cancer cell. As such, in some embodiments a tumor-associated antigen or epitope is an antigen or epitope that is present on a tumor cell or a cancer cell or a cell that results from the presence of a tumor or cancer (e.g., an endothelial cell that results from tumor-associated angiogenesis).

[0034] As used herein, the phrases “tumor-specific” and “tumor-exclusive” refer to an antigen or an epitope thereof that is expressed by a tumor cell but that is substantially or completely absent from a normal cell from which the tumor cell was derived. In some embodiments, a tumor-specific or tumor-exclusive antigen or epitope is one that is overexpressed in tumor cells relative to normal cells. In some embodiments, a tumor-specific or tumor-exclusive antigen or epitope can be targeted by genetically modified cells as disclosed herein.

[0035] As used herein, the phrases “genetically modified cells”, “redirected cells”, “genetically engineered cells”, and “modified cells” refer to cells that have been genetically

altered in order to modulate the expression of one or more genes identified as being involved in T-cell infiltration and/or activation in a solid tumor as disclosed herein.

[0036] Immune Cells and Responses

[0037] As used herein, the phrase “immune cell” refers to the cells of a mammalian immune system including but not limited to antigen presenting cells, B-cells, basophils, cytotoxic T-cells, dendritic cells, eosinophils, granulocytes, helper T-cells, leukocytes, lymphocytes, macrophages, mast cells, memory cells, monocytes, natural killer cells, neutrophils, phagocytes, plasma cells and T-cells. Immune cells include immune cells that have been explanted from a subject, genetically modified (*e.g.*, CAR T cells) and reintroduced into the subject (*i.e.*, where the T cells are allogeneic).

[0038] As used herein, the phrase “immune response” refers to immunities including but not limited to innate immunity, humoral immunity, cellular immunity, immunity, inflammatory response, acquired (adaptive) immunity, autoimmunity, and/or overactive immunity.

[0039] As used herein, the phrase “target cell” refers to any cell that is associated with a disease, disease state, or disorder that can be targeted by the genetically modified cells disclosed herein (including but not limited to genetically modified T-cells, NK cells, hematopoietic stem cells, pluripotent stem cells, and embryonic stem cells). In some embodiments, a target cell is a tumor cell, a cancer cell, or a cancer stem cell that expresses an epitope associated with a solid tumor.

[0040] As used herein, the terms “T-cell” and “T-lymphocyte” are interchangeable and used synonymously. Examples include, but are not limited to, naive T cells, central memory T cells, effector memory T cells, and combinations thereof.

[0041] Polypeptides

[0042] Reference is made herein to peptides, polypeptides, proteins and compositions comprising peptides, polypeptides, and proteins. As used herein, a polypeptide and/or protein is defined as a polymer of amino acids, typically of length ≥ 100 amino acids (Garrett & Grisham,

Biochemistry, 2nd edition, 1999, Brooks/Cole, 110). A peptide is defined as a short polymer of amino acids, of a length typically of 20 or less amino acids, and more typically of a length of 12 or less amino acids (Garrett & Grisham, Biochemistry, 2nd edition, 1999, Brooks/Cole, 110).

[0043] As disclosed herein, exemplary peptides, polypeptides, proteins may comprise, consist essentially of, or consist of any reference amino acid sequence disclosed herein, or variants of the peptides, polypeptides, and proteins may comprise, consist essentially of, or consist of an amino acid sequence having at least about 80%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to any amino acid sequence disclosed or contemplated herein. Variant peptides, polypeptides, and proteins may include peptides, polypeptides, and proteins having one or more amino acid substitutions, deletions, additions and/or amino acid insertions relative to a reference peptide, polypeptide, or protein. Also disclosed are nucleic acid molecules that encode the disclosed peptides, polypeptides, and proteins (*e.g.*, polynucleotides that encode any of the peptides, polypeptides, and proteins disclosed herein and variants thereof).

[0044] The term “amino acid,” includes but is not limited to amino acids contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues. The term “amino acid residue” also may include amino acid residues contained in the group consisting of homocysteine, 2-Aminoadipic acid, N-Ethylasparagine, 3-Aminoadipic acid, Hydroxylysine, β -alanine, β -Amino-propionic acid, allo-Hydroxylysine acid, 2-Aminobutyric acid, 3-Hydroxyproline, 4-Aminobutyric acid, 4-Hydroxyproline, piperidinic acid, 6-Aminocaproic acid, Isodesmosine, 2-Aminoheptanoic acid, allo-Isoleucine, 2-Aminoisobutyric acid, N-Methylglycine, sarcosine, 3-Aminoisobutyric acid, N-Methylisoleucine, 2-Aminopimelic acid, 6-N-Methyllysine, 2,4-Diaminobutyric acid, N-Methylvaline, Desmosine, Norvaline, 2,2'-Diaminopimelic acid, Norleucine, 2,3-Diaminopropionic acid, Ornithine, and N-Ethylglycine. Typically, the amide linkages of the peptides are formed from an amino group of the backbone of one amino acid and a carboxyl group of the backbone of another amino acid.

[0045] The amino acid sequences contemplated herein may include conservative amino acid substitutions relative to a reference amino acid sequence. For example, a variant peptides, polypeptides, and proteins as contemplated herein may include conservative amino acid substitutions relative to an amino acid sequence of a reference peptide, polypeptide, or protein. “Conservative amino acid substitutions” are those substitutions that are predicted to interfere least with the properties of the reference peptide, polypeptide, or protein. In other words, conservative amino acid substitutions substantially conserve the structure and the function of the reference peptide, polypeptide, or protein. The following table provides a list of exemplary conservative amino acid substitutions.

[0046] Table of Conservative Amino Acid Substitutions

Original Residue	Conservative Substitution
Ala	Gly, Ser
Arg	His, Lys
Asn	Asp, Gln, His
Asp	Asn, Glu
Cys	Ala, Ser
Gln	Asn, Glu, His
Glu	Asp, Gln, His
Gly	Ala
His	Asn, Arg, Gln, Glu
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	His, Met, Leu, Trp, Tyr
Ser	Cys, Thr
Thr	Ser, Val
Trp	Phe, Tyr
Tyr	His, Phe, Trp
Val	Ile, Leu, Thr

[0047]

[0048] “Non-conservative amino acid substitutions” are those substitutions that are predicted to interfere most with the properties of the reference peptide, polypeptide, or protein. For example, a non-conservative amino acid substitution might replace a basic amino acid at physiological pH such as Arg, His, or Lys, with a non-basic or acidic amino acid at physiological pH such as Asp or Glu. A non-conservative amino acid substitution might replace a non-polar

amino acid at physiological pH such as Ala, Gly, Ile, Leu, Phe, or Val, with a polar amino acid at physiological pH such as Arg, Asp, Glu, His, or Lys.

[0049] Variants comprising deletions relative to a reference amino acid sequence or nucleotide sequence are contemplated herein. A “deletion” refers to a change in a reference amino acid sequence that results in the absence of one or more amino acid residues. A deletion removes at least 1, 2, 3, 4, 5, 10, 20, 50, 100, or 200 amino acids residues or a range of amino acid residues bounded by any of these values (*e.g.*, a deletion of 5-10 amino acids). A deletion may include an internal deletion or a terminal deletion (*e.g.*, an N-terminal truncation or a C-terminal truncation of a reference polypeptide). A “variant” of a reference polypeptide sequence may include a deletion relative to the reference polypeptide sequence (*e.g.*, relative to any of SEQ ID NOs:2-5 and 7-9).

[0050] The words “insertion” and “addition” refer to changes in an amino acid sequence resulting in the addition of one or more amino acid residues. An insertion or addition may refer to 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, or 200 amino acid residues or a range of amino acid residues bounded by any of these values (*e.g.*, an insertion or addition of 5-10 amino acids).. A “variant” of a reference polypeptide sequence may include an insertion or addition relative to the reference polypeptide sequence (*e.g.*, relative to any of SEQ ID NOs:2-5 and 7-9).

[0051] A “fusion polypeptide” refers to a polypeptide comprising at the N-terminus, the C-terminus, or at both termini of its amino acid sequence a heterologous amino acid sequence, for example, a heterologous amino acid sequence that extends the half-life of the fusion polypeptide in serum. A “variant” of a reference polypeptide sequence may include a fusion polypeptide comprising the reference polypeptide.

[0052] A “fragment” is a portion of an amino acid sequence which is identical in sequence to but shorter in length than a reference sequence (*e.g.*, a fragment of any of SEQ ID NOs:2-5 and 7-9). A fragment may comprise up to the entire length of the reference sequence, minus at least one amino acid residue. For example, a fragment may comprise from 5 to 1000 contiguous amino acid residues of a reference polypeptide. In some embodiments, a fragment

may comprise at least 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 150, 250, or 500 contiguous amino acid residues of a reference polypeptide; or a fragment may comprise no more than 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 150, 250, or 500 contiguous amino acid residues of a reference polypeptide; or a fragment may comprise a range of contiguous amino acid residues of a reference polypeptide bounded by any of these values (e.g., 40-80 contiguous amino acid residues). Fragments may be preferentially selected from certain regions of a molecule. The term “at least a fragment” encompasses the full length polypeptide. A “variant” of a reference polypeptide sequence may include a fragment of the reference polypeptide sequence.

[0053] “Homology” refers to sequence similarity or, interchangeably, sequence identity, between two or more polypeptide sequences. Homology, sequence similarity, and percentage sequence identity may be determined using methods in the art and described herein.

[0054] The phrases “percent identity” and “% identity,” as applied to polypeptide sequences, refer to the percentage of residue matches between at least two polypeptide sequences aligned using a standardized algorithm. Methods of polypeptide sequence alignment are well-known. Some alignment methods take into account conservative amino acid substitutions. Such conservative substitutions, explained in more detail above, generally preserve the charge and hydrophobicity at the site of substitution, thus preserving the structure (and therefore function) of the polypeptide. Percent identity for amino acid sequences may be determined as understood in the art. (*See, e.g.*, U.S. Patent No. 7,396,664, which is incorporated herein by reference in its entirety). A suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul, S. F. et al. (1990) J. Mol. Biol. 215:403-410), which is available from several sources, including the NCBI, Bethesda, Md., at its website. The BLAST software suite includes various sequence analysis programs including “blastp,” that is used to align a known amino acid sequence with other amino acids sequences from a variety of databases.

[0055] Percent identity may be measured over the length of an entire defined polypeptide sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined

polypeptide sequence, for instance, a fragment of at least 15, at least 20, at least 30, at least 40, at least 50, at least 100, at least 150, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450, at least 500, at least 550, at least 600, at least 650, or at least 700 contiguous amino acid residues; or a fragment of no more than 15, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, or 700 amino acid residues; or over a range bounded by any of these values (*e.g.*, a range of 500-600 amino acid residues) Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in the tables, figures or Sequence Listing, may be used to describe a length over which percentage identity may be measured.

[0056] In some embodiments, a “variant” of a particular polypeptide sequence may be defined as a polypeptide sequence having at least 20% sequence identity to the particular polypeptide sequence over a certain length of one of the polypeptide sequences using blastp with the “BLAST 2 Sequences” tool available at the National Center for Biotechnology Information’s website. (*See* Tatiana A. Tatusova, Thomas L. Madden (1999), “Blast 2 sequences - a new tool for comparing protein and nucleotide sequences”, FEMS Microbiol Lett. 174:247-250). Such a pair of polypeptides may show, for example, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% or greater sequence identity over a certain defined length of one of the polypeptides, or range of percentage identity bounded by any of these values (*e.g.*, range of percentage identity of 80-99%).

[0057] The disclosed fusion polypeptides may comprise an amino acid sequence fused directly to a heterologous amino acid sequence or fused indirectly via a linker sequence. Suitable linker sequences may include amino acid sequences of 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acids or more, or a range bounded by any of these values (*e.g.*, a linker of 5-25 amino acids). In some embodiments, the linker sequence comprises only glycine and serine residues.

[0058] Fusion polypeptide disclosed herein may include an amino acid tag sequence, for example, which may be utilized for purifying and or identifying the fusion polypeptide. Suitable

amino acid tag sequences may include, but are not limited to, histidine tag sequences comprising 5-10 histidine residues.

[0059] A variant polypeptide may have substantially the same functional activity as a reference polypeptide. For example, a variant polypeptide may exhibit or more biological activities associated with binding a ligand, exhibiting fluorescence, and/or enzymatic activity.

[0060] Polynucleotides

[0061] Reference also is made herein to nucleic acid and nucleic acid sequences. The terms “nucleic acid” and “nucleic acid sequence” refer to a nucleotide, oligonucleotide, polynucleotide (which terms may be used interchangeably), or any fragment thereof. These phrases also refer to DNA or RNA of genomic or synthetic origin (which may be single-stranded or double-stranded and may represent the sense or the antisense strand).

[0062] The term “polynucleotide” as used herein includes but is not limited to DNA, RNA, complementary DNA (cDNA), messenger RNA (mRNA), ribosomal RNA (rRNA), small hairpin RNA (shRNA), small nuclear RNA (snRNA), short nucleolar RNA (snoRNA), microRNA (miRNA), genomic DNA, synthetic DNA, synthetic RNA, and/or tRNA.

[0063] The terms “percent identity” and “% identity,” as applied to polynucleotide sequences, refer to the percentage of residue matches between at least two polynucleotide sequences aligned using a standardized algorithm (e.g., aligned with a reference sequence such as SEQ ID NO:1 or SEQ ID NO:6). Such an algorithm may insert, in a standardized and reproducible way, gaps in the sequences being compared in order to optimize alignment between two sequences, and therefore achieve a more meaningful comparison of the two sequences. Percent identity for a nucleic acid sequence may be determined as understood in the art. (*See, e.g.,* U.S. Patent No. 7,396,664, which is incorporated herein by reference in its entirety). A suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul, S. F. et al. (1990) J. Mol. Biol. 215:403-410), which is available from several sources, including the NCBI, Bethesda, Md., at its website. The BLAST software suite

includes various sequence analysis programs including “blastn,” that is used to align a known polynucleotide sequence with other polynucleotide sequences from a variety of databases. Also available is a tool called “BLAST 2 Sequences” that is used for direct pairwise comparison of two nucleotide sequences. “BLAST 2 Sequences” can be accessed and used interactively at the NCBI website. The “BLAST 2 Sequences” tool can be used for both blastn and blastp (discussed above).

[0064] Percent identity may be measured over the length of an entire defined polynucleotide sequence or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined sequence (*e.g.* SEQ ID NO:1 or SEQ ID NO:6), for instance, a fragment of at least 20, at least 30, at least 40, at least 50, at least 70, at least 100, or at least 200 contiguous nucleotides. Such lengths are exemplary only, and it is understood that any fragment length may be used to describe a length over which percentage identity may be measured.

[0065] A “full length” polynucleotide sequence of a gene is one containing at least a translation initiation codon (*e.g.*, methionine) followed by an open reading frame and a translation termination codon. A “full length” polynucleotide sequence encodes a “full length” polypeptide sequence (*e.g.* SEQ ID NO:1 or SEQ ID NO:6).

[0066] A “variant,” “mutant,” or “derivative” of a particular nucleic acid sequence may be defined as a nucleic acid sequence having at least 50% sequence identity to the particular nucleic acid sequence over a certain length of one of the nucleic acid sequences using blastn with the “BLAST 2 Sequences” tool available at the National Center for Biotechnology Information’s website. (*See* Tatiana A. Tatusova, Thomas L. Madden (1999), “Blast 2 sequences - a new tool for comparing protein and nucleotide sequences”, FEMS Microbiol Lett. 174:247-250). In some embodiments a variant polynucleotide may show, for example, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% or greater sequence identity over a certain defined length relative to a reference polynucleotide (*e.g.* relative to SEQ ID NO:1 or SEQ ID NO:6).

[0067] Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences due to the degeneracy of the genetic code. It is understood that changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequences that all encode substantially the same protein.

[0068] A “recombinant nucleic acid” is a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two or more otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, *e.g.*, by genetic engineering techniques such as those described in Sambrook, J. *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1 3, Cold Spring Harbor Press, Plainview N.Y. The term recombinant includes nucleic acids that have been altered solely by addition, substitution, or deletion of a portion of the nucleic acid. Frequently, a recombinant nucleic acid may include a nucleic acid sequence operably linked to a promoter sequence. Such a recombinant nucleic acid may be part of a vector that is used, for example, to transform a cell.

[0069] Transgene Expression

[0070] The genes disclosed herein that are involved in cell trafficking and/or activation in solid tumors may be overexpressed in cells, for example via transfection and/or transformation with an expression cassette. “Transfection” and “transformation” describe a process by which exogenous DNA is introduced into a recipient cell. Transfection and transformation may occur under natural or artificial conditions according to various methods well known in the art, and may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method for transfection or transformation is selected based on the type of host cell being transformed and may include, but is not limited to, bacteriophage or viral infection, electroporation, heat shock, lipofection, and particle bombardment. The terms “transfected cells” and “transformed cells” include stably transfected cells or transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome, as well as transiently transfected cells or transformed cells which express the inserted DNA or RNA for limited

periods of time. The introduced gene or sequence can also be called a “cloned”, “foreign”, or “heterologous” gene or sequence or a “transgene”, and can include regulatory and/or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell's genetic machinery. The gene or sequence can include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been “transformed” and is a “transformant” or a “clone”, and is “transgenic”. The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species

[0071] The polynucleotide sequences contemplated herein may be present in expression cassettes and/or expression vectors (*e.g.*, an expression vector comprising an expression cassette). For example, the vectors may comprise a polynucleotide encoding an ORF of a recombinant protein (*e.g.*, an exogenous sensor as disclosed herein). The polynucleotide present in the vector may be operably linked to a promoter (*e.g.*, a eukaryotic promoter or prokaryotic promoter). “Operably linked” refers to the situation in which a first nucleic acid sequence is placed in a functional relationship with a second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Operably linked DNA sequences may be in close proximity or contiguous and, where necessary to join two protein coding regions, in the same reading frame. Vectors contemplated herein may comprise a heterologous promoter (*e.g.*, a eukaryotic or prokaryotic promoter) operably linked to a polynucleotide that encodes a protein. A “heterologous promoter” refers to a promoter that is not the native or endogenous promoter for the protein or RNA that is being expressed. For example, a heterologous promoter for a LAMP may include a eukaryotic promoter or a prokaryotic promoter that is not the native, endogenous promoter for the LAMP.

[0072] As used herein, “expression” refers to the process by which a polynucleotide is transcribed from a DNA template (such as into and mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as “gene

product.” If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

[0073] As used herein, an “expression cassette” minimally refers to a recombinant polynucleotide comprising a promoter operably linked to a recombinant coding sequence. An expression cassette may be present in a vector (*e.g.*, an episomal vector which is transfected into a cell and remains episomal and/or which recombines into the genome of the cell). A vector may include one or more expression cassettes which express one or more coding sequences (*e.g.*, one or more coding sequences for sensors as disclosed herein).

[0074] Compositions

[0075] A “composition comprising a given polypeptide” and a “composition comprising a given polynucleotide” refer broadly to any composition containing the given polynucleotide or amino acid sequence. The composition may comprise a dry formulation or an aqueous solution. The compositions may be stored in any suitable form including, but not limited to, freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. The compositions may be aqueous solution containing salts (*e.g.*, NaCl), detergents (*e.g.*, sodium dodecyl sulfate; SDS), and other components.

[0076] “Substantially isolated or purified” nucleic acid or amino acid sequences are contemplated herein. The term “substantially isolated or purified” refers to nucleic acid or amino acid sequences that are removed from their natural environment, and are at least 60% free, preferably at least 75% free, and more preferably at least 90% free, even more preferably at least 95% free from other components with which they are naturally associated.

[0077] Vectors

[0078] The term “vector” refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, bacteriophages, cosmids, viruses, and bacteria. As used herein, a “viral vector” (*e.g.*, an adenovirus, Sendai virus, or measles virus vector) refers to recombinant viral nucleic acid that has been engineered to express a heterologous polypeptide. The recombinant viral nucleic acid

typically includes *cis*-acting elements for expression of the heterologous polypeptide. The recombinant viral nucleic acid typically is capable of being packaged into a helper virus that is capable of infecting a host cell. For example, the recombinant viral nucleic acid may include *cis*-acting elements for packaging. Typically, the viral vector is not replication competent or is attenuated. An “attenuated recombinant virus” refers to a virus that has been genetically altered by modern molecular biological methods (*e.g.*, restriction endonuclease and ligase treatment, and rendered less virulent than wild type), typically by deletion of specific genes. For example, the recombinant viral nucleic acid may lack a gene essential for the efficient production or essential for the production of infectious virus.

[0079] Any of the conventional vectors used for expression in eukaryotic cells may be used for directly introducing DNA into a cell. Expression vectors containing regulatory elements from eukaryotic viruses may be used in eukaryotic expression vectors (*e.g.*, vectors containing SV40, CMV, or retroviral promoters or enhancers). Exemplary vectors include those that express proteins under the direction of such promoters as the SV40 early promoter, SV40 later promoter, metallothionein promoter, human cytomegalovirus promoter, murine mammary tumor virus promoter, and Rous sarcoma virus promoter. Therapeutic quantities of plasmid DNA can be produced for example, by fermentation in *E. coli*, followed by purification. Aliquots from the working cell bank are used to inoculate growth medium, and grown to saturation in shaker flasks or a bioreactor according to well known techniques. Plasmid DNA can be purified using standard bioseparation technologies such as solid phase anion-exchange resins. If required, supercoiled DNA can be isolated from the open circular and linear forms using gel electrophoresis or other methods. Purified plasmid DNA can be prepared for injection using a variety of formulations (*e.g.*, lyophilized DNA which may be reconstituted in sterile phosphate-buffered saline (PBS)). The purified DNA may be introduced to a subject by any suitable method (*e.g.*, intramuscular (IM) or intradermal (ID) administration).

[0080] The term “vector” refers to some means by which nucleic acid (*e.g.*, DNA) can be introduced into a host organism or host tissue. There are various types of vectors including plasmid vector, bacteriophage vectors, cosmid vectors, bacterial vectors, and viral vectors. As used herein, a “vector” may refer to a recombinant nucleic acid that has been engineered to

express a heterologous polypeptide (*e.g.*, the fusion proteins disclosed herein). The recombinant nucleic acid typically includes *cis*-acting elements for expression of the heterologous polypeptide.

[0081] Any of the conventional vectors used for expression in eukaryotic cells may be used for directly introducing DNA into a subject. Expression vectors containing regulatory elements from eukaryotic viruses may be used in eukaryotic expression vectors (*e.g.*, vectors containing SV40, CMV, or retroviral promoters or enhancers). Exemplary vectors include those that express proteins under the direction of such promoters as the SV40 early promoter, SV40 later promoter, metallothionein promoter, human cytomegalovirus promoter, murine mammary tumor virus promoter, and Rous sarcoma virus promoter. Expression vectors as contemplated herein may include eukaryotic or prokaryotic control sequences that modulate expression of a heterologous protein (*e.g.* the fusion protein disclosed herein). Prokaryotic expression control sequences may include constitutive or inducible promoters (*e.g.*, T3, T7, Lac, trp, or phoA), ribosome binding sites, or transcription terminators.

[0082] The vectors contemplated herein may be introduced and propagated in a prokaryote, which may be used to amplify copies of a vector to be introduced into a eukaryotic cell or as an intermediate vector in the production of a vector to be introduced into a eukaryotic cell (*e.g.* amplifying a plasmid as part of a viral vector packaging system). A prokaryote may be used to amplify copies of a vector.

[0083] The presently disclosed methods may include delivering one or more polynucleotides, such as or one or more vectors as described herein, one or more transcripts thereof, and/or one or proteins transcribed therefrom, to a host cell. Further contemplated are host cells produced by such methods, and organisms (such as animals, plants, or fungi) comprising or produced from such cells. The disclosed extracellular vesicles may be prepared by introducing vectors that express mRNA encoding a fusion protein as contemplated herein. Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids in mammalian cells or target tissues. Non-viral vector delivery systems include DNA plasmids, RNA (*e.g.* a transcript of a vector described herein), naked nucleic acid, and nucleic acid complexed with a delivery vehicle, such as a liposome. Viral vector delivery systems

include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell.

[0084] In the methods contemplated herein, a host cell may be transiently or non-transiently transfected (*i.e.*, stably transfected) with one or more vectors described herein. In some embodiments, a cell is transfected as it naturally occurs in a subject (*i.e.*, in situ). In some embodiments, a cell that is transfected is taken from a subject (*i.e.*, explanted). In some embodiments, the cell is derived from cells taken from a subject, such as a cell line. Suitable cells may include stem cells (*e.g.*, embryonic stem cells and pluripotent stem cells). A cell transfected with one or more vectors described herein may be used to establish a new cell line comprising one or more vector-derived sequences. In the methods contemplated herein, a cell may be transiently transfected with the components of a system as described herein (such as by transient transfection of one or more vectors, or transfection with RNA), and modified through the activity of a complex, in order to establish a new cell line comprising cells containing the modification but lacking any other exogenous sequence.

[0085] Genetic Modification Using CRISPR/Cas9 Systems

[0086] Cells comprising genes identified herein that are involved in T cell trafficking and/or activation in solid tumors may be genetically modified in order to delete or otherwise inactivate the genes. The cells may be modified in particular using CRISPR/Cas9 systems as known in the art, *e.g.*, to delete or otherwise inactivate one or more genes identified herein that are involved in T cell trafficking and/or activation in solid tumors. (*See, e.g.*, Wang *et al.*, CRISPR/Cas9 in Genome Editing and Beyond,” Ann Rev Biochem. Vol. 85:227-264 (June 2016); the content of which is incorporated herein by reference in its entirety.

[0087] Identification of Genes Involved in T Cell Trafficking and Activation in Solid Tumors via the Sleeping Beauty (SB) Transposon System

[0088] The disclosed compositions, kits, and methods may include or utilize systems for identify genes associated with T-cell trafficking and activation in solid tumors. In some embodiments, the disclosed compositions, kits, and methods may include or utilize Sleeping

Beauty transposon system in order to identify genes that are involved in T-cell trafficking and/or T-cell infiltration into tumors. The Sleeping Beauty (SB) transposon system comprises the Sleeping Beauty (SB) transposase, which is a Tc1/mariner-type transposase, and a transposon that is designed to insert specific sequences of DNA into genomic DNA at a target sequence. (See Plasterk RH (September 1993). “Molecular mechanisms of transposition and its control.” *Cell* 74 (5): 781–786. doi:10.1016/0092-8674(93)90458-3). In the SB transposon system, the transposon is translocated from one DNA site to another in a simple, cut-and-paste manner (in which a defined DNA segment is excised from one DNA molecule and moved to another site in the same or different DNA molecule or genome. Like all Tc1/mariner-type transposases, the SB transposase inserts a transposon into a “TA” dinucleotide base pair in a recipient DNA sequence. (See Plasterk RH, Izsvák Z, Ivics Z (August 1999). “Resident aliens: the Tc1/mariner superfamily of transposable elements.” *Trends Genet.* 15 (8): 326–332. doi:10.1016/S0168-9525(99)01777-1). The insertion site can be elsewhere in the same DNA molecule, or in another DNA molecule or chromosome. In mammalian genomes, including humans, there are approximately 200 million TA dinucleotide sites which provide targets for SB transposition. The TA dinucleotide insertion site is duplicated in the process of transposon integration, which is the hallmark of SB transposition. The transposase can be encoded either within the transposon or the transposase can be provided in *trans*, in which case the transposon becomes a non-autonomous element. Non-autonomous SB transposons are useful as genetic tools because after insertion they cannot independently continue to excise and re-insert. As such, non-autonomous SB transposons can be used to create gain-of-function mutations or loss-of-function mutations in order to identify genes associated with a desired biological activity. For example, in the present application, non-autonomous SB transposons were used to identify genes that are involved in T-cell trafficking and/or T-cell infiltration into tumors.

[0089] Others have used shRNA libraries targeting portions of the genome to successfully identify a gene *in vivo* that increased T cell proliferation in a different murine tumor model. (See Zhou *et al.*, “*In vivo* discovery of immunotherapy targets in the tumour microenvironment.” *Nature* 2014 Feb 6;506(7466):52-7). Key improvements in the screening strategy disclosed herein include, but are not limited to, whole genome coverage and gain- and loss-of-function mutation capabilities. Additionally, we have successfully identified three genes

in our preliminary studies, and have potential to identify more upon experimental expansion. Additional research plans include expanding our current studies to identify additional gene candidates, validation experiments of gene candidates we discover, and determining whether these candidates augment current therapeutic strategies *in vivo*.

[0090] The disclosed methods may be utilized to identify genes associated with T cell trafficking and/or infiltration into a variety of tumors and/or T cell activation against tumors, which may include but are not limited to melanomas, adenocarcinoma, sarcomas, and teratocarcinomas. In particular, the disclosed methods may be utilized to identify genes associated with T cell trafficking and/or infiltration into a variety of tumors and/or T cell activation against tumors such as tumors of the skin, adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, colon, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, prostate, testis, thymus, and uterus.

[0091] Genes involved in T-cell trafficking and activation in solid tumors may include, but are not limited to: *Aak1*, *1300002E11Rik*, *Ehhadh*, *Map7d1*, *Ptprd*, *Smr3a*, *Susd4*, *Wdr59*, *Astn2*, *Gpatch8*, *Cpa6*, *Cpne4*, *Crtc3*, *Dab1*, *Grid2*, *Itga2b*, *Kdm4c*, *Lingo2*, *Nfu1*, *Opcml*, *Pappa2*, *Skint6*, *Stau2*, *Thsd7b*, *2310015D24Rik*, *2610307P16Rik*, *2810055G20Rik*, *4732456N10Rik*, *4930444G20Rik*, *4930521E06Rik*, *4930544M13Rik*, *6330409D20Rik*, *9330175M20Rik*, *A330023F24Rik*, *Abca12*, *Abcg2*, *Actn2*, *Adam19*, *Adk*, *Agbl4*, *Ago3*, *Agtr1a*, *Aldoat1*, *Alg6*, *Ammecr1*, *Ankrd13c*, *Ankrd44*, *Ankrd6*, *Antxr2*, *Apaf1*, *Arhgap15*, *Arhgap25*, *Armc9*, *At13*, *Atp1b1*, *Atp2c1*, *Bag1*, *Bai3*, *Bcl1*, *Bckdhh*, *Bcl2*, *Birc2*, *Bnc2*, *Bre*, *Brinp3*, *Brwd1*, *C430002N11Rik*, *Cachd1*, *Cadps*, *Camk1g*, *Ccdc19*, *Ccdc23*, *Cd46*, *Cdc5l*, *Cdk5rap2*, *Cdyl2*, *Cep162*, *Cga*, *Chd1*, *Chm*, *Chmp5*, *Clvs1*, *Cntn5*, *Cntnap5b*, *Col5a2*, *Csmd2*, *Ctnna3*, *D030040B21Rik*, *Dag1*, *Dennd1a*, *Dip2c*, *Dis3l2*, *Disp1*, *Dlx5*, *Dnajc6*, *Dnm3*, *Dock1*, *Dopey1*, *Dpp10*, *Dpy19l4*, *Dsp*, *E030011O05Rik*, *E130309D14Rik*, *E130309F12Rik*, *Edem3*, *Eif4g3*, *Elavl1*, *Elavl4*, *Emr4*, *Eomes*, *Epha4*, *Epha6*, *Erc2*, *Ermap*, *Esp34*, *Etohd2*, *Exoc6b*, *Faf1*, *Faim*, *Fam172a*, *Fbxl17*, *Fhit*, *Fip1l1*, *Foxj3*, *Frmd3*, *Fyb*, *Gad2*, *Gli3*, *Glycam1*, *Gm11487*, *Gm4847*, *Gm9054*, *Gnb1*, *Gpatch2*, *Gpatch2l*, *Gpc5*, *Gpr63*, *Grhl3*, *Gtdc1*, *Gulp1*, *Hcn1*, *Hdac4*, *Hecw2*, *Hmgcll1*, *Hoxc4*, *Htr1d*, *Htr5b*, *Hykk*, *Ibtk*, *Igfbpl1*, *Il23r*, *Iqcf5*, *Isca1*, *Jam3*, *Kcnd2*, *Kcnd3*, *Kcnh1*, *Kcnk5*, *Krt75*, *Lama2*, *Lnp*, *Lrch1*, *Lrrfip2*, *Lsamp*, *Lypd6b*, *March1*, *Mdga2*, *Mir297c*,

Mir29b-2, Mir29c, Mir669a-1, Mir669a-10, Mir669a-11, Mir669a-12, Mir669a-4, Mir669a-5, Mir669a-6, Mir669a-7, Mir669a-8, Mir669a-9, Mir669e, Mir669p-1, Mir669p-2, Mmp16, Mogat1, Msh3, Mtrf1, Mtr, Mycbp2, Naa15, Naalad2, Nav1, Ncam2, Nckap5, Ndst4, Nfx1, Nkain3, Npas2, Ntm, Olfr1259, Olfr1367, Olfr250, Olfr846, Olfr887, Olfr899, Ormdl1, Osgin2, Pappa, Pard3b, Pax3, Pax5, Pbx1, Pcca, Pcdh11x, Pcdh15, Pde6c, Pde8a, Pde8b, Pdpn, Pdzn4, Peak1, Pik3r3, Pitpnm2, Pkhd1, Pknx2, Pla2g7, Plcl1, Pms1, Ppapdc1b, Ppp1r9b, Prex2, Prg4, Primpol, Prkag2, Prkdc, Ptpnc, Ptpn22, Pum1, Pvr, Rab3b, Ralgs2, Rasa3, Rasal2, Rassf3, Rblcc1, Rbm6, Reep1, Rfx7, Rgs7, Ric3, Rims1, Rims2, Riok2, Rngt, Rora, Rpgrip1l, Rtn1, Rtn3, Sall1, Serpinb3d, Sf3a1, Sgca, Sgpp2, Skint1, Skint3, Skint5, Slc25a21, Slc41a2, Slitrk6, Smad1, Smarcal1, Sorcs1, Spata17, Srgap2, Ssbp3, Stat1, Tab2, Tacc2, Tancl, Tbc1d10a, Tbx20, Tcaim, Tceb1, Tek, Tenm2, Tll1, Tmem163, Tmem65, Top2b, Tor2a, Tox, Tox3, Tppp, Tpst2, Trpm3, Ttc39b, Txln, Ubtd2, Ugg1, Ulk4, Unc13c, Unc80, Utp20, Vcp, Vti1b, Vwc2, Wdfy3, Whsc1l1, Zbtb7a, Zcchc2, Zfp292, Zfp62, Zfp692, Zfp827, Zmym4, Zswim6, Syndig1, Ak1, Cntnap5a, Cpa5, Dhdds, Eng, Fbxl5, Filip1, Gm10439, Gm13490, Golt1b, Lin28a, Magi2, Me2, Mtus2, Nfia, Orc3, Recql, St8sia6, Ube2cbp, 1700019G24Rik, 4930527F14Rik, Aff2, Aff3, Arhgef10l, Astn1, Cd2ap, Cd99l2, Chl1, Cyp2b10, Dync1i1, Esr1, Fam19a5, Fermt2, Fmn2, Foxn2, Frmpd4, Il1rap1l, Itga6, Klf13, Lepr, Mir1933, Myo3a, Nacc2, Olfr850, Olfr851, Pgam2, Phf21a, Raf1, Rictor, Ugp2, and Wbscr27.

[0092] Modulating Expression of T-Cell Genes to Enhance Immunotherapy

[0093] The methods disclosed herein may be utilized to identify genes that are involved in T-cell trafficking and activation in solid tumors. The expression of the identified genes may be modulated in T-cells to enhance immunotherapy, for example, CAR T-cell immunotherapy for treating a cell proliferative disorder in a subject in need thereof. In some embodiments, the disclosed methods may include genetically modifying a T-cell to modulate the expression of a gene involved in T-cell trafficking and activation in solid tumors and/or administering a therapeutic agent to the subject that modulates the expression and/or the biological activity of a gene involved in T-cell trafficking and activation in solid tumors.

[0094] The disclosed therapeutic methods may include administering chimeric antigen receptor (CAR) T-cell therapy to a subject wherein the CAR T-cells administering in the therapy

target an antigen expressed on a solid tumor. In some embodiments, the CAR T-cells may be further genetically modified in a manner that results in increased or decreased expression of a gene that is involved in T-cell trafficking or activation, which may include, but is not limited to *Aak1*, *Crct3*, or both of *Aak1* and *Crct3* (and/or gene products encoded by *Aak1* or *Crct3*). In further embodiments, the CAR T-cells may be administered with a therapeutic agent that results in increased or decreased expression or biological activity of a gene involved in T-cell trafficking or activation, which may include, but is not limited to *Aak1*, *Crct3*, or both of *Aak1* and *Crct3* (and/or gene products encoded by *Aak1* or *Crct3*). As such, the therapeutic agent administered in the disclosed methods, which modulates the expression and/or biological activity of the gene products of the identified genes, may include a therapeutic agent that is utilized for administering chimeric antigen receptor (CAR) T-cell therapy to the subject.

[0095] In particular, the disclosed therapeutic methods may include methods for treating cancer such as melanoma, adenocarcinoma, sarcoma, teratocarcinoma, and lymphoma. In some embodiments, the disclosed therapeutic methods may be utilized for treating tumors of tissues selected from adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, colon, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, prostate, skin, testis, thymus, and uterus.

[0096] The disclosed therapeutic methods may include administering a therapeutic agent that modulates the activity of T cells. In some embodiments, the therapeutic agent modulates T cell trafficking and/or infiltration into a tumor and/or the therapeutic agent modulates T cell stimulation and/or activation (*e.g.*, against a tumor).

[0097] The disclosed compositions, kits, and methods may be utilized to identify genes that are involved in T-cell trafficking and/or T-cell infiltration into tumors. For example, the disclosed compositions, kits, and methods may be utilized to identify immune checkpoint regulators in order to develop new immunotherapies such as immune checkpoint inhibitor therapies. Immune checkpoint inhibitors are known in the art and include anti CTLA-4 antibodies (*e.g.*, Ipilimumab or Tremelimumab), anti PD-1 antibodies (MDX-1106, BMS-936558, MK3475, CT-011, AMP-224), anti PD-L1 antibodies (*e.g.*, MDX-1105), anti IDO-1 antibodies, anti IDO-2 antibodies, anti KIR antibodies, anti CD70 antibodies, anti LAG-3

antibodies (*e.g.*, IMP321), anti B7-H3 antibodies (*e.g.*, MGA271), anti B7-H4 antibodies, anti TIM3 antibodies, and combinations thereof.

[0098] The disclosed therapeutic methods may include administering immunotherapy to a subject. As such, the therapeutic agent administered in the disclosed methods, which modulates the expression and/or biological activity of the gene productions of the identified genes, may include a therapeutic agent that is utilized for administering immunotherapy to the subject. Optionally, the disclosed methods may include administering another therapeutic agent that is utilized for administering immunotherapy to the subject (*e.g.*, an immune checkpoint blockade modulator such as an anti-PD-1 therapeutic agent to the subject (*e.g.*, an anti-PD-1 therapeutic agent comprising an anti-PD-1 antibody or an antigen binding fragment thereof)).

[0099] The disclosed therapeutic methods typically include administering a therapeutic agent that modulates the expression and/or biological activity of the gene productions of the identified genes. In some embodiments, the therapeutic agent is an antibody or an antigen binding fragment thereof (*e.g.*, a single chain variable fragment (scFv)) that binds to the gene product and modulates (*e.g.*, inhibits) the biological activity of the gene product. A suitable antibody or an antigen binding fragment thereof may include a monoclonal antibody or an antigen binding fragment thereof (*e.g.*, a humanized monoclonal antibody or an antigen binding fragment thereof).

[00100] In some embodiments, the therapeutic agent that is administered in the therapeutic methods is a therapeutic agent that is a small molecule that inhibits the biological activity of the gene products of the identified genes. In some embodiments, a gene product of an identified gene has an enzymatic activity (*e.g.*, kinase activity), and the small molecule inhibits the enzymatic activity of the gene product.

[00101] In some embodiments, the therapeutic agent that is administered in the therapeutic methods is a therapeutic agent that inhibits expression of the gene product. For example, the disclosed therapeutic methods may utilize RNA interference to inhibit expression of a gene product of an identified gene. Methods of utilizing RNA interference are known in the art and

may include, but are not limited to, the use of small interfering RNA (siRNA) and/or small hairpin RNA (shRNA).

[00102] Experimental Mice and Screening Methods

[00103] Also disclosed herein are methods for identifying T cell genes that may be associated with T cell biological activities such as trafficking and/or infiltration into a tumor and/or T cell stimulation and/or activation against a tumor. In some embodiments, the methods include: (a) isolating T cells from a syngeneic tumor in mice comprising endogenous T cells that have been mutagenized using a Sleeping Beauty transposon system; and (b) identifying in genomic DNA of the isolated T cells genes that have been mutagenized by the Sleeping Beauty transposon system (*e.g.*, by sequencing the genomic DNA). The T cells isolated in the method may include, for example, CD4+ cells and/or CD48+ cells. In some embodiments, the mice will be treated with an immunotherapeutic agent prior to isolating T cells from the tumors of the mice. For example, the mice may be treated with an anti-PD-1 therapeutic agent prior to isolating T cells from the tumors of the mice. The identified genes further may be tested to determine whether the identified genes are associated with T cell biological activities such as trafficking and/or infiltration into a tumor and/or T cell stimulation and/or activation against a tumor. Suitable tumors for use in the disclosed methods may include, but are not limited to, tumors of melanoma cells, adenocarcinoma cells, sarcoma cells, or teratocarcinoma cells. Suitable tumors for use in the disclosed methods may include tumors comprising cancer cells of a tissue selected from, but not limited to, adrenal gland, bladder, bone, bone marrow, brain, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, prostate, skin, testis, thymus, and uterus.

[00104] Also contemplated herein are experimental mice, optionally cohorts of mice, comprising endogenous T cells that have been mutagenized using a Sleeping Beauty transposon system. The contemplated experimental mice may be utilized in the disclosed methods by inserting a syngeneic tumor in the mice, optionally treating the mice with an immunotherapeutic agent (*e.g.*, an anti-PD-1 therapeutic agent), isolating T cells from the syngeneic tumor, and identifying in genomic DNA of the T cells genes which have been mutagenized using a Sleeping Beauty transposon system.

[00105] *Aak1* Gene

[00106] Herein the *Aak1* has been shown to be involved in T cell trafficking and/or activation in solid tumors. As such, expression of the *Aak1* gene including any polypeptide encoded therein may be modified in T cells in order to modulate trafficking and/or activation in solid tumors.

[00107] The nucleic acid sequence of the *Aak1* gene and the amino acid sequence of the Aak1 protein and variants are known in the art. The *Aak1* gene is present on chromosome 2 of the human genome and its nucleic acid sequence is reported in the Genome Reference Consortium Human Build 38 patch release 12 (GRCh38.p12). (See NC_000002.12 (69457995..69643845, complement). SEQ ID NO:1 provides the sequence of the *Aak1* gene. Transcription variants are expressed from the *Aak1* gene and encode a long form Aak1 polypeptide having 961 amino acids (SEQ ID NO:2) and a short form Aak1 polypeptide having 823 amino acids (SEQ ID NO:3), wherein the short form Aak1 polypeptide lacks the 138 C-terminal amino acids present in the long form Aak1 polypeptide.

[00108] Using the methods disclosed herein, the present inventors have identified a variant of the Aak1 long form polypeptide (SEQ ID NO:4). The variant is translated from a variant mRNA having a start codon positioned at amino acid position 126 of the Aak1 long form polypeptide precursor and amino acid position 80 of the processed Aak1 long form polypeptide precursor having the signal peptide removed. As such, the variant includes an 80 N-terminal amino acid truncation relative to the Aak1 full-length processed polypeptide and may be referred to herein as dN80, signifying the 80 amino acid N-terminal truncation relative to the Aak1 full-length processed polypeptide. The dN80 variant also has a C-terminal truncation relative to the Aak1 full-length processed polypeptide and lacks the 139 C-terminal amino acids that are present in the Aak1 full-length processed polypeptide and terminates with a GGS amino acid sequence. The dN80 variant, therapeutic compositions comprising the dN80 variant, and uses thereof for modulating T cell trafficking and/or activation in solid tumors are specifically contemplated herein.

[00109] The *Aak1* gene is hypothesized to encode a further polypeptide having 302 amino acids. (See SEQ ID NO:5).

[00110] *Crct3* Gene

[00111] Herein the *Crct3* has been shown to be involved in T cell trafficking and/or activation in solid tumors. As such, expression of the *Crct3* gene including any polypeptide encoded therein may be modified in T cells in order to modulate trafficking and/or activation in solid tumors.

[00112] The nucleic acid sequence of the *CREB Regulated Transcription Coactivator 3* (*Crct3*) *Aak1* gene and the amino acid sequence of the Crct3 protein and variants are known in the art. The *Crct3* gene is present on chromosome 15 of the human genome and its nucleic acid sequence is reported in the Genome Reference Consortium Human Build 38 patch release 12 (GRCh38.p12). (See NC_000015.10 (90529886..90645345)). SEQ ID NO:6 provides the sequence of the *Crct3* gene. Transcription variants are expressed from the *Crct3* gene and encode a long form Crct3 polypeptide having 619 amino acids (SEQ ID NO:7), a medium form *Crct3* polypeptide having 586 amino acids (SEQ ID NO:8), and a short form *Crct3* polypeptide having 428 amino acids (SEQ ID NO:9).

[00113] Chimeric Antigen Receptor (CAR) T-cells

[00114] Chimeric Antigen Receptor (CAR) T-cells and their use in immunotherapy for diseases such as cancer are known in the art. (See, e.g., June *et al.*, "CAR T cell immunotherapy for human cancer," *Science* 2018 Mar 23;359(6382):1361-1365; Xu *et al.*, "The development of CAR design for tumor CAR-T cell therapy," *Oncotarget*. 2018 Jan 12;9(17):13991-14004; Chen *et al.*, "Driving CARs on the uneven road of antigen heterogeneity in solid tumors," *Curr Opin Immunol*. 2018 Mar 16;51:103-110; Vormittag *et al.*, "A guide to manufacturing CAR T cell therapies," *Curr Opin Biotechnol*. 2018 Feb 17;53:164-181; D'Aloia *et al.*, "CAR-T cells: the long and winding road to solid tumors," *Cell Death Dis*. 2018 Feb 15;9(3):282; Ye *et al.*, "Engineering chimeric antigen receptor-T cells for cancer treatment," *Mol Cancer*. 2018 Feb 15;17(1):32; Li *et al.*, "Chimeric antigen receptor T cell (CAR-T) immunotherapy for solid

tumors: lessons learned and strategies for moving forward,” J Hematol Oncol. 2018 Feb 13;11(1):22; Yoon *et al.*, “Incorporation of Immune Checkpoint Blockade into Chimeric Antigen Receptor T cells (CAR-Ts): Combination or Built-In CAR-T,” Int J Mol Sci. 2018 Jan 24;19(2); Wang *et al.*, “New Chimeric Antigen Receptor Design for Solid Tumors,” Frontiers in Immunol. 2017 Dec; 3:1934; the contents of which are incorporated herein by reference in their entireties).

[00115] Methods for preparing CAR-T cells and their use in treating cancer have been described in patent documents. (*See, e.g.*, U.S. Patent Nos. 9,932,572; 9,932,406; 9,932,405; 9,931,347; 9,920,132; 9,914,909; 9,913,882; 9,907,842; 9,889,161; 9,889,160; 9,868,774; 9,856,322; 9,855,298; 9,855,297; 9,845,362; 9,834,545; 9,828,435; 9,822,340; 9,821,012; 9,821,011; 9,815,901; 9,809,581; 9,803,022; 9,790,282; 9,790,278; 9,783,591; 9,777,064; 9,777,061; 9,765,342; 9,765,142; 9,745,368; 9,725,519; 9,714,278; 9,701,758; 9,688,760; 9,688,740; 9,670,281; 9,663,763; 9,662,405; 9,657,105; 9,650,428; 9,636,388; 9,629,877; 9,624,306; 9,624,276; 9,598,489; 9,597,357; 9,587,237; 9,587,020; 9,580,685; 9,573,988; 9,572,837; 9,572,836; 9,562,087; 9,540,445; 9,522,955; 9,518,123; 9,499,855; 9,492,499; 9,487,800; 9,481,728; 9,464,140; 9,453,075; 9,447,194; 9,446,105; 9,422,351; 9,416,190; 9,409,994; 9,409,992; 9,402,865; 9,394,368; 9,393,268; 9,365,641; 9,359,447; 9,328,156; 9,315,585; 9,273,283; 9,272,002; 9,266,960; 9,220,728; 9,181,527; 9,175,308; 9,169,328; 9,163,258; 9,156,915; 9,102,761; 9,102,760; 9,101,584; 9,040,669; 8,975,071; 8,956,828; 8,916,381; 8,911,993; 8,906,682; 8,822,647; 8,822,196; 8,802,374; 8,486,911; and 8,465,743; the contents of which are incorporated herein by reference in their entireties.

[00116] CAR-T cells having receptors that are targeted to antigens present on solid tumors have been designed and are being tested in clinical trials. (*See* Wang *et al.*, “New Chimeric Antigen Receptor Design for Solid Tumors,” Frontiers in Immunol. 2017 Dec; 3:1934). Antigenic targets of these CAR-T cells for solid tumors include epidermal growth factor receptor (EGFR) (*e.g.*, for targeting glioblastoma and non-small cell lung cancer), mesothelin (*e.g.*, for targeting pancreatic cancer, mesotheliomas, ovarian cancers, and lung cancers), glypican-3 (*e.g.*, for targeting hepatocellular carcinoma (HCC), malignant rhabdoid tumors (MRTs), embryonal sarcoma, and lung cancers), receptor tyrosine-protein kinase erbB-2 (*e.g.*, for targeting lung cancer ovarian cancer, and breast cancer), prostate specific membrane antigen (PMSA) (*e.g.*, for

targeting prostate cancer), human epidermal growth factor receptor 2 (HER2) (*e.g.*, for targeting breast cancer and sarcoma), mucin 1 (MUC1) (*e.g.*, for targeting hepatocellular carcinoma (HCC), non-small cell lung cancer, and triple-negative breast cancer), carcinoembryonic antigen (CEA) (*e.g.*, for targeting liver metastases), and IL-13 receptor alpha (*e.g.*, for targeting glioblastoma). As such, suitable antigenic targets for the modified CAR-T cells as contemplated herein may include, but are not limited to epidermal growth factor receptor (EGFR), mesothelin, receptor tyrosine-protein kinase erbB-2, prostate specific membrane antigen (PMSA), human epidermal growth factor receptor 2 (HER2), mucin 1 (MUC1), carcinoembryonic antigen (CEA), and IL-13 receptor alpha.

[00117] CAR-T cells as contemplated herein may include, but are not limited to so-called first generation, second generation, and third generation CAR-T cells. (*See, e.g.*, Hartmann *et al.*, “Clinical development of CAR T-cells—challenges and opportunities in translating innovative treatment concepts,” *EMBO Molec Medic.* (2017) e201607485; the contents of which is incorporated herein by reference in its entirety).

[00118] CAR-T cells as contemplated herein express a chimeric antigen receptor that typically comprises a “single chain variable fragment.” As used herein, the phrases “single chain variable fragment”, “single-chain antibody variable fragments”, and “scFv” antibodies refer to forms of antibodies comprising the variable regions of only the heavy and light chains, connected by a linker peptide.

[00119] Single-chain antibody fragments can overcome some of the problems associated with the use of antibodies containing a part or all of a constant domain. For example, single-chain antibody fragments tend to be free of undesired interactions between biological molecules and the heavy-chain constant region, and/or other unwanted biological activities. Additionally, single-chain antibody fragments are considerably smaller than whole antibodies and can therefore be characterized by greater capillary permeability than whole antibodies, allowing single-chain antibody fragments to localize and bind to target antigen-binding sites more efficiently. Also, antibody fragments can be produced on a relatively large scale in prokaryotic cells, thus facilitating their production. Furthermore, the relatively small size of single-chain antibody fragments makes them less likely than whole antibodies to provoke an immune

response in a recipient. The single-chain antibody fragments of the presently disclosed subject matter include, but are not limited to single chain fragment variable (scFv) antibodies and derivatives thereof such as, but not limited to tandem di-scFv, tandem tri-scFv, diabodies, triabodies, tetrabodies, miniantibodies, and minibodies.

EXAMPLES

[00120] The following examples are illustrative and are not intended to limit the disclosed and claimed subject matter.

[00121] Example 1 – T-Cell Trafficking Genes Identification Using the Sleeping Beauty Transposon

[00122] Reference is made to the Invention Disclosure Form submitted in 2016 and entitled “T-Cell Trafficking Genes Identification Using the Sleeping Beauty Transposon,” by George Weiner, Laura Rogers, and Adam Dupuy.

[00123] Immune checkpoint blockade therapies, including anti-PD1 and anti-PDL1, have been hugely beneficial in treating late-stage malignant melanoma. However, therapeutic success is only achieved in about 30% of patients. One important determinant of immune checkpoint blockade success is intratumoral T cell infiltration. Thus, increasing T cell infiltration is a viable approach to enhance immunotherapy response. We developed an in vivo screen-based approach to identify novel candidate genes involved in T cell trafficking. Our genetic screen approach uses the Sleeping Beauty (SB) transposon system to randomly mutagenize the genome of endogenous T cells, followed by subcutaneous injection of B16F0 melanoma cells. We hypothesize that mutations in T cells that are over-represented in intratumoral T cells compared to splenic T cells will reveal candidate genes biologically responsible for T cell trafficking and expansion within tumors. In initial studies, we identified three candidate genes (*Crtc3*, *Astn2*, *Gpatch8*) that, when disrupted, could increase intratumoral T cell trafficking. These three genes have not been well characterized in the literature, but what is known about them suggests they could impact on intratumoral trafficking or growth. Additional clonal insertion sites were identified in genes with

known functions involved in T cell activity, but these were not statistically significant due to the cohort size of these preliminary experiments. We expect to be able to identify additional candidate genes as the studies progress. These genes could be used as therapeutic targets to enhance the efficacy of checkpoint blockade therapy. For example, the genes identified in the disclosed methods could have therapeutic and diagnostic applications in the fields of immunology, oncology, and cancer research, and be used to overcome a drawback to current immunotherapies, specifically that current therapies have limited efficacy in poorly-infiltrated tumors. The genes we have identified have the potential to augment intratumoral T cell infiltration, and extensive literature searches have not produced any publications that previously implicated these as functionally contributing to tumor infiltrating lymphocyte behavior.

[00124] Example 2 – Using *Sleeping Beauty* mutagenesis to identify novel immunotherapy targets in a syngeneic mouse model of melanoma

[00125] Reference is made to the Abstract entitled “Using *Sleeping Beauty* mutagenesis to identify novel immunotherapy targets in a syngeneic mouse model of melanoma,” which refers to a presentation given at the 2016 University of Iowa Melanoma Symposium.

[00126] Background and Rationale: Immune checkpoint blockade therapies, including anti-PD1 and anti-PDL1, have been hugely beneficial in treating late-stage malignant melanoma. However, therapeutic success is only achieved in about 30% of patients, and understanding response determinants has become a major area of research. It is becoming clear that at least one important determinant of immune checkpoint blockade success is intratumoral T cell infiltration. A recent publication demonstrated that treating tumors with an agent that increased T cell numbers in the tumor enhanced the efficacy of anti-PDL1 in their mouse model (Tang et al., 2016). Thus, increasing T cell infiltration is a viable approach to enhance immunotherapy response. We have designed an *in vivo* screen-based approach to identify novel candidate genes involved in T cell trafficking using the B16F0 syngeneic mouse model of melanoma. Our rationale for using the B16F0 melanoma model is that subcutaneous tumors are poorly infiltrated by T cells and, perhaps as a result, respond poorly to immune checkpoint blockade. Our genetic

screen approach uses the *Sleeping Beauty* (SB) transposon system to randomly mutagenize the genome of endogenous T cells, followed by subcutaneous injection of B16F0 melanoma cells. We hypothesize that mutations in T cells that are over-represented in intratumoral T cells compared to splenic T cells will reveal candidate genes biologically responsible for T cell trafficking and expansion within tumors. These candidate genes can then be validated in subsequent experiments to determine whether they impact on T cell function, increase T cell infiltration and enhance the efficacy of checkpoint blockade therapy.

[00127] Methods: We performed a forward genetic screen to identify genes that impact on infiltration of T cells into melanoma using SB transposon insertional mutagenesis in T cells. By carrying out this screen *in vivo*, the complexity of the tumor microenvironment is preserved. Other advantages of using the SB system include the ability to cause both gain- and loss-of-function mutations, as well as unbiased mutagenesis of the entire genome followed by easy identification of insertion sites using an Illumina sequencing-based method. Adult mice with mutagenized T cells were injected subcutaneously with B16F0 melanoma cells bilaterally, and tumors were allowed to grow for three weeks (n=70). At that time, left and right tumors, as well as the spleen, from each animal were harvested and submitted for high throughput sequencing. Bioinformatic analyses were performed to determine which insertions were present in both tumors but absent from the spleen within an individual mouse.

[00128] Results: In initial studies, we identified three candidate genes that, when disrupted, could increase intratumoral T cell trafficking. These three genes have not been well characterized in the literature, but what is known about them suggests they could impact on intratumoral trafficking or growth. Additional clonal insertion sites were identified in genes with known functions involved in T cell activity, but these were not statistically significant due to the cohort size of these preliminary experiments. We conclude that *Sleeping Beauty* mutagenesis can be used to identify novel immunotherapy targets using the B16 model.

[00129] Future studies: We plan to use our approach to identify additional candidate genes by repeating our protocol in additional mice. We will simultaneously begin validation experiments using various knockdown or knockout approaches to investigate the impact of the identified candidate genes on relevant T cell phenotypes both *in vitro* and *in vivo*. We

hypothesize that modulation of our candidate genes will result in enhanced intratumoral T cell trafficking. Finally, we aim to determine whether modulating these targets enhances checkpoint blockade efficacy in mice. Any that are found to enhance T cell infiltration and/or checkpoint blockade efficacy will be considered promising novel therapeutic targets for melanoma treatment. Submission of this research for NIH and other peer-reviewed funding is planned.

[00130] Relevance to Melanoma: Our preliminary data have identified three candidate genes that may be involved in intratumoral T cell trafficking, which is a prerequisite for optimal immune checkpoint blockade therapeutic success. Further identification and characterization of our gene candidates may provide novel immunotherapeutic drug targets that could be used to enhance the efficacy of current immunotherapies. This has the potential to be highly beneficial for melanoma patients that experience suboptimal response to anti-CTLA4, anti-PD1, or anti-PDL1.

[00131] Example 3 – Use of Sleeping Beauty Mutagenesis to Identify Genes that Influence Intratumoral T Cell Infiltration

[00132] Reference is made to the Abstract entitled “Use of Sleeping Beauty Mutagenesis to Identify Genes that Influence Intratumoral T Cell Infiltration,” which was accepted for poster presentation at the American Association for Cancer Research (AACR) 2016 Meeting, April 16-20, 2016.

[00133] Immune checkpoint blockade antibodies (*e.g.* anti-CTLA-4 and anti-PD-1) enhance T cell anti-tumor activity and have produced exciting and durable results in treatment of a number of cancers including melanoma. Unfortunately, response to first generation checkpoint blockade therapy is limited to a subset of patients. A more comprehensive understanding of the genes and molecules involved in T cell checkpoint control and other aspects of anti-tumor T cell activity may allow a wider patient population to benefit from this exciting and new approach to cancer therapy.

[00134] To this end, we performed a forward genetic screen to identify gene pathways that influence intratumoral T cell infiltration using *Sleeping Beauty* (SB) mutagenesis. More specifically, this genetic T cell screen was designed to identify additional genes and molecules responsible for selection and expansion of intratumoral T cells via a variety of T cell processes including T cell receptor (TCR)-mediated activation, clonal expansion of tumor-specific T cells, T cell trafficking into the tumor, and maintenance of prolonged viability once there. Advantages of using SB in a genetic screen include the ability to cause both gain- and loss-of-function mutations, as well as mutagenesis of the entire genome followed by easy identification of insertion sites. In addition, SB screens are performed *in vivo*, and thus preserve the complexity of the tumor microenvironment.

[00135] We generated a pilot cohort of mice (n=12) with SB-mutagenized endogenous T cells. These mice were challenged with syngeneic B16F0 melanoma cells subcutaneously. After tumor development (21 days after tumor challenge), tumor-infiltrating CD4+ and CD8+ T cells were harvested. Splenic T cells representing unselected CD4+ and CD8+ populations were also isolated from the same animals at time of tumor harvest. Harvested T cells were evaluated by high throughput sequencing to identify SB insertion sites. T cells harvested from tumors demonstrated a decrease in clonal insertion sites compared to splenic T cells suggesting intratumoral clonal selection. Moreover, clonal insertions in intratumoral T cells were significantly enriched in or near genes, signifying likely selection for insertions impacting gene function ($p < 0.000003$, Fisher's exact test). Clonal insertion sites in T cells from tumors that were absent from spleens, representing potential immunotherapy targets and including insertion sites in genes known to be associated with the T cell response, were identified. We are currently in the process of expanding this experimental cohort using a larger number of mice.

[00136] We conclude T cell specific SB mutagenesis has the potential to identify novel molecules that influence intratumoral T cell infiltration and can be used to identify additional genes that may contribute to the anti-tumor T cell response.

[00137] Example 4 – Modulating T Cell Genes to Enhance Immunotherapy

[00138] Reference is made to the Invention Disclosure Form submitted in 2017 and entitled “Modulating T Cell Genes to Enhance Immunotherapy,” by George Weiner, Laura Rogers, and Adam Dupuy.

[00139] T cells in particular, and the immune system in general, have amazing potential to eliminate tumor cells throughout the body. This therapeutic potential is evidenced by the clinical success of immunotherapies designed to enhance T cell function, such as immune checkpoint blockade and chimeric antigen receptor T cell (CAR-T) therapies. Despite the remarkable progress to date, expanding success to a broader number of patients is a top priority in the field. T cell infiltration into tumors appears to be an important prerequisite for the success of both immune checkpoint blockade and CAR-T therapies. For example, patients with inflamed tumors have a better response rate to anti-PD-1 than patients whose tumors are immune excluded (T cells present at tumor border, but blocked from entry) or immune deserts (very few, or no, intratumoral T cells). Preclinical models have also demonstrated that increasing intratumoral T cell number enhances immune checkpoint blockade efficacy. Thus, increasing T cell infiltration into the tumor could have beneficial therapeutic impact.

[00140] To this end, we developed an innovative, systematic method to screen for novel therapeutic targets in T cells that impact T cell infiltration into tumors. We have identified 386 T cell genes using various cancer models, most of which have no previously described role in T cells or immunotherapy. The disclosed T cell genes include the following genes: *Aak1*, *1300002E11Rik*, *Ehhadh*, *Map7d1*, *Ptprd*, *Smr3a*, *Susd4*, *Wdr59*, *Astn2*, *Gpatch8*, *Cpa6*, *Cpne4*, *Crtc3*, *Dab1*, *Grid2*, *Itga2b*, *Kdm4c*, *Lingo2*, *Nfu1*, *Opcml*, *Pappa2*, *Skint6*, *Stau2*, *Thsd7b*, *2310015D24Rik*, *2610307P16Rik*, *2810055G20Rik*, *4732456N10Rik*, *4930444G20Rik*, *4930521E06Rik*, *4930544M13Rik*, *6330409D20Rik*, *9330175M20Rik*, *A330023F24Rik*, *Abca12*, *Abcg2*, *Actn2*, *Adam19*, *Adk*, *Agbl4*, *Ago3*, *Agtr1a*, *Aldoart1*, *Alg6*, *Ammecr1*, *Ankrd13c*, *Ankrd44*, *Ankrd6*, *Antxr2*, *Apaf1*, *Arhgap15*, *Arhgap25*, *Armc9*, *Atl3*, *Atp1b1*, *Atp2c1*, *Bag1*, *Bai3*, *Bcl1*, *Bckdhhb*, *Bcl2*, *Birc2*, *Bnc2*, *Bre*, *Brinp3*, *Brwd1*, *C430002N11Rik*, *Cachd1*, *Cadps*, *Camk1g*, *Ccdc19*, *Ccdc23*, *Cd46*, *Cdc5l*, *Cdk5rap2*, *Cdyl2*, *Cep162*, *Cga*, *Chd1*, *Chm*, *Chmp5*, *Clvs1*, *Cntn5*, *Cntnap5b*, *Col5a2*, *Csmd2*, *Ctnna3*, *D030040B21Rik*, *Dag1*, *Dennd1a*, *Dip2c*, *Dis3l2*, *Disp1*, *Dlx5*, *Dnajc6*, *Dnm3*, *Dock1*, *Dopey1*, *Dpp10*, *Dpy19l4*, *Dsp*, *E030011O05Rik*,

E130309D14Rik, E130309F12Rik, Edem3, Eif4g3, Elavl1, Elavl4, Emr4, Eomes, Eph4, Eph6, Erc2, Ermap, Esp34, Etohd2, Exoc6b, Faf1, Faim, Fam172a, Fbxl17, Fhit, Fip1l1, Foxj3, Frmd3, Fyb, Gad2, Gli3, Glycam1, Gm11487, Gm4847, Gm9054, Gnb1, Gpatch2, Gpatch2l, Gpc5, Gpr63, Grhl3, Gtdc1, Gulp1, Hcn1, Hdac4, Hecw2, Hmgcll1, Hoxc4, Htr1d, Htr5b, Hykk, Iltk, Igfbpl1, Il23r, Iqcf5, Isca1, Jam3, Kcnd2, Kcnd3, Kcnh1, Kcnk5, Krt75, Lama2, Lnp, Lrch1, Lrrfip2, Lsamp, Lypd6b, March1, Mdga2, Mir297c, Mir29b-2, Mir29c, Mir669a-1, Mir669a-10, Mir669a-11, Mir669a-12, Mir669a-4, Mir669a-5, Mir669a-6, Mir669a-7, Mir669a-8, Mir669a-9, Mir669e, Mir669p-1, Mir669p-2, Mmp16, Mogat1, Msh3, Mtrf1, Mtr, Mycbp2, Naa15, Naalad2, Nav1, Ncam2, Nckap5, Ndst4, Nfx1, Nkain3, Npas2, Ntm, Olfr1259, Olfr1367, Olfr250, Olfr846, Olfr887, Olfr899, Ormdl1, Osgin2, Pappa, Pard3b, Pax3, Pax5, Pbx1, Pcca, Pcdh11x, Pcdh15, Pde6c, Pde8a, Pde8b, Pdpn, Pdzn4, Peak1, Pik3r3, Pitpnm2, Pkhd1, Pknox2, Pla2g7, Plcl1, Pms1, Ppapdc1b, Ppp1r9b, Prex2, Prg4, Primpol, Prkag2, Prkdc, Ptprc, Ptptrt, Pum1, Pvr, Rab3b, Ralgs2, Rasa3, Rasal2, Rassf3, Rblcc1, Rbm6, Reep1, Rfx7, Rgs7, Ric3, Rims1, Rims2, Riok2, Rngtt, Rora, Rpgr1l, Rtn1, Rtn3, Sall1, Serpinb3d, Sf3a1, Sgca, Sgpp2, Skint1, Skint3, Skint5, Slc25a21, Slc41a2, Slitrk6, Smap1, Smarcal1, Sorcs1, Spata17, Srgap2, Ssbp3, Stat1, Tab2, Tacc2, Tanc1, Tbc1d10a, Tbx20, Tcaim, Tceb1, Tek, Tenm2, Tll1, Tmem163, Tmem65, Top2b, Tor2a, Tox, Tox3, Tppp, Tpst2, Trpm3, Ttc39b, Txlng, Ubtd2, Ugg1, Ulk4, Unc13c, Unc80, Utp20, Vcp, Vti1b, Vwc2, Wdfy3, Whsc1l1, Zbtb7a, Zcchc2, Zfp292, Zfp62, Zfp692, Zfp827, Zmym4, Zswim6, Syndig1, Ak1, Cntnap5a, Cpa5, Dhdds, Eng, Fbxl5, Filip1, Gm10439, Gm13490, Golt1b, Lin28a, Magi2, Me2, Mtus2, Nfia, Orc3, Recql, St8sia6, Ube2cbp, 1700019G24Rik, 4930527F14Rik, Aff2, Aff3, Arhgef10l, Astn1, Cd2ap, Cd99l2, Chl1, Cyp2b10, Dync1i1, Esr1, Fam19a5, Fermt2, Fmn2, Foxn2, Frmpd4, Il1rap1l, Itga6, Klfl3, Lepr, Mir1933, Myo3a, Nacc2, Olfr850, Olfr851, Pgam2, Phf21a, Raf1, Rictor, Ugp2, and Wbscr27.

[00141] Uses for this disclosed technology include, but are not limited to: 1) Using these genes to develop a diagnostic platform to predict immunotherapy success in patients, 2) Using these genes as the basis for developing novel co-therapies to existing immunotherapies including immune checkpoint blockade and CAR-T technologies, and 3) Using these genes as targets for novel single-agent immunotherapies--all of which would aim to improve our ability to treat human disease.

[00142] We have verified that at least one of the identified genes (*Crtc3*) impacts T cell production of cytokines *in vitro*, which are important signaling molecules within the tumor microenvironment that influence T cell anti-tumor activity. Further, these genes were identified in tumors that displayed an increased % T cells inside the tumor, suggesting they may positively impact this process *in vivo*. Further testing is needed to determine whether manipulating the activities of these genes directly enhances existing immunotherapies, or alone as a therapeutic agent, in preclinical models. Further testing is also required using human expression data to assess the predictive value of these genes as biomarkers for immunotherapy clinical success.

[00143] Example 5 – Rationally improving T cell-mediated cancer immunotherapy using *Sleeping Beauty* mutagenesis

[00144] Reference is made to the Abstract and Poster entitled “Rationally improving T cell-mediated cancer immunotherapy using *Sleeping Beauty* mutagenesis,” which will be presented at the University of Iowa Immunology Retreat on August 17, 2017.

[00145] Background: T cells have amazing potential to eliminate tumor cells throughout the body, evidenced by the clinical success of immunotherapies designed to enhance T cell function like immune checkpoint blockade and chimeric antigen receptor T cell (CAR-T) therapies. Expanding success to a broader number of patients is a top priority in the field. T cell infiltration into tumors is an important prerequisite for the success of both immune checkpoint blockade and CAR-T therapies, thus, increasing T cell infiltration into tumors could have beneficial therapeutic impact.

[00146] Methods: We designed a forward genetic screen to identify genes that contribute to intratumoral T cell accumulation using *Sleeping Beauty* (SB) transposon mutagenesis in T cells. The genes we identified have the potential to modify important T cell functions including trafficking to the tumor, clonal expansion, and sustained viability once inside the tumor.

[00147] Results: We identified 312 tumor-enriched genes that were mutated in tumors, but not the spleen, of individual mice. Twenty of these were detected in more than one mouse,

representing strong gene candidates for validation. We demonstrated that one gene candidate is functionally associated with T cell response to activation signals. Specifically, CRISPR-mediated knockout of this gene in a murine T cell line resulted in cytokine production defects after T cell receptor stimulation. Additional screening has begun using other tumor models and anti-PD-1 therapy. Preliminary data from these indicate that few genes are conserved across tumor models and treatment groups.

[00148] Conclusions: We are currently investigating the role of candidate genes in intratumoral T cell accumulation and immunotherapy enhancement using an *in vivo* approach. Together, these experiments have the potential to expand our understanding of T cell infiltration into tumors and may provide previously unexplored strategies to rationally enhance immunotherapy efficacy.

[00149] Example 6 – Rationally Improving T Cell-Mediated Immunotherapy Using Sleeping Beauty Mutagenesis

[00150] Reference is made to the Application for Federal Assistance dated June 8, 2017, and entitled “Rationally Improving T Cell-Mediated Immunotherapy Using Sleeping Beauty Mutagenesis,” principal investigator Laura M. Rogers.

[00151] Research Strategy

[00152] Significance. T cell-mediated immunotherapies, including CAR-T and anti-PD-1, have clear clinical activity in many tumor types, but there is considerable room for improvement^{3,4}. The determinants of success are only now being defined, but they likely require a pre-existing anti-tumor T cell response⁵⁻⁷. Tumors that have minimal T cells infiltrating into tumors, called “immune deserts”, have poor response to immunotherapy in general. At least one group has demonstrated that enhancing T cell infiltration can increase checkpoint blockade efficacy¹. Our unbiased screen approach is designed to identify genes that can be modified in T cells to enhance the efficacy of T-cell cancer immunotherapy. Our work is well suited to address one of the most pressing issues of cancer immunotherapy: rationally identifying promising

combinatorial approaches to provide therapeutic success to a broader patient population. This could lead directly to practical approaches to enhancing T cell therapeutics including CAR-T and immune checkpoint blockade therapies such as anti-PD-1.

[00153] Innovation. We have designed an innovative, systematic method to screen for novel therapeutic targets in T cells that impact T cell infiltration into tumors. This screen allows us to evaluate both loss-of-function and gain-of-function mutations in genes across the entire genome. In studies of intratumoral T cell infiltration in melanoma and lymphoma, we have identified genes that were not previously known to be involved in tumor-associated T cell biology. Thus, both our genetic screening method and the immunotherapeutic potential of the novel genes we have identified are highly innovative.

[00154] Approach

[00155] Introduction. T cells in particular, and the immune system in general, have amazing potential to eliminate tumor cells throughout the body. This therapeutic potential is evidenced by the clinical success of immunotherapies designed to enhance T cell function, such as immune checkpoint blockade⁴ and chimeric antigen receptor T cell (CAR-T) therapies³. Despite the remarkable progress to date, expanding success to a broader number of patients is a top priority in the field.

[00156] T cell infiltration into tumors appears to be an important prerequisite for the success of both immune checkpoint blockade and CAR-T therapies⁷⁻¹⁰. For example, patients with inflamed tumors have a better response rate to anti-PD-1 than patients whose tumors are immune excluded (T cells present at tumor border, but blocked from entry) or immune deserts (very few, or no, intratumoral T cells)⁷. Thus, increasing T cell infiltration into the tumor could have beneficial therapeutic impact.

[00157] We have designed a forward genetic screen to identify T cell genes that contribute to intratumoral T cell accumulation using *Sleeping Beauty* (SB) transposon insertional mutagenesis in T cells (Fig. 1). The SB transposon mutagenesis system has traditionally been used as a cancer gene discovery tool and model of a variety of malignancies¹¹. SB insertional

mutagenesis is accomplished in mice by engineering strains that carry both the DNA transposon and the SB transposase. The SB system was further refined by the design of a Cre-inducible transposase allele. This allows for tissue-specific expression of transposase, thus limiting mutagenesis to a desired cell type. With respect to this proposal, the application of the SB technology to immunotherapy target discovery is entirely unique.

[00158] Preliminary Data

[00159] SB screen to identify novel candidate immunotherapy targets. While SB screens have been used as cancer gene discovery tools in the past, we have begun using the system instead to identify T cell genes involved in the intratumoral accumulation of T cells. The genes we identified have the potential to modify important T cell functions including trafficking to the tumor, clonal expansion, and sustained viability once inside the tumor. We generated a large screen cohort of mice with SB-mutagenized T cells by crossing SB transgenic mice with the CD4-Cre strain (Fig.1A). In this way, we induced mutation of both CD4+ and CD8+ T cell subsets, as CD8+ T cells also express CD4 during the double positive stage of T cell development. Adult offspring with mutagenized T cells were then injected bilaterally with syngeneic melanoma cells (B16F0). This was done using young mice that harbor up to hundreds of mutations in each T cell, but which have not had adequate time to develop T cell malignancies (that emerge at 49 months on average¹²). B16F0 melanomas developed untreated for three weeks to allow for spontaneous T cell infiltration before tissues were collected. High throughput sequencing was performed on the tumors (two tumors per mouse) and spleens from each mouse to identify genes in the T cells mutated by transposon insertions. Sequencing was performed on amplicon libraries generated using primers specific for transposon sequences, enabling rapid identification of the insertion sites. Note that SB insertions arise only in the T cells from these tissue samples due to CD4-Cre mediated tissue specificity. Thus, the malignant cells themselves do not contribute to the genetic signature.

[00160] Significance of candidate genes was determined through subsequent bioinformatics analysis, carried out in two steps (Fig. 1B,C). The first step identified insertion sites that were present in T cells in both tumors, but absent from the spleen, of an individual mouse. By subtracting out background splenic T cell mutation signatures, we are able to focus

specifically on tumor T cell-enriched genetic signatures. Moreover, we expect bona-fide, tumor-specific T cells to be present systemically in both tumors from an individual animal, and so we filtered out mutations that occurred in only T cells from one of the two tumors. These analyses resulted in a list of tumor T cell-enriched insertions: genes that were significantly mutated in intratumoral, but not splenic, T cells from an individual mouse. The second bioinformatics step identified recurring, significantly mutated genes across all mice in the experimental cohort. We hypothesize that tumor-associated T cell genes significantly mutated in more than one mouse are most likely to functionally contribute to intratumoral T cell accumulation and are strong candidates for therapeutic intervention to enhance intratumoral T cell infiltration.

[00161] The gene candidates identified impact T cell function. We observed that clonal insertions in T cells from tumor samples were significantly enriched in or near genes, signifying likely selection for insertions impacting gene function ($P < 0.000003$, Fisher's exact test). Importantly, the mice in which we identified strong genetic signatures also had the highest percentage of T cells inside the tumors (Fig. 2A), supporting their role in enhancing intratumoral T cell accumulation.

[00162] As designed, we expect our screen to identify T cell genes that increase trafficking into the tumor, enhance clonal expansion after activation, and sustain viability within the tumor microenvironment. In fact, a number of gene candidates that we identified do have known functions impacting various T cell processes including metabolic programming of T cells, and T cell receptor (TCR) signaling. In total, we identified 312 tumor-enriched T cell genes that were mutated in both tumors, but not in the spleen of individual mice. Twenty of these were detected in more than one mouse, representing strong gene candidates for validation. Further, we demonstrated that one gene candidate, *Crtc3* ($P = 1.40 \times 10^{-32}$), is functionally associated with T cell response to activation signals. Specifically, CRISPR-mediated knockout of *Crtc3* in a murine T cell line resulted in cytokine production defects after T cell receptor stimulation (Fig. 2B, manuscript in preparation). Together, these data highlight the power and legitimacy of our screen approach.

[00163] Additional screens and identification of Aak1 as a promising immunotherapy target candidate. Given that different tumor types can have different immune

microenvironments, we felt it important to perform a second SB screen using a different tumor model. We selected the EL4 lymphoma model. The lymphoma screen was performed as depicted in Fig. 1, with the exception that EL4 cells were injected instead of B16F0 cells. While this cohort was smaller (n=22 mice versus 98 in the melanoma cohort), we identified 65 tumor-enriched T cell genes and 23 of these were observed in more than one mouse. This experiment is ongoing, with the goal of generating a similar size cohort to that of the melanoma screen.

[00164] We reasoned that genes identified in both tumor models would represent the best candidates to pursue, as they might contribute to T cell accumulation despite differences in the tumor microenvironments. However, genes that passed both bioinformatics steps 1 and 2 (enriched in the tumor versus spleen AND observed in more than one individual) were not shared between tumor models. We did, however, identify 11 genes shared between tumor models that were present in only one mouse in each cohort (Fig. 3A). It is possible that with additional mice in the EL4 cohort, that this list could strengthen.

[00165] In addition to assessing multiple tumor types, we reasoned that perhaps a better approach to identify T cell genes that specifically synergize with immune checkpoint blockade would be to do a sensitized screen with anti-PD-1 treatment. Thus, we generated a small pilot cohort of mice (n=13) injected with B16F0 melanoma and treated twice weekly (10mg/kg doses, injected intraperitoneally) with anti-PD-1 (clone RMP1-14) until tumors were harvested 3 weeks later. Despite having only a small cohort to analyze, we identified 8 tumor-enriched insertions, with 3 in two or more individuals. The top gene candidate, *Aak1* (*AP2-associated kinase 1*), is particularly notable. It was overwhelmingly identified in the anti-PD-1 treated cohort, observed in 9 of the 13 mice (69%). *Aak1* was also the top gene candidate in the untreated melanoma cohort mutated in 6 of 98 mice (6%), and it was the only gene identified in all 3 screen cohorts, appearing in 1 of 22 mice (5%) in the lymphoma cohort. The selection for *Aak1* mutation in 3 screens using 2 distinct tumor models strengthens its potential as a candidate to broadly augment immunotherapy efficacy. Thus, *Aak1* (described in more detail in Aim 1) is a major focus of this research proposal.

[00166] As briefly mentioned, SB mutagenesis can cause both gain- and loss-of-function mutations due to promoter and stop/poly-A signals carried within the transposon itself. Thus,

when inserted in the forward orientation, it can promote expression of downstream exons. If inserted in the reverse orientation, it can introduce a premature stop. All insertions within *Aak1* are located in intron 2, with the majority in the reverse orientation (Fig. 3B). As the coding sequence for the functional kinase domain of Aak1 spans exons 2-3, we would predict that the SB insertions in intron 2 introduce a loss-of-function mutation. Because of this, we propose studying *Aak1* knock out T cells in our initial experiments.

[00167] Summary and hypothesis. In order to identify T cell genes that could enhance existing immunotherapies by increasing intratumoral T cell infiltration, we designed an unbiased forward genetic screen approach using SB. We have completed a screen using an immunocompetent model of melanoma and are well into generating a complementary screen cohort in a model of lymphoma. We have successfully identified a number of promising gene candidates, one of which (*Crtc3*) we have demonstrated impacts cytokine production by activated T cells. Finally, we completed a small pilot study, introducing anti-PD-1 treatment to our screen design to identify mechanisms that synergize specifically with checkpoint blockade. One notable gene candidate, *Aak1*, was identified in mice from all 3 screen cohorts, and *Aak1* mutations were extremely frequent in the anti-PD-1 treated group. Thus, we hypothesize that the genes we identify, including *Aak1*, will enhance intratumoral T cell accumulation and augment anti-PD-1 therapeutic efficacy.

[00168] In Aim 1, we will investigate the role of *Aak1* in T cell trafficking into tumors (Aim 1A) and the anti-tumor activity of modified T cells (Aim 1B) using an *in vivo* adoptive transfer experimental approach. Positive results would be of interest to the CAR-T field. In Aim 2, we propose continuation of our screening approach using additional tumor models and anti-PD-1 treatment. Based on the success of our initial screens, we are confident we will identify additional T cell intrinsic mechanisms that represent rational combinatorial pathways. We will begin by testing *Aak1*, with *Crtc3* as a second candidate, but any newly identified gene candidates from Aim 2 can undergo testing similar to that proposed in Aim 1. We will focus on those candidates that are relevant to more than one tumor model. Together, these experiments have the potential to expand our understanding of T cell infiltration into tumors and may provide previously unexplored strategies to rationally enhance immunotherapy efficacy.

[00169] Experimental Design

[00170] Aim 1. Assess whether modulating gene candidate *Aak1* impacts T cell infiltration or anti-tumor activity. The top candidate we identified in our screens was *Aak1*, which is an AP2-associated kinase expressed in T cells and involved in clathrin-mediated endocytosis¹³. This process impacts a number of T cell signaling proteins, including Notch and CTLA4^{14,15}. Gene expression data from the Immunological Genome Project shows *Aak1* expression correlates with PD-1 (*Pdcd1*) expression (ImmGen.org Data Browser)¹⁶. Together, these data support the functional relevance of *Aak1* in modulating T cell anti-tumor activity and its promise as a candidate for additional validation.

[00171] Importantly, *Aak1* was mutated in tumors, but not spleens, from SB mice in two independent tumor models (Fig. 3). It was prevalent in the B16 melanoma cohort (mutated in 6 of 98 mice), and the EL4 lymphoma cohort (mutated in 1 of 22 mice). Importantly, *Aak1* was mutated in tumor infiltrating T cells in 9 of 13 mice treated with anti-PD-1. All SB insertions were located in *Aak1* intron 2, disrupting the kinase domain and likely causing loss-of-function of *Aak1*. Thus, we hypothesize that disrupting *Aak1* expression will result in enhanced T cell accumulation into tumors.

[00172] Our experimental setup for both Aim 1A and Aim 1B will make use of the same lentiviral CRISPR-Cas9 vector previously used to knock out *Crtc3* in T cells (Fig. 2B). We have engineered this vector to include a fluorescent reporter (EGFP or mCherry) that allows us to select transduced cells using FACS live sorting and will also allow us to quantify modified T cells in tumors. For this experiment, we have generated two CRISPRCas9 lentiviral constructs: an *Aak1* KO vector with both an mCherry or EGFP reporter, and a control non-targeting CRISPR vector with an EGFP reporter.

[00173] We have successfully transduced and adoptively transferred primary CD8+ T cells using the following approach: primary murine CD8+ T cells are isolated from pooled spleens of donor mice (1 donor mouse per recipient mouse) using a negative selection kit (Miltenyi). CD8+ T cells are then activated *in vitro* for one day prior to transduction using anti-CD3/anti-CD28 beads (Dynabeads, Invitrogen) and cultured for 2-4 days posttransduction in

media containing 50U/ml rIL-2 (PeproTech). EGFP or mCherry positive cells are collected using live FACS sorting (FACS Aria, BD), resuspended in saline. A portion of modified T cells will be withheld to confirm *Aak1* knock out by Western blot (anti-AAK1, Abcam). The remaining modified T cells will be injected intravenously into recipient mice as indicated in Sub Sims 1A and 1B.

[00174] Aim 1A: Assessing intratumoral T cell infiltration. To test whether *Aak1* KO enhances intratumoral T cell accumulation, tumor-bearing recipient mice will be generated by injecting wild type C57BL/6J mice (Jackson) with B16F0, or B16F10-Ova, tumor cells subcutaneously on each rear flank. The B16F0 melanoma cell line was used in the original screen, so it is our first choice for initial validation experiments. We will also use the ovalbumin expressing B16F10 (B16F10-Ova) in these studies. Ovalbumin is a model antigen specifically recognized by T cells from OT-1 T cell receptor transgenic mice¹⁷. Thus, by adoptively transferring OT-1 T cells, we not only ensure all donor cells are antigen-specific, but we also mimic the CAR-T approach that has been successful in human clinical trials. Limitations of this approach are discussed below.

[00175] Tumors will be allowed to grow for 7-10 days, and then tumor-bearing mice (n=10 per experimental replicate) will receive 1×10^6 transduced C57BL/6J or OT-1 T cells intravenously. We will mix FACS-sorted *Aak1* KO (mCherry) and control cells (EGFP) 1:1 and adoptively transfer this mixture into tumor-bearing mice, such that each tumor-bearing mouse receives both experimental and control cells (Fig. 4a). Tumor-bearing mice will be euthanized 1, 3, and 5 days posttransfer, and flow cytometry will be performed to assess the percentage of CD8+, *Aak1* KO (mCherry+), and control KO (EGFP+) T cells inside each tumor and spleen from each mouse.

[00176] Data will be analyzed as ratios of percent *Aak1* KO (mCherry+) to percent control (EGFP+) in tumors and spleen (Fig. 4B) using a 2-way ANOVA with multiple comparisons and Sidak correction to determine if there is a significant difference in infiltration between *Aak1* KO and control KO cells at each time point. Splenic data will help us determine what effects are tumor microenvironment-specific and what are universal (such as engraftment differences). Based on our hypothesis that disruption of *Aak1* in T cells will increase their intratumoral

accumulation, we expect to see an increased ratio of *Aak1* (mCherry) to control (EGFP) cells in the tumor compared to the same ratio in the spleen, indicating enhanced T cell infiltration with *Aak1* KO. In subsequent experiments, we will inject mice with wild type B16F0 tumor on one flank and B16F10-Ova tumor cells on the other to assess the relative tumor infiltration of *Aak1* modified OT-1 cells into antigen positive and antigen negative tumors.

[00177] Mouse numbers for Aim 1A were calculated such that candidate genes may be tested in at least 2 technical replicates of each experiment, with 10 mice per experimental group per replicate, and using a 1:1 donor to recipient ratio. Experimental group sample size was determined using a 2-sample 2-tailed power calculation to detect a ~2-fold difference in T cell infiltration with 80% power. The use of dual fluorescent reporters allows us to assess relative numbers during flow cytometric analysis of tissues after euthanization and will minimize technical variation.

[00178] Our screen results indicate that different tumor models impact which genes are identified as important for T cell infiltration. In fact, we chose to validate *Aak1* specifically because it was identified in more than one tumor model. Thus, it is important to assess the effect of *Aak1* on T cell infiltration using a second tumor model. Our lab also possesses an ovalbumin-expressing EL4 lymphoma cell line (EG7), and we plan an analogous set of experiments using EG7 tumor-bearing mice as modified-OT-1 recipients.

[00179] The SB system was developed on the C57BL/6 strain background, and all our screens were performed on this background. However, it is widely appreciated that strain background strongly influences the anti-tumor immune response. It will therefore be valuable to assess the impact of modifying *Aak1* in other genetic backgrounds as well. Our laboratory has extensive experience studying immunotherapy, including anti-PD-1 therapy, in the A20 B cell lymphoma model that originated on the BALB/c background. We will therefore assess the effect of knocking out *Aak1* on infiltration of adoptively transferred BALB/c T cells into A20 tumors.

[00180] Aim 1B: Assessing T cell anti-tumor activity. We will also test whether knockout of *Aak1* in CD8⁺ T cells has therapeutic anti-tumor effects, alone or in combination with anti-PD-1. We hypothesize that loss of *Aak1* will enhance CD8⁺ T cell anti-tumor activity,

particularly if *Aak1* impacts intratumoral infiltration. To directly test this, we plan to adoptively transfer *Aak1* KO cells or control cells into tumor-bearing recipients, along with anti-PD-1 therapy or isotype control, and measure tumor growth (Fig. 5). The specific tumor model to used (melanoma or lymphoma) for these therapy studies will be selected based on which model demonstrated the greatest increase in intratumoral T cell infiltration after *Aak1* knockout.

[00181] Given the robust immune response by OT-1 cells to ovalbumin antigen, it is likely that B16F10-Ova or EG7 tumor-bearing mice receiving *Aak1* KO OT-1 cells will exhibit tumor enhanced regression when combined with anti-PD-1 treatment. However, it should be noted that a recent shRNA screen successfully identified at least one novel therapeutic gene that, when knocked down in adoptively transferred T cells, had a therapeutic anti-tumor effect in the absence of other treatments². Therefore, it is important to include treated and untreated subgroups in both the *Aak1* KO and control KO groups, so that we will be able to measure the anti-tumor effect *Aak1* KO alone has, and whether additional therapeutic effect is achieved in combination with anti-PD-1.

[00182] Thus, our experimental design is as follows: recipient mice will receive subcutaneous tumor 7-10 days before adoptive T cell transfer in order to establish tumors before treatment. Tumor-bearing recipient mice (n=15 per treatment group) will then receive *Aak1* KO (EGFP positive, live sorted) CD8+ cells *or* control KO (also EGFP positive, live sorted) CD8+ cells and treated with anti-PD-1 or isotype control antibody. Tumor growth will be measured twice weekly once tumors become palpable and monitored for signs of tumor regression. Survival will also be followed, graphed as a Kaplan-Meier plot and evaluated for significant differences using a log-rank test. Mouse numbers for Aim 1B were calculated such that candidate genes will be tested in at least 2 technical replicates of each experiment using a 1:1 donor to recipient ratio. The experimental group sample size (n=15 mice per experimental group per replicate) was determined using a Cox proportional hazards 2-sided model to assess survival with a hazard ratio of ~3 with 80% power.

[00183] Expected results and alternative approaches

[00184] Aim 1A. Gene modification effect on intratumoral T cell accumulation. We hypothesize that knocking out *Aak1* in T cells will enhance their accumulation in the tumor. Therefore, we expect that *Aak1* KO cells will be present at higher percentages than control KO cells in tumors (Fig. 4B), such that the mCherry:EGFP ratio will be above 1, and higher than the ratio in the spleen. It is possible that knocking out *Aak1* in T cells will not enhance their intratumoral accumulation. Results indicating a marked reduction in intratumoral T cells (opposite to our hypothesis) might suggest that testing *Aak1* overexpression (or gain-of-function) is more appropriate. Results indicating no change in intratumoral accumulation would suggest that we should choose one of our many other gene candidates, such as *Crtc3*, to focus our efforts on instead.

[00185] While widely used to study antigen-specific immunological responses, the OT-1/Ova system does not fully recapitulate the tumor antigen specific immune response. We chose to begin our experimentation using this system because it is so well studied, induces a robust antigen-specific T cell response, and is likely to achieve measureable T cell infiltration even by control “knock out” cells, thus allowing us to provide proof-of-concept data for further study of *Aak1* as a therapeutic target. Additional studies could make use of a second, more physiologically relevant, TCR transgenic/tumor antigen combination such as the Pmel-1/gp100 model¹⁸ or murine CAR-T cells.

[00186] Finally, we have chosen to focus on CD8+ T cells, rather than CD4+ T cells, due to preliminary sequence data on sorted cell populations suggesting that the SB insertions were primarily derived from the CD8+ subpopulation (data not shown). Moreover, many adoptive transfer therapies have focused on using CD8+ T cells due to their cytotoxic potential. However, the role of *Aak1* in CD4+ T cell function may also be important to study.

[00187] Aim 1B. Gene modification effect on anti-tumor activity. The proposed experimental design will allow us to determine whether *Aak1* KO in adoptively transferred T cells enhances the therapeutic effect of the T cells. If *Aak1* KO enhances the anti-tumor efficacy of T cells, with or without anti-PD-1, we would have strong evidence supporting the validity of our hypothesis. Either finding will be useful in enhancing T cell mediated immunotherapies. If

tumor regression is not enhanced with adoptive transfer alone or in combination with anti-PD-1, we have other candidate genes produced by our screens, including *Crtc3*, which we have already shown impacts cytokine production upon T cell activation *in vitro*. Finally, we expect to generate additional gene candidates in Aim 2 that could be validated *in vivo* using the same experimental approaches outlined in Aim 1.

[00188] Aim 2. Identify additional genes that influence intratumoral T cell infiltration and anti-tumor effects in combination with anti-PD-1 across tumor types. Preliminary studies suggest the combination of CAR-T therapy with anti-PD-1 may improve CAR-T efficacy¹⁹. There are likely many as yet undefined molecular mechanisms that may act synergistically with these immunotherapeutic approaches. Aim 2 focuses on identifying additional gene candidates with a focus on those that might be effective in combination with anti-PD-1 and can eventually be tested with retargeted T cells. We hypothesize that SB mutagenesis of T cells in additional tumor models and with anti-PD-1 treatment will identify additional genes that act synergistically with anti-PD-1.

[00189] As described in the preliminary data section, we performed a pilot screen (n=13) similar to that in Fig. 1 to assess how anti-PD-1 treatment impacts genetic selection of SB insertions in intratumoral T cells in the B16F0 model. *Aak1* was the top gene candidate, mutated at a significantly higher rate with anti-PD-1 treatment (9 of 13 mice) than without treatment (Fisher's Exact, $P < 0.0001$). This suggests that anti-PD-1 has a large impact on the selection of T cell genes that are important for intratumoral T cell accumulation, even if it does not have a large therapeutic effect as a single-agent therapy in the B16F0 melanoma model. These results are encouraging, and provide rationale for expanding our pilot screen to include a larger number of mice in order to identify additional T cell genes that may specifically synergize with anti-PD-1 therapy.

[00190] Further, few gene candidates were shared between the melanoma and lymphoma screen cohorts, suggesting that additional tumors models are needed to identify conserved mechanisms that could be broadly applicable to more than one tumor type. Therefore, we propose applying our screen approach to two additional immunocompetent mouse models: MC38 colon carcinoma and LLC lung carcinoma, both of which respond poorly to immune

checkpoint blockade alone^{20,21}. As described in our preliminary data, we will generate mice with SB-mutagenized T cells by breeding CD4-Cre transgenic mice with SB strains, with and without anti-PD-1 treatment (n=70 each model, calculated to allow us to determine significance of a 4-fold difference in prevalence between cohorts with 80% power).

[00191] Candidate genes identified in more than one (and ideally, all) tumor models will be considered the strongest candidates for development as potential immunotherapeutic targets. Further, genes identified in the presence of anti-PD-1 will be compared to the candidate genes from the untreated cohort (n=98) using a Fisher's Exact approach to strengthen the specificity for anti-PD-1 action. For example, clonally expanded insertion sites present in a higher proportion of the anti-PD-1 treated cohort (like *Aak1*) represent gene candidates that could functionally impact anti-PD-1 therapy. We anticipate that this approach will allow us to identify key signaling pathways mediating anti-PD-1 blockade, perhaps including molecules that participate in other immune checkpoint pathways. Promising gene candidates will be targeted in T cells as outlined in Aim 1 to assess their impact on T cell infiltration into tumors (Aim 1A) and therapeutic effect (Aim 1B).

[00192] Expected results and alternative approaches

[00193] Based on our pilot data, we anticipate that expanding our screen to include other tumor models treated with anti-PD-1 will allow us to successfully identify additional gene candidates that are either unique to, or overrepresented in, the anti-PD-1 treatment cohort. These genes will be tested similarly to *Aak1* in for roles in T cell infiltration and therapeutic synergy with immune checkpoint blockade. Given the differences observed between the tumor models, it may be informative to compare the gene candidate lists from untreated counterparts as well.

[00194] Strain background also strongly influences the anti-tumor immune response. Currently, our tumor model choices are limited to the C57BL/6 strain background, since the SB transgenic mice are not available on other strain backgrounds. Thus, it will be important to validate promising candidates using diverse strain background as described in Aim 1.

[00195] Future studies and long-range objectives

[00196] Our studies are designed to identify T cell genes that can be modified to enhance T cell-mediated immunotherapies such as CAR-T and immune checkpoint blockade. Preliminary studies have confirmed the SB model can be used to identify candidate genes that impact T cell function, and we have already identified *Aak1* as the most promising to test in preclinical models. Successful completion of the proposed studies could lead to three different avenues of investigation:

[00197] 1. Modifying *AAK1* (and alternative genes identified in our SB screen) in human T cells could be explored as a way to enhance the efficacy of CAR-T cells or other adoptive T cell therapies.

[00198] 2. AAK1 and CRTC3—another candidate identified in the melanoma screen—are kinases for which small molecule inhibitors have been developed^{22,23}. While their protein expression is not limited to T cells, further studies could be done to assess whether such small molecules impact on T cell infiltration into tumors.

[00199] 3. Further studies could be done to understand the biology of how modification of the genes identified in our SB screens impact on T cell infiltration into tumors. Understanding whether modification enhances T cell trafficking, expansion or sustained viability within the tumor could be particularly important. This would include both laboratory studies and correlative science exploring expression of the identified gene (such as *Aak1*) in tumor infiltrating lymphocytes using immunohistochemistry on human tumor biopsies.

[00200] References

[00201] 1. Tang, H. *et al.* Facilitating T Cell Infiltration in Tumor Microenvironment Overcomes Resistance to PDL1 Blockade. *Cancer cell* 29, 285-296, doi:10.1016/j.ccell.2016.02.004 (2016).

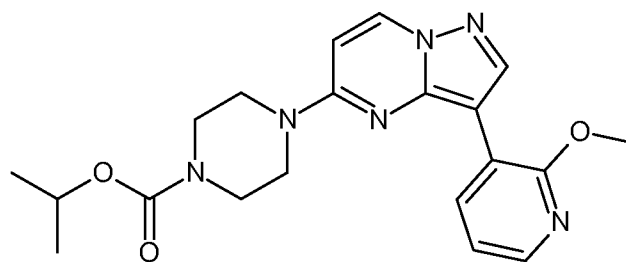
[00202] 2. Zhou, P. *et al.* In vivo discovery of immunotherapy targets in the tumour microenvironment. *Nature* 506, 52-57, doi:10.1038/nature12988 (2014).

- [00203]** 3. Wang, Z., Guo, Y. & Han, W. Current status and perspectives of chimeric antigen receptor modified T cells for cancer treatment. *Protein & cell*, doi:10.1007/s13238-017-0400-z (2017).
- [00204]** 4. Iwai, Y., Hamanishi, J., Chamoto, K. & Honjo, T. Cancer immunotherapies targeting the PD-1 signaling pathway. *Journal of biomedical science* 24, 26, doi:10.1186/s12929-017-0329-9 (2017).
- [00205]** 5. Gajewski, T. F., Louahed, J. & Brichard, V. G. Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. *Cancer journal* 16, 399-403, doi:10.1097/PPO.0b013e3181eacbd8 (2010).
- [00206]** 6. Ji, R. R. *et al.* An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer immunology, immunotherapy : CII* 61, 1019-1031, doi:10.1007/s00262-011-1172-6 (2012).
- [00207]** 7. Chen, D. S. & Mellman, I. Elements of cancer immunity and the cancer-immune set point. *Nature* 541, 321-330, doi:10.1038/nature21349 (2017).
- [00208]** 8. Kershaw, M. H. *et al.* A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 12, 6106-6115, doi:10.1158/1078-0432.CCR-06-1183 (2006).
- [00209]** 9. Papa, S., van Schalkwyk, M. & Maher, J. Clinical Evaluation of ErbB-Targeted CAR T-Cells, Following Intracavity Delivery in Patients with ErbB-Expressing Solid Tumors. *Methods in molecular biology* 1317, 365-382, doi:10.1007/978-1-4939-2727-2_21 (2015).
- [00210]** 10. Enblad, G., Karlsson, H. & Loskog, A. S. CAR T-Cell Therapy: The Role of Physical Barriers and Immunosuppression in Lymphoma. *Human gene therapy* 26, 498-505, doi:10.1089/hum.2015.054 (2015).

- [00211]** 11. Mann, M. B., Jenkins, N. A., Copeland, N. G. & Mann, K. M. Sleeping Beauty mutagenesis: exploiting forward genetic screens for cancer gene discovery. *Current opinion in genetics & development* 24, 16-22, doi:10.1016/j.gde.2013.11.004 (2014).
- [00212]** 12. Berquam-Vrieze, K. E. *et al.* Cell of origin strongly influences genetic selection in a mouse model of TALL. *Blood* 118, 4646-4656, doi:10.1182/blood-2011-03-343947 (2011).
- [00213]** 13. Conner, S. D. & Schmid, S. L. Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis. *The Journal of cell biology* 156, 921-929, doi:10.1083/jcb.200108123 (2002).
- [00214]** 14. Gupta-Rossi, N. *et al.* The adaptor-associated kinase 1, AAK1, is a positive regulator of the Notch pathway. *The Journal of biological chemistry* 286, 18720-18730, doi:10.1074/jbc.M110.190769 (2011).
- [00215]** 15. Shiratori, T. *et al.* Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with clathrin-associated adaptor complex AP-2. *Immunity* 6, 583-589 (1997).
- [00216]** 16. Heng, T. S., Painter, M. W. & Immunological Genome Project, C. The Immunological Genome Project: networks of gene expression in immune cells. *Nature immunology* 9, 1091-1094, doi:10.1038/ni1008-1091 (2008).
- [00217]** 17. Clarke, S. R. *et al.* Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. *Immunology and cell biology* 78, 110-117, doi:10.1046/j.1440-1711.2000.00889.x (2000).
- [00218]** 18. Overwijk, W. W. *et al.* Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *The Journal of experimental medicine* 198, 569-580, doi:10.1084/jem.20030590 (2003).

- [00219]** 19. John, L. B. *et al.* Anti-PD-1 antibody therapy potentially enhances the eradication of established tumors by gene-modified T cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* 19, 5636-5646, doi:10.1158/1078-0432.CCR-13-0458 (2013).
- [00220]** 20. Nagato, T., Lee, Y. R., Harabuchi, Y. & Celis, E. Combinatorial immunotherapy of polyinosinicpolycytidylic acid and blockade of programmed death-ligand 1 induce effective CD8+ T-cell responses against established tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research* 20, 1223-1234, doi:10.1158/1078-0432.CCR-13-2781 (2014).
- [00221]** 21. Kodumudi, K. N. *et al.* Immune Checkpoint Blockade to Improve Tumor Infiltrating Lymphocytes for Adoptive Cell Therapy. *PloS one* 11, e0153053, doi:10.1371/journal.pone.0153053 (2016). Contact PD/PI: Rogers, Laura M. References Cited Page 8
- [00222]** 22. Clark, K. *et al.* Phosphorylation of CRTCL3 by the salt-inducible kinases controls the interconversion of classically activated and regulatory macrophages. *Proceedings of the National Academy of Sciences of the United States of America* 109, 16986-16991, doi:10.1073/pnas.1215450109 (2012).
- [00223]** 23. Kostich, W. *et al.* Inhibition of AAK1 Kinase as a Novel Therapeutic Approach to Treat Neuropathic Pain. *The Journal of pharmacology and experimental therapeutics* 358, 371-386, doi:10.1124/jpet.116.235333 (2016).
- [00224]** Example 7 – Aak1 as a Modulator of T cell Infiltration in Solid Tumors
- [00225]** The role of Aak1 in T cell infiltration and activation was further studied in vitro and in vivo. Figure 6 provides a schematic representation of *Aak1* gene. In the SB assay, we observed that transposon insertion were clustered in intron 2.

[00226] Aak1 or is a protein kinase shown to interact with AP2, from which interaction its name is derived, i.e., “AP2-associated protein kinase.” Aak1 is known to positively regulate Notch signaling, which can have pro-proliferation activity. Aak1 also is involved generally in clathrin-mediated endocytosis and endosomal recycling. Specifically, Aak1 is involved in major histocompatibility complex I (MHCI) and epithelial growth factor receptor (EGFR) internalization. AP-2 interacts with the T cell receptor (TCR), so Aak1 may modulate the TCR through its interaction with AP-2. A small molecule inhibitor of Aak1’s kinase activity called LP-935509 has been described and is under preclinical models to prevent neuropathic pain.



LP-935509.

[00228] Aak1 has a predicted molecular weight of 103,885 Da. The Aak1 polypeptide includes an N-terminal serine/threonine kinase domain, followed by a QPA rich domain, following by a C-terminal α -interacting domain (see Figure 6), through which Aak1 interacts with AP-2 and clathrin. Aak1 autophosphorylates as well as phosphorylates AP-1, AP-2 and Numb, that latter of which is required for IL-2R recycling. Aak1 is localized at the membrane in early endosomes and regulates endocytosis of the transferrin receptor, EGFR, and Notch.

[00229] The human and mouse forms of Aak1 exhibit 91% sequence identity. As such, mouse models of Aak1 activity are expected to correlate well with human AAK1 activity.

[00230] Aak1 has a long form having 961 amino acids (SEQ ID NO:2) and a short form having 823 amino acids (SEQ ID NO:3), which is truncated at the C-terminus relative to the long form. The long and short forms have equivalent kinase activity. However, the extended C-terminus of the long form may facilitate additional clathrin binding. A monoclonal antibody that binds both of the long and short form via binding an epitope nearer the N-terminus have been described (Abcam #173329). As well, a monoclonal antibody that is specific for the long form

via binding to a C-terminal epitope not present in the short form also has been described (Abcam #59740). Figure 7 illustrates a Western blot of cell lysates using Aak1 monoclonal antibody specific for the long form of Aak1 (Abcam #59740). Expression of the long form of Aak1 was detected in mouse tissue lysate, rat kidney lysate, and human CD8+ T cells. Figure 8 illustrates a Western blot of cell lysates using Aak1 monoclonal antibody specific for the long form of Aak1 (Abcam #59740) and a monoclonal antibody for both of the long form of Aak1 and the short form of Aak1 (Abcam #173329). The long and short forms of Aak1 were detected in ms brain tissue lysate whereas the long form of Aak1 was detected in human CD8+ T cells.

[00231] Figure 9 illustrates the results of in vitro treatment of primary mouse CD8+ T cells with Aak1 inhibitor (iAak1 aka LP-935509) on PD-1 expression. Primary mouse CD8+ T cells were treated with the indicated concentration of LP-935509 and PD-1 expression was measured at days 6, 7, 8, and 9. A transient increase in PD-1 expression was observed when activated CD8+ T cells were treated with the Aak1 inhibitor.

[00232] Figure 10 illustrates the results of in vitro treatment of primary mouse CD8+ T cells with Aak1 inhibitor (iAak1) on cell proliferation. Primary mouse CD8+ T cells were treated on day 0 with the indicated concentration of LP-935509 and optionally activated. Cell proliferation was assessed at days 1, 2, 3, 4, 5, 6, and 7. A modest enhancement in proliferation was observed when the CD8+ T cells were administered the Aak1 inhibitor.

[00233] Figure 11 illustrates the results of in vitro treatment of primary mouse splenocytes with Aak1 inhibitor (iAak1) on PD-1 expression. Mouse splenocytes were treated with 2 μ M concentration of iAak1 and PD-1 expression was assayed in CD8+ T cells. A modest increase in PD-1 expression was observed in CD8+ T cells derived from splenocytes.

[00234] Figure 12 illustrates the results of Aak1 inhibitor (iAak1) on the growth of EG7 tumors in mice. Mice were inoculated with EG7 tumor cells. Treatment began three (3) days post tumor inoculation. All mice received twice weekly IP injections of aPD1. Five (5) mice received DMSO, and five (5) mice received iAak1 via oral gavage on same days as aPD1 treatment. Treatment with iAak1 resulted in increased EG7 tumor growth in vivo.

[00235] Figure 13 illustrates the results of Aak1 inhibitor (iAak1) on PD-1 expression in tumor-infiltrating versus splenic CD8+ T cells in an EG7 tumor model. Mice were inoculated with EG7 tumor cells. Treatment began three (3) days post tumor inoculation. All mice received twice weekly IP injections of aPD1. Five (5) mice received DMSO, and five (5) mice received Aak1inh via oral gavage on same days as aPD1 treatment. The Aak1 inhibitor (iAak1) was observed to increase PD-1 expression in CD8+ T cells isolated from tumors but not in CD8+ cells that were isolated from the spleen.

[00236] Figure 14 illustrates the results of Aak1 inhibitor (iAak1) on growth of EG7 cells in vitro. The Aak1 inhibitor (iAak1) was not observed to directly increase EG7 cell proliferation in vitro at concentrations as high as 2 μ M.

[00237] Figure 15 illustrates the results of Aak1 inhibitor (iAak1) on the growth of A20 tumors in mice. Mice were inoculated with A20 tumor cells. Treatment began ten (10) days post tumor inoculation. All mice received twice weekly IP injections of aPD1. Ten (10) mice received DMSO, and ten (10) mice received iAak1 via oral gavage daily. Treatment with iAak1 was observed to result in increased A20 tumor growth in vivo and to moderate inhibit CD3+ T cell infiltration of the tumor.

[00238] It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention. Thus, it should be understood that although the present invention has been illustrated by specific embodiments and optional features, modification and/or variation of the concepts herein

disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00239] Citations to a number of patent and non-patent references are made herein. The cited references are incorporated by reference herein in their entireties. In the event that there is an inconsistency between a definition of a term in the specification as compared to a definition of the term in a cited reference, the term should be interpreted based on the definition in the specification..

CLAIMS

We claim:

1. A T cell that has been genetically modified in a manner that results in increased or decreased expression of a gene product encoded by a gene selected from *Aak1*, *Crct3*, or both of *Aak1* and *Crct3*.
2. The T cell of claim 1, wherein the T cell is a CD8+ T cell.
3. The T cell of claim 1, wherein the T cell is a CD4+ T cell.
4. The T cell of claim 1, wherein the T cell has been that has been genetically modified in a manner that results in increased expression of the gene product.
5. The T cell of claim 4, wherein the T cell has been genetically modified by inserting into the genomic DNA of the T cell an expression cassette for overexpressing the gene product.
6. The T cell of claim 5, wherein the expression cassette is inserted by administering to the T cell a retroviral vector comprising the expression cassette for overexpressing gene product.
7. The T cell of claim 1, wherein the T cell has been genetically modified in a manner that results in decreased expression of a gene product encoded by a gene selected from *Aak1* and *Crct3*.
8. The T cell of claim 7, wherein the T cell has been genetically modified to lack expression of the gene product.
9. The T cell of claim 8, wherein the T cell has been genetically modified to delete at least a portion of the *Aak1* gene or the *Crct3* gene.
10. The T cell of claim 1, wherein the T cell expresses a chimeric antigen receptor (CAR) in addition to having been genetically modified in a manner that results in

increased or decreased expression of a gene selected from *Aak1*, *Crct3*, or both of *Aak1* and *Crct3*.

11. The T cell of claim 10, wherein the CAR is targeted to an antigen expressed on the surface of a solid tumor.

12. The T cell of claim 11, wherein the antigen is selected from the group consisting of epidermal growth factor receptor (EGFR), mesothelin, receptor tyrosine-protein kinase erbB-2, prostate specific membrane antigen (PMSA), human epidermal growth factor receptor 2 (HER2), mucin 1 (MUC1), carcinoembryonic antigen (CEA), and IL-13 receptor alpha.

13. A therapeutic composition comprising a population of T cells that have been genetically modified in a manner that results in increased or decreased expression of a gene product encoded by a gene selected from *Aak1*, *Crct3*, or both of *Aak1* and *Crct3*.

14. The therapeutic composition claim 13, wherein the T cells are CD8+ T cells.

15. The therapeutic composition claim 13, wherein the T cells are CD4+ T cells.

16. The therapeutic composition claim 13, wherein the T cells have been genetically modified in a manner that results in increased expression of the gene product.

17. The therapeutic composition claim 16, wherein the T cells have been genetically modified by inserting into the genomic DNA of the T cells an expression cassette for overexpressing the gene product.

18. The therapeutic composition claim 17, wherein the expression cassette is inserted by administering to the T cells a retroviral vector comprising the expression cassette for overexpressing the gene product.

19. The therapeutic composition claim 13, wherein the T cells have been genetically modified in a manner that results in decreased expression of the gene product.

20. The therapeutic composition claim 19, wherein the T cells have been genetically modified to lack expression of the gene product.

21. The therapeutic composition claim 20, wherein the T cells have been genetically modified to delete at least a portion of the *Aak1* gene or the *Crc13* gene.

22. The therapeutic composition claim 13, wherein the T cells express a chimeric antigen receptor (CAR).

23. The therapeutic composition claim 22, wherein the CAR is targeted to an antigen expressed on the surface of a solid tumor.

24. The therapeutic composition claim 23, wherein the antigen is selected from the group consisting of epidermal growth factor receptor (EGFR), mesothelin, receptor tyrosine-protein kinase erbB-2, prostate specific membrane antigen (PMSA), human epidermal growth factor receptor 2 (HER2), mucin 1 (MUC1), carcinoembryonic antigen (CEA), and IL-13 receptor alpha.

25. A method for treating a subject having cancer characterized by a solid tumor, the method comprising administering to the subject the composition of claim 13.

26. The method of claim 25, wherein the cancer is selected from brain cancer, pancreatic cancer, ovarian cancer, lung cancer, liver cancer, breast cancer, and prostate cancer.

27. A method comprising:

- (a) isolating T cells from a syngeneic tumor in mice comprising endogenous T cells that have been mutagenized using a Sleeping Beauty transposon system; and
- (b) identifying in genomic DNA of the isolated T cells genes that have been mutagenized by the Sleeping Beauty transposon system.

28. The method of claim 27, wherein the mice have been treated with an anti-PD-1 therapeutic agent.

29. The method of claim 27, wherein step (b) comprises sequencing the genomic DNA.

30. The method of claim 27, further comprising identifying genes that regulate T cell trafficking and/or infiltration into a tumor and/or genes that regulate T cell activation against a tumor.

31. The method of claim 27, wherein the isolated T cells comprise CD4+ cells.

32. The method of claim 27, wherein the isolated T cells comprise CD8+ cells.

33. The method of claim 27, wherein the syngeneic tumor comprises melanoma cells, adenocarcinoma cells, sarcoma cells, or teratocarcinoma cells.

34. The method of claim 27, wherein the syngeneic tumor comprises cancer cells of a tissue selected from adrenal gland, bladder, bone, bone marrow, brain, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, prostate, skin, testis, thymus, and uterus

35. A mouse comprising endogenous T cells that have been mutagenized using a Sleeping Beauty transposon system.

Fig. 1

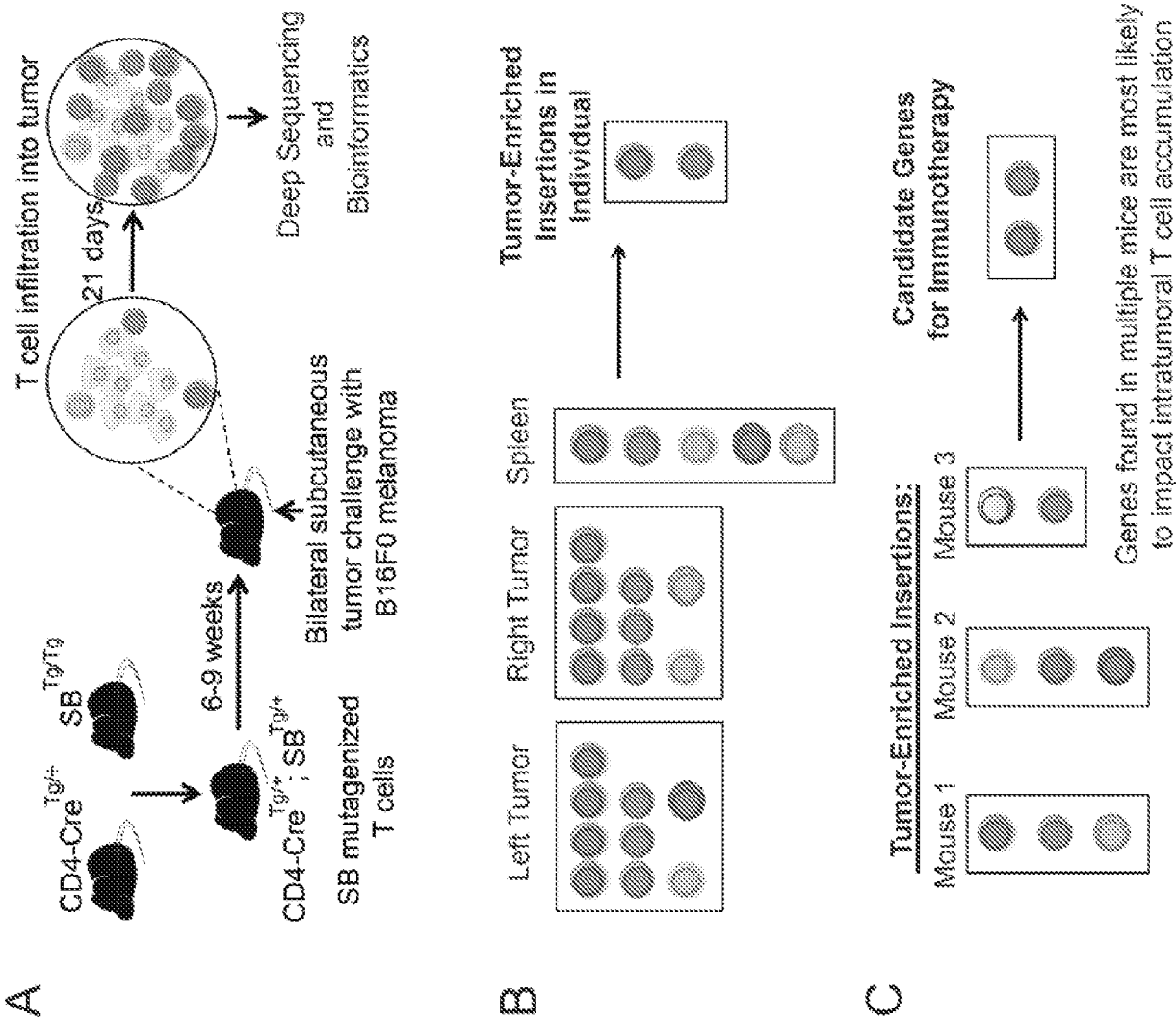
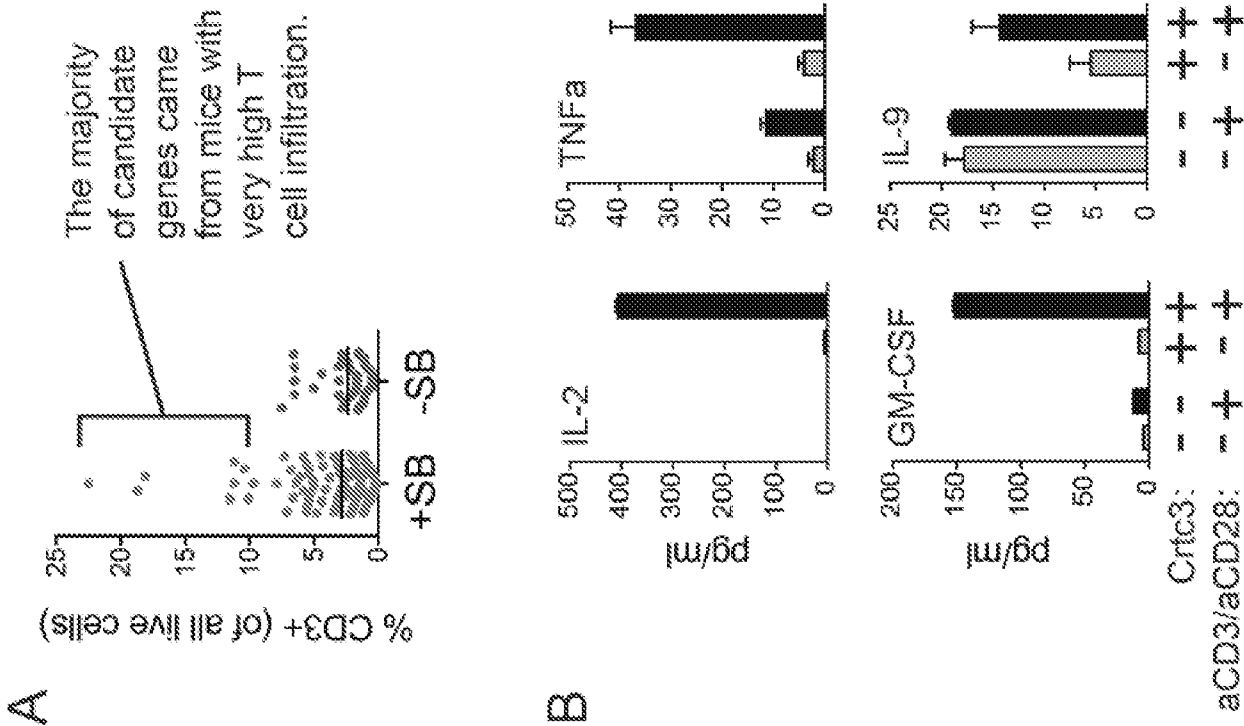
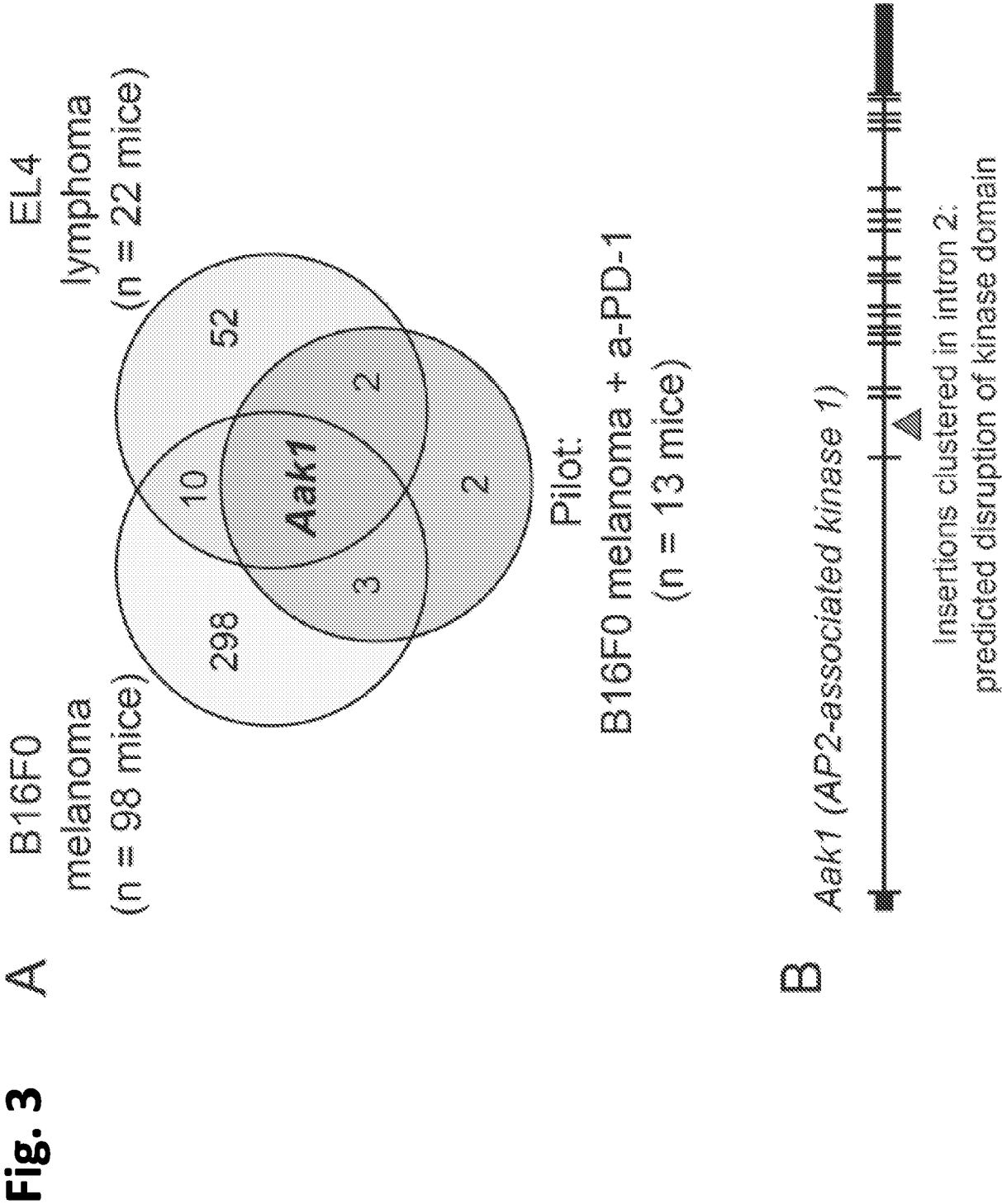
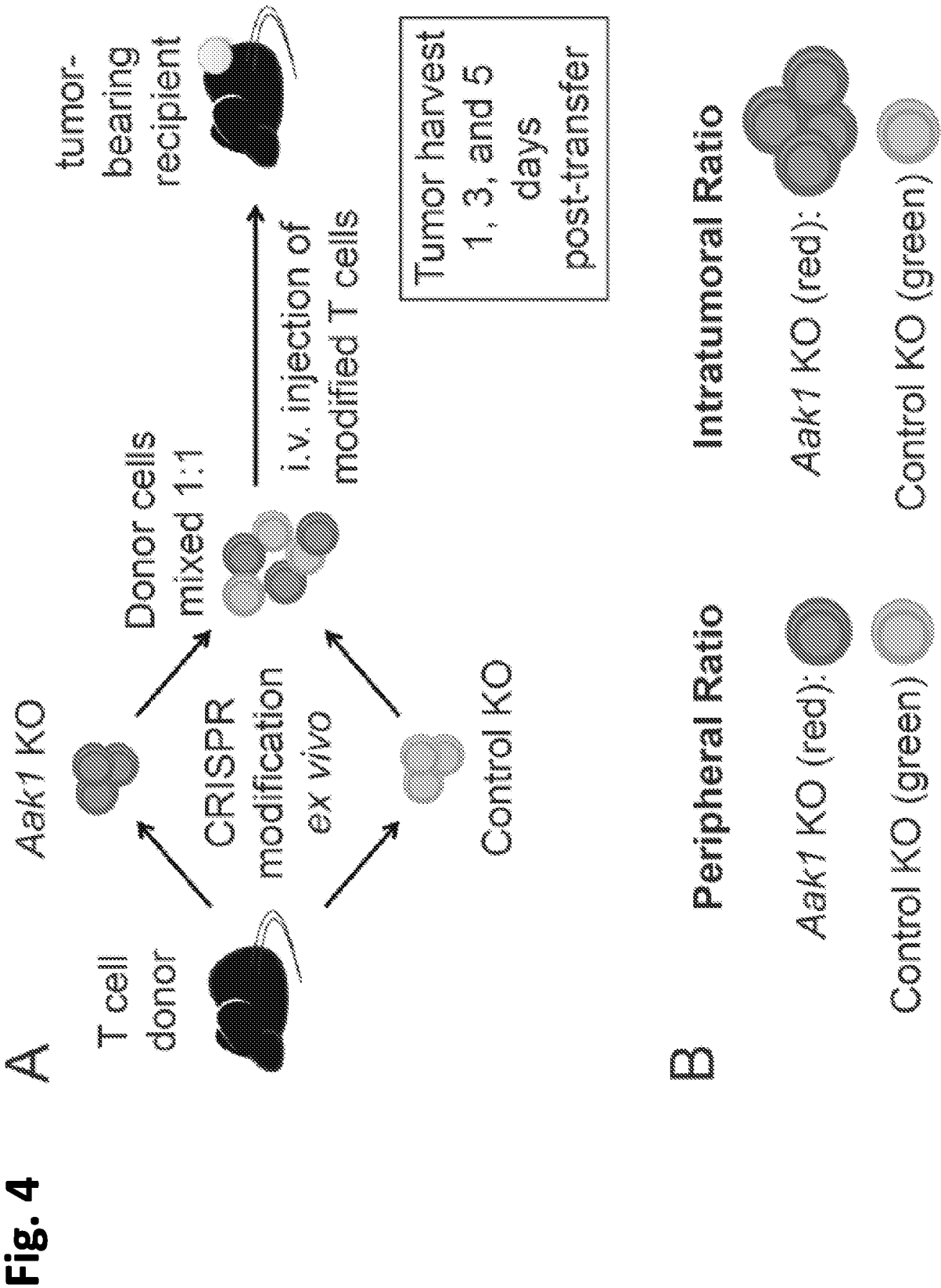
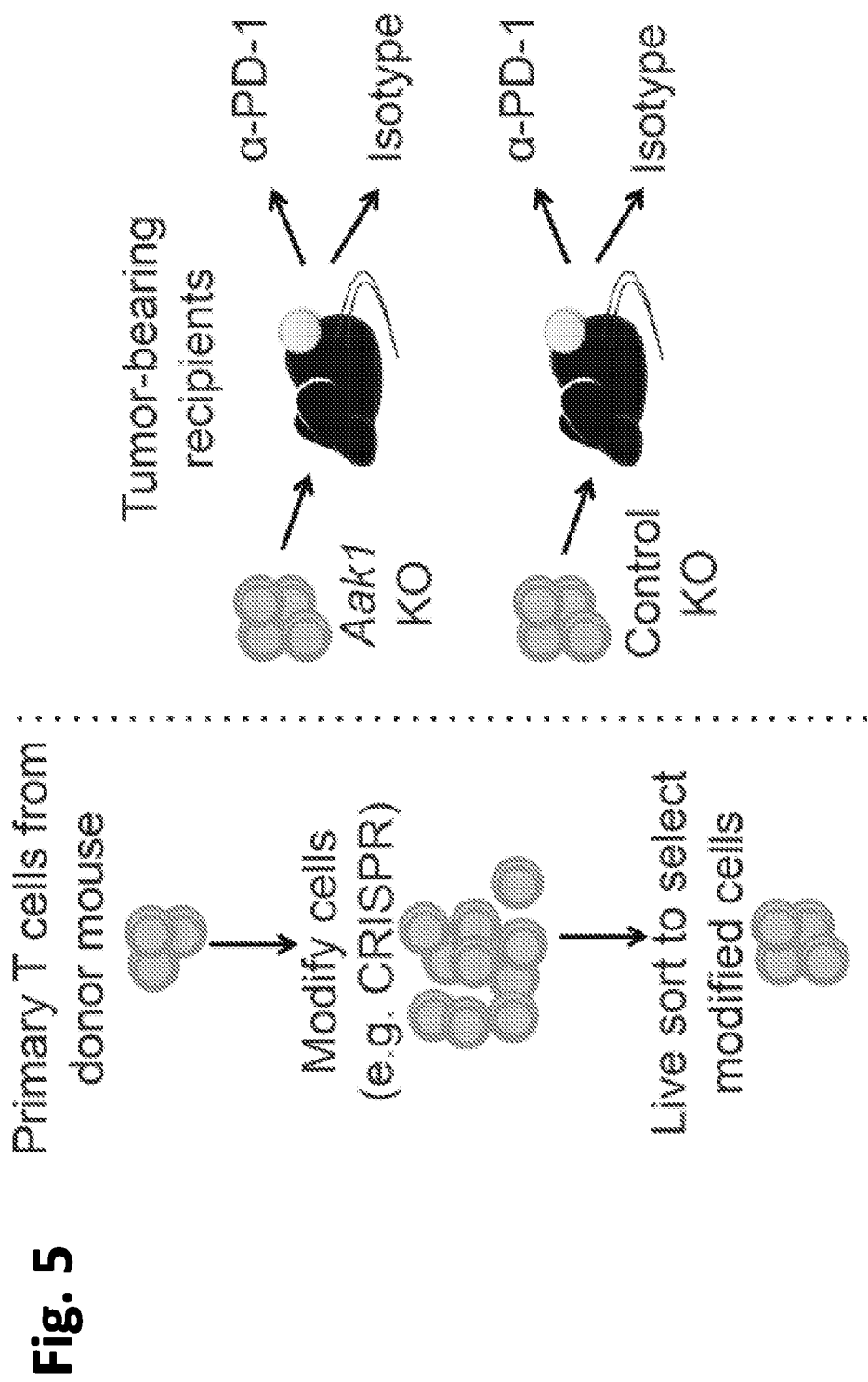


Fig. 2









Primary experimental objective:
Serial tumor measurements to assess whether *Aak1* knock out results in enhanced tumor regression in combination with (and without) anti-PD-1 treatment.

Fi. 6

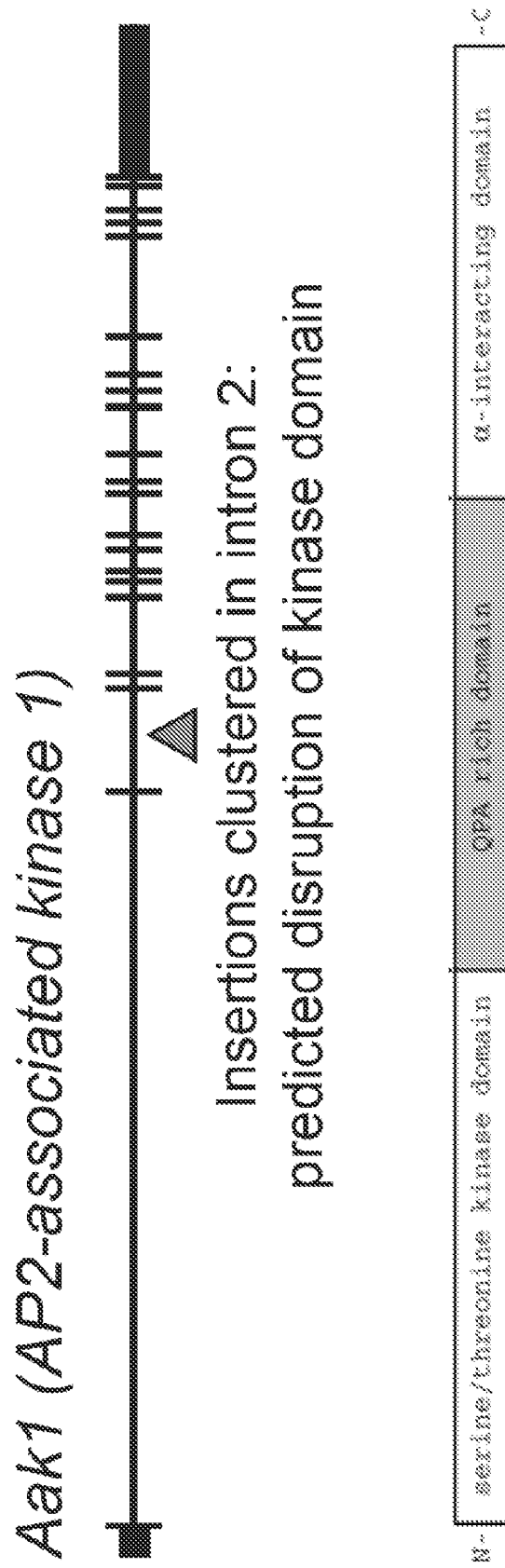


Fig. 7

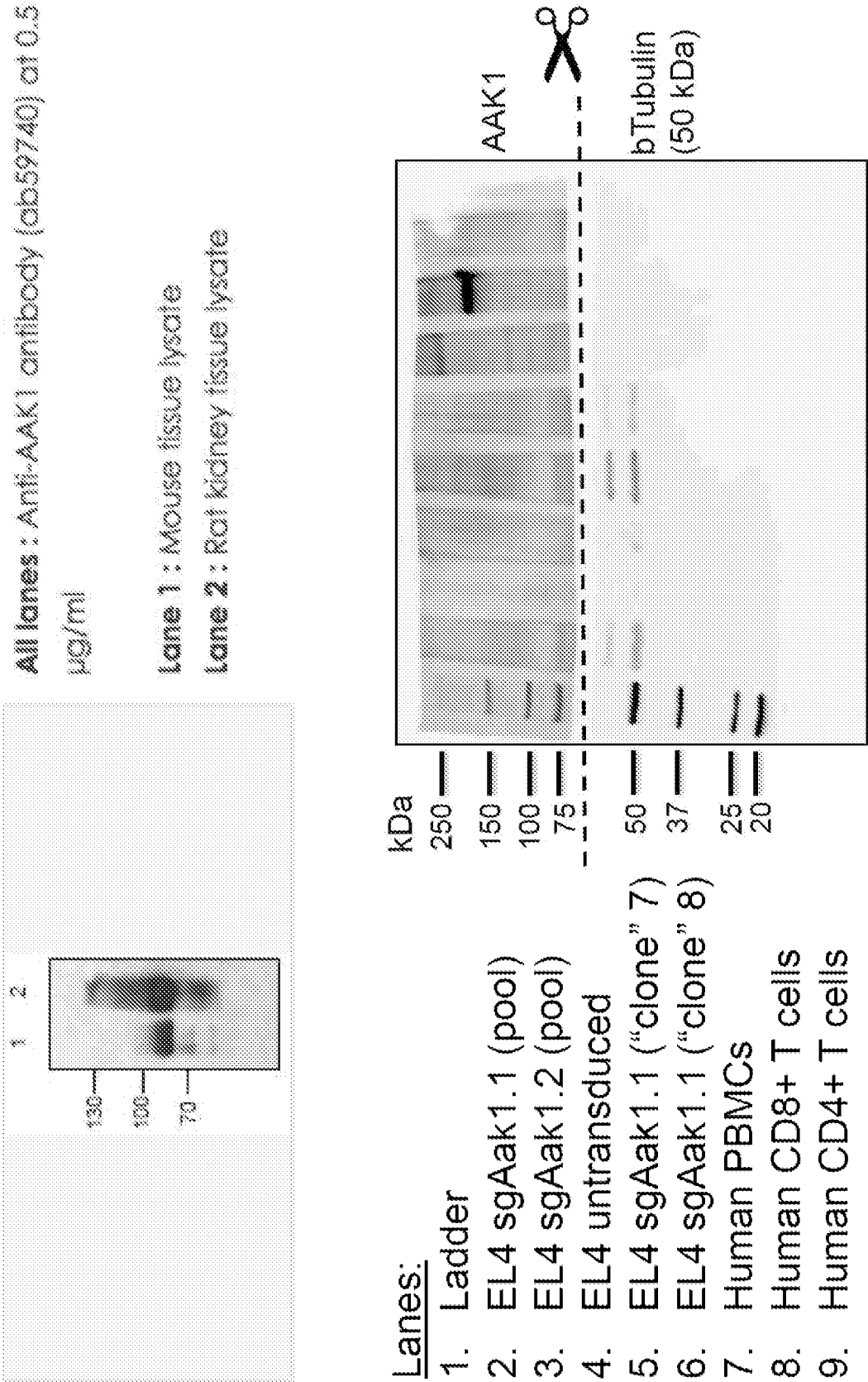


Fig. 8

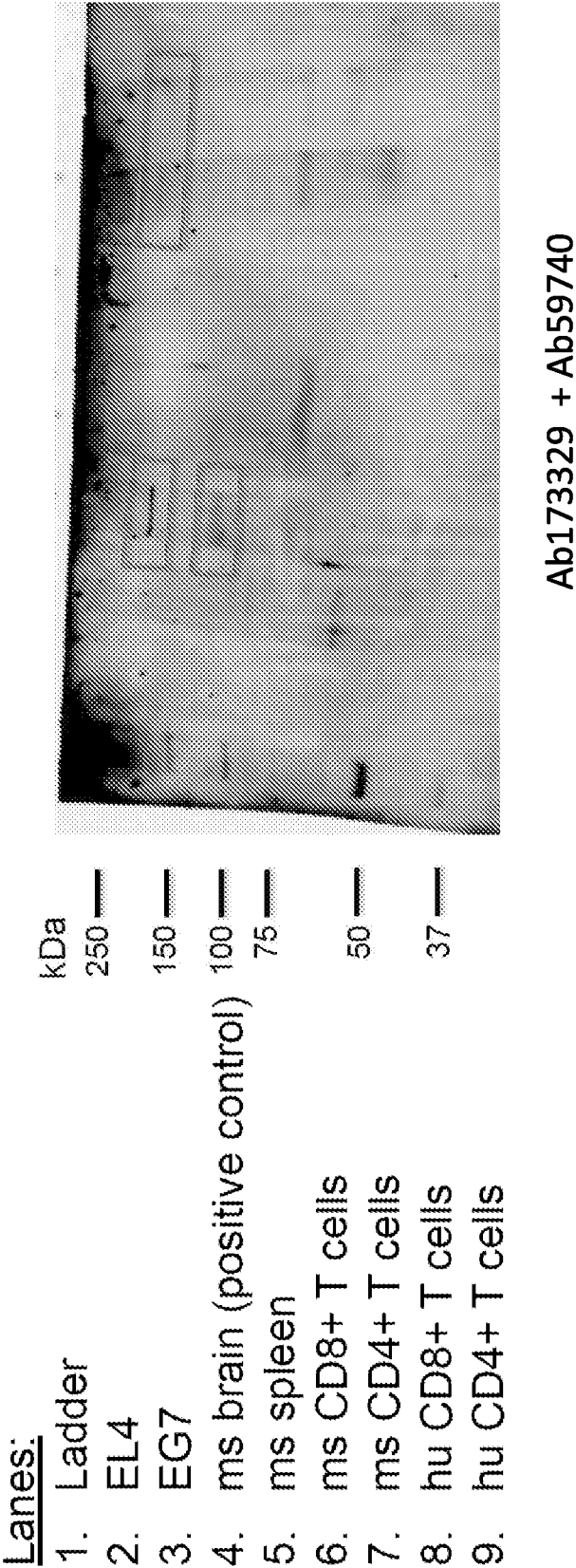
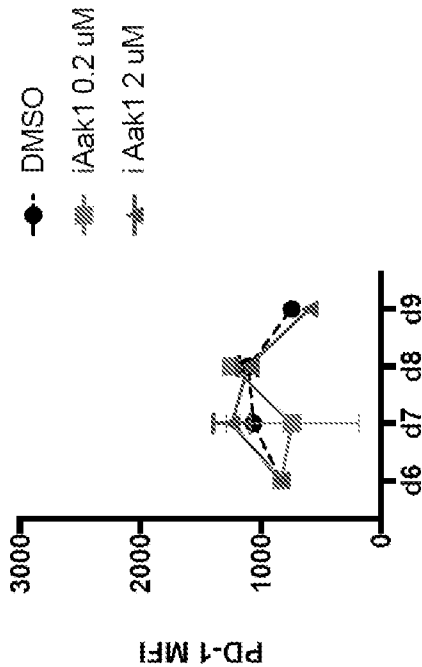
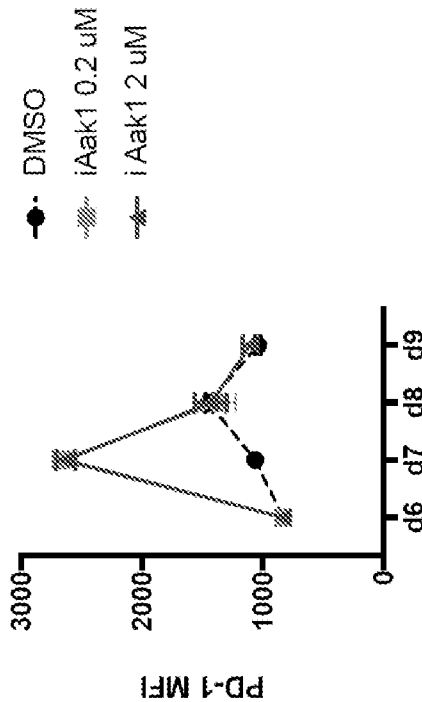


Fig. 9

PD-1 Expression on Resting CD8+ T Cells



PD-1 Expression on Activated CD8+ T Cells



P = 0.0003
Two way ANOVA with
multiple comparisons

Fig. 10

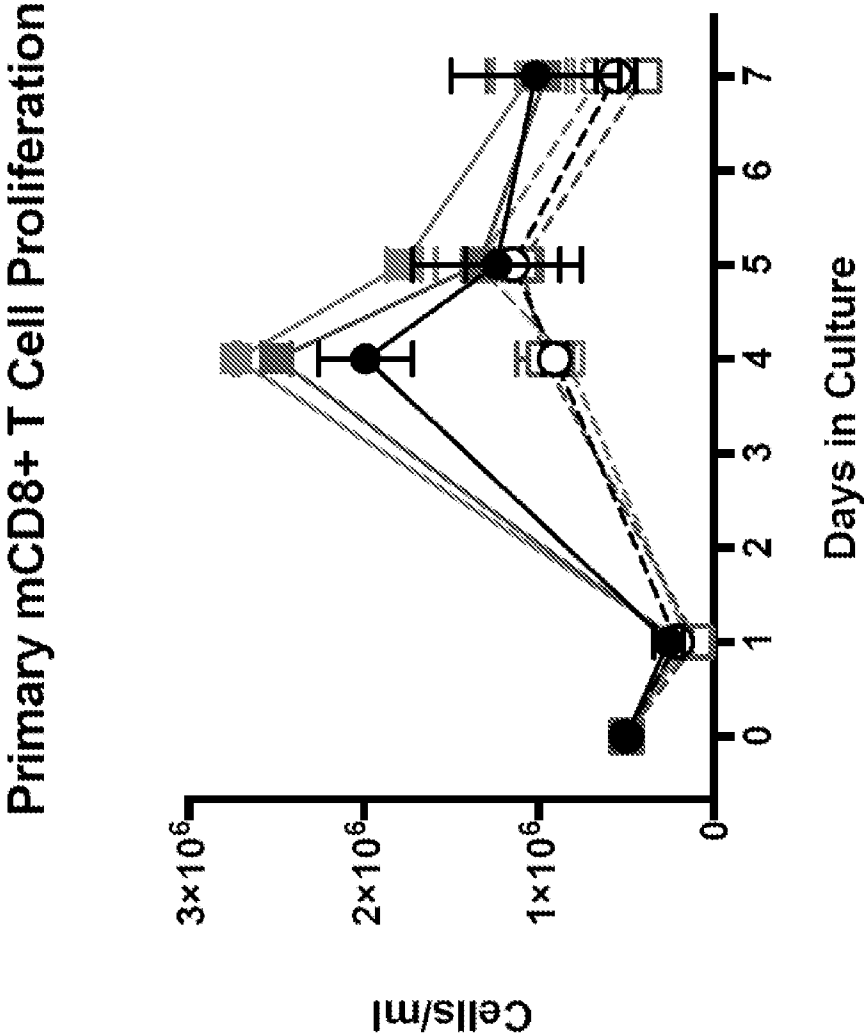


Fig. 11

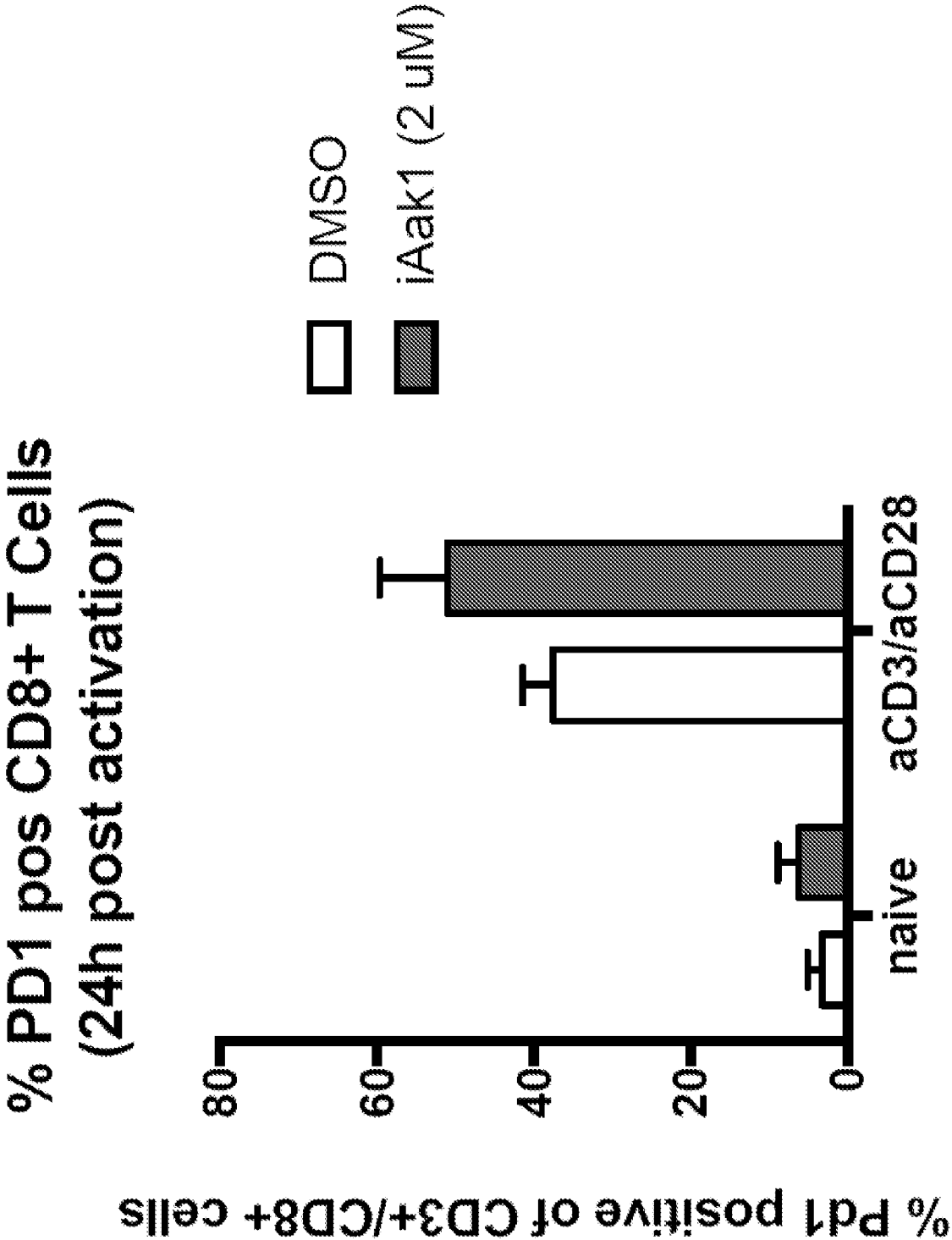


Fig. 12

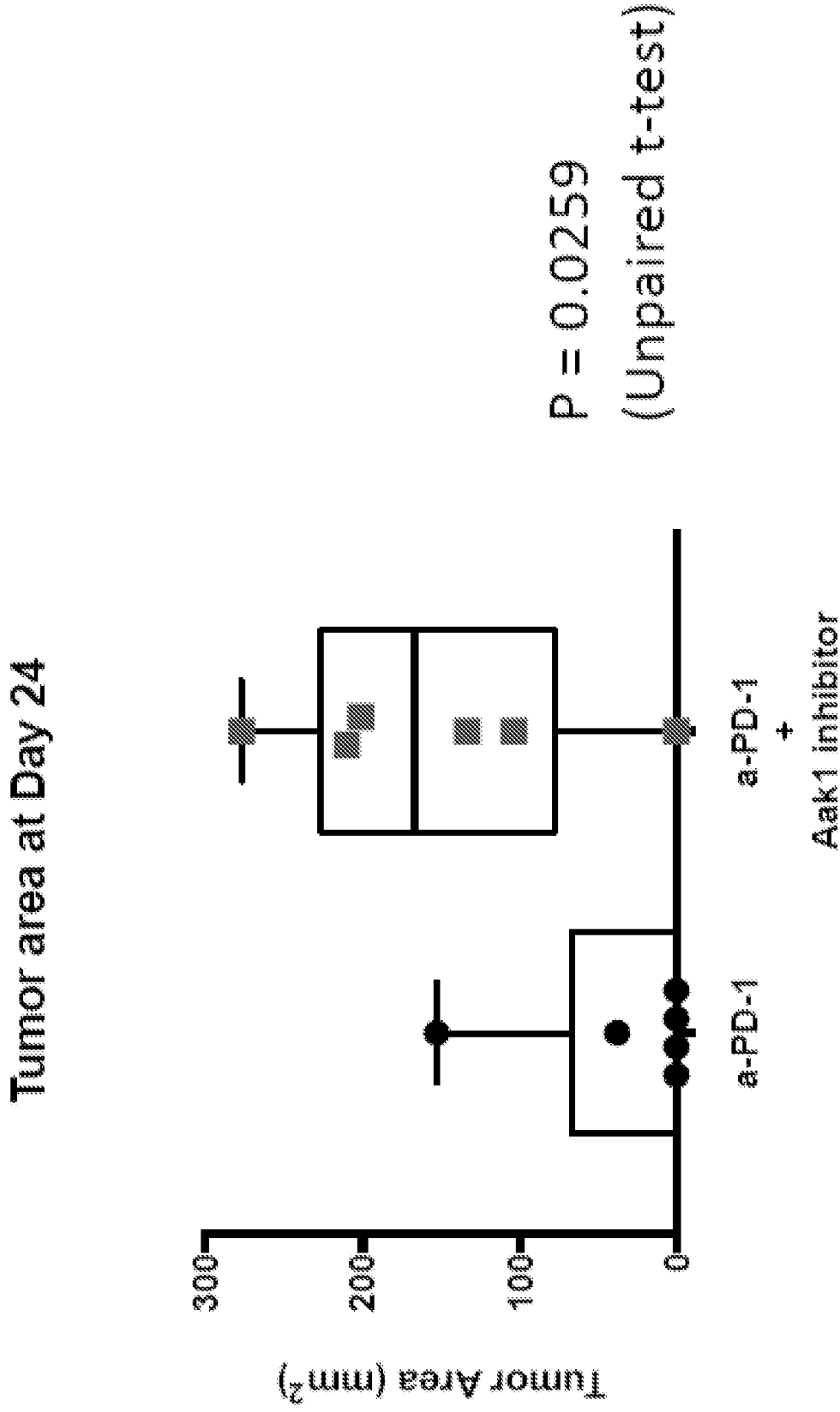


Fig. 13

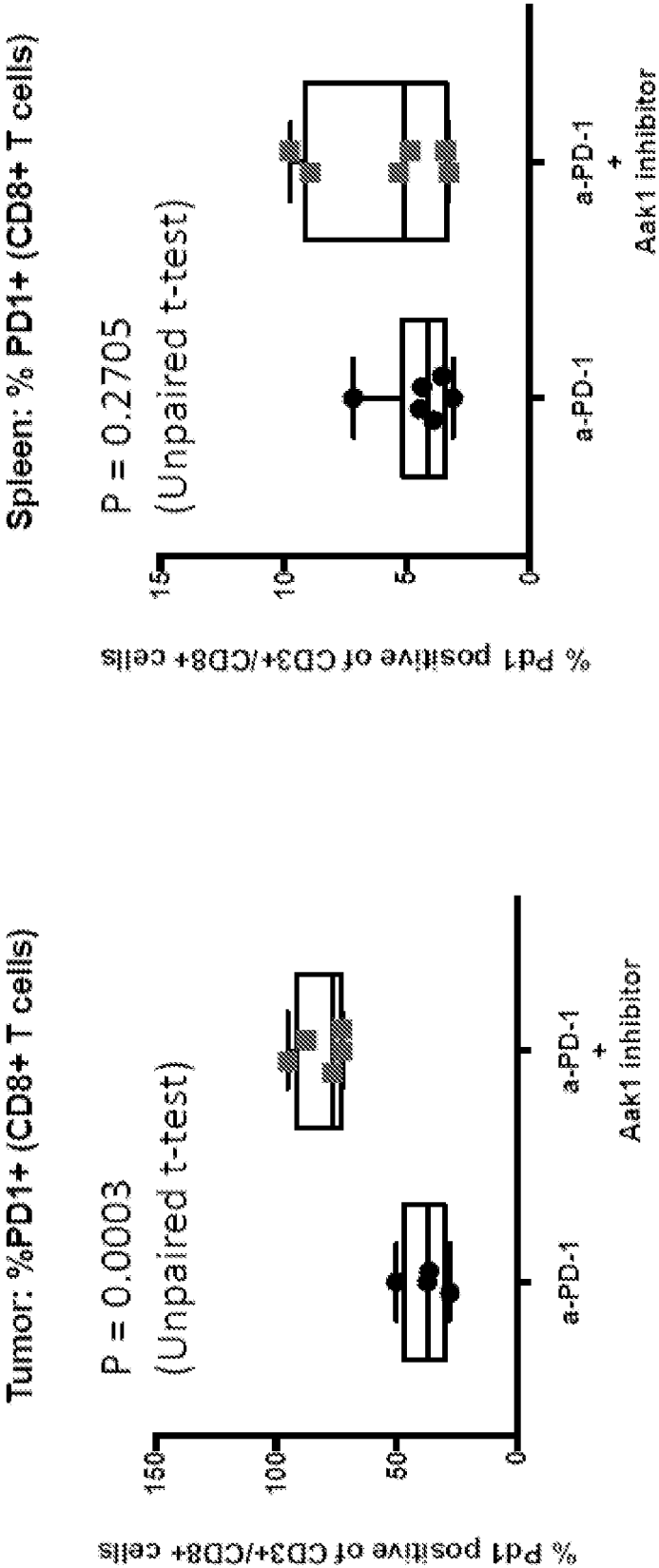


Fig. 14

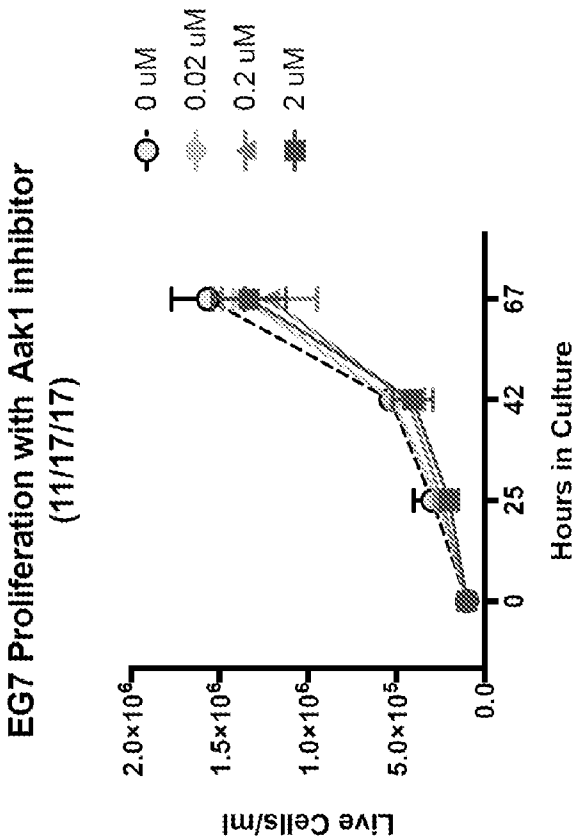
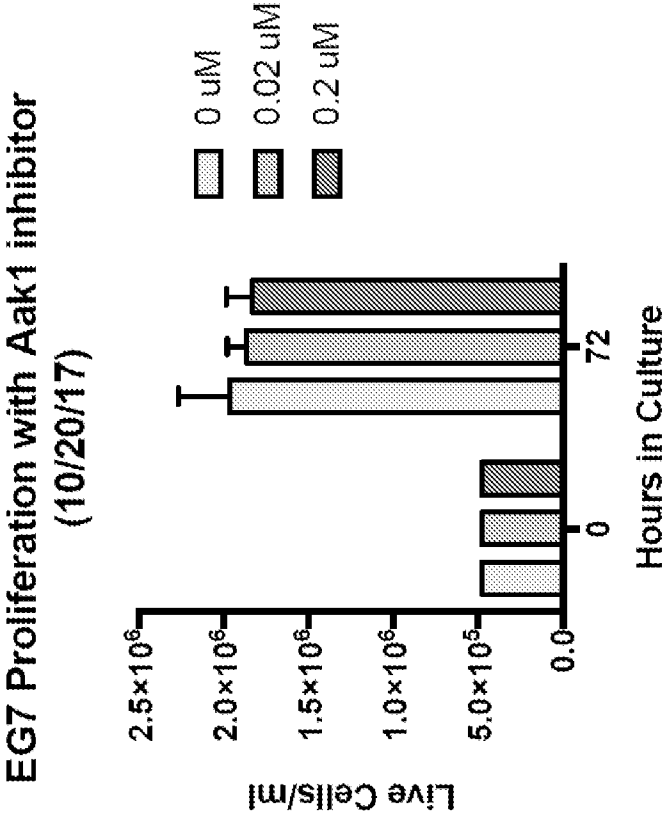
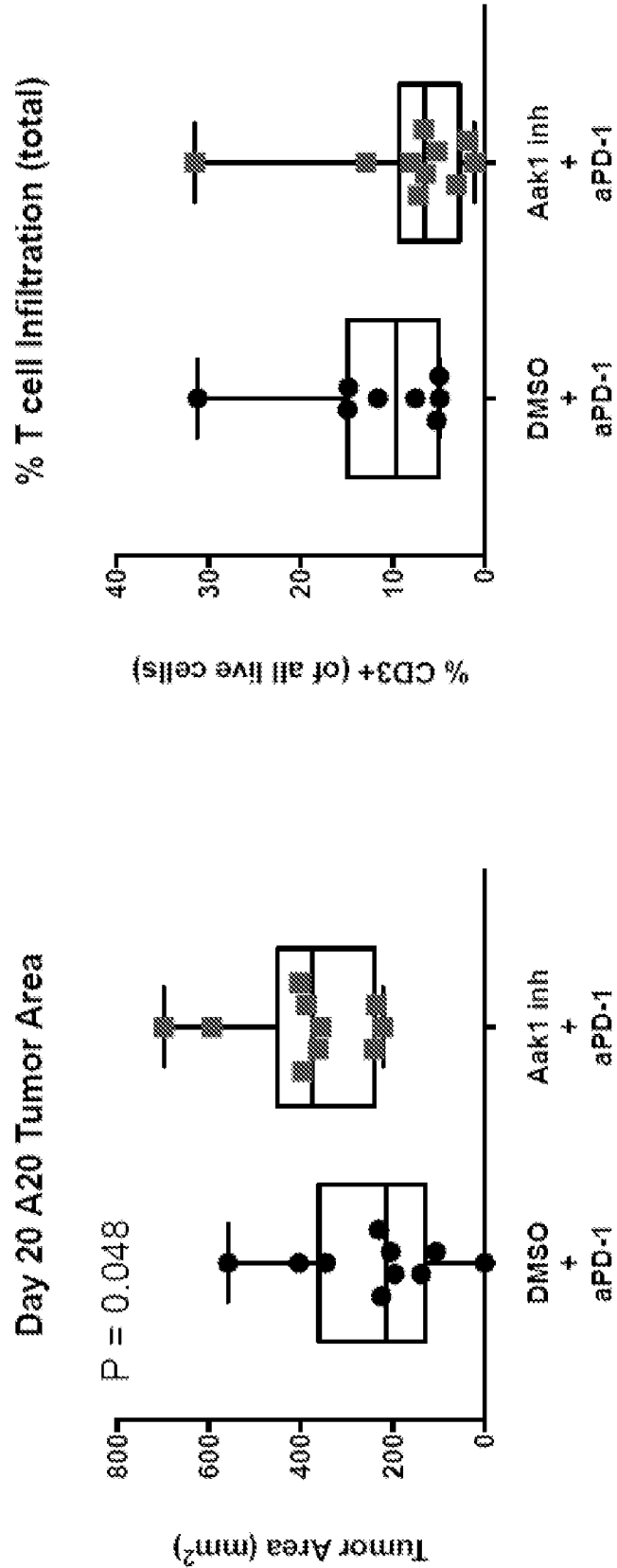


Fig. 15



INTERNATIONAL SEARCH REPORT	International application No. PCT/US 2018/028071												
A. CLASSIFICATION OF SUBJECT MATTER (see extra sheet) 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) C12N 5/0783, 5/10, A61K 35/17, 48/00, A61P 35/00 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) PatSearch, EMBL, NCBI, Espacenet, DWPI, PCT Online, USPTO DP, CIPO (Canada PO), SIPO DB													
C. DOCUMENTS CONSIDERED TO BE RELEVANT <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category*</th> <th style="width: 70%;">Citation of document, with indication, where appropriate, of the relevant passages</th> <th style="width: 20%;">Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X Y</td> <td>WO 2014/134351 A2 (BROAD INST. INC. et al.) 04.09.2014, paragraphs [005], [0025], [0060], [0067], [0085] - [0099], [0101] - [0108], [0115] - [0118], examples 4, 6, Table 11, claims</td> <td style="text-align: center; vertical-align: top;">1-9 10-26, 31, 32</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>WO 2017/015427 A1 (NOVARTIS AG et al.) 26.01.2017, p.23-39, 159-179, 191-194, 210-215, 244-265, claims</td> <td style="text-align: center; vertical-align: top;">10-34</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X Y</td> <td>HUDECEK M. et al. Going non-viral: the Sleeping Beauty transposon system breaks on through to the clinical side. Crit Rev Biochem Mol Biol., 2017, Vol.52, no.4, p.355-380, published online: 12.04.2017, especially c.364-365, 368, 370-372, fig .6</td> <td style="text-align: center; vertical-align: top;">35 27-43</td> </tr> </tbody> </table>		Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	X Y	WO 2014/134351 A2 (BROAD INST. INC. et al.) 04.09.2014, paragraphs [005], [0025], [0060], [0067], [0085] - [0099], [0101] - [0108], [0115] - [0118], examples 4, 6, Table 11, claims	1-9 10-26, 31, 32	Y	WO 2017/015427 A1 (NOVARTIS AG et al.) 26.01.2017, p.23-39, 159-179, 191-194, 210-215, 244-265, claims	10-34	X Y	HUDECEK M. et al. Going non-viral: the Sleeping Beauty transposon system breaks on through to the clinical side. Crit Rev Biochem Mol Biol., 2017, Vol.52, no.4, p.355-380, published online: 12.04.2017, especially c.364-365, 368, 370-372, fig .6	35 27-43
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☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.													
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Date of the actual completion of the international search 15 June 2018 (15.06.2018)	Date of mailing of the international search report 02 August 2018 (02.08.2018)												
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INTERNATIONAL SEARCH REPORT
Classification of subject matter

International application No.

PCT/US 2018/028071

C12N 5/0783 (2010.01)

C12N 5/10 (2006.01)

A61K 35/17 (2015.01)

A61K 48/00 (2006.01)

A61P 35/00 (2006.01)