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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 December 2019 (12.12.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/237042 A1

(51) International Patent Classification:
A61K 38/02 (2006.01) *C07K 16/28* (2006.01)
A61K 39/395 (2006.01)

(21) International Application Number:
PCT/US2019/036121

(22) International Filing Date:
07 June 2019 (07.06.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/682,698 08 June 2018 (08.06.2018) US

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

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

(54) Title: TARGETING JAML-CAR INTERACTIONS FOR TUMOR IMMUNOTHERAPY

Expression of JAML and CAR is associated with improved survival in many epithelial cancers (The Human Protein Atlas Database)

	5-year Survival Rates Based on JAML Expression (The Human Protein Atlas)						
	Cancer Type	Colorectal	Head and Neck	Lung	Cervical	Uterine	Melanoma*
JAML Expression	High Expression	74%	48%	47%	89%	80%	40%
	Low Expression	54%	36%	33%	59%	66%	0%
	5-year Survival Rates Based on CAR Expression (The Human Protein Atlas)						
	Cancer Type	Colorectal	Head and Neck	Lung	Urothelial	Stomach	Melanoma*
CAR Expression	High Expression	65%	51%	51%	49%	40%	38%
	Low Expression	50%	39%	38%	35%	25%	0%

* 3-year survival rate

FIG. 1

(57) Abstract: This invention provides compositions and methods for promoting binding of JAML on T cells to CAR on tumor cells. The invention also provides methods for treating various types of tumors.

[Continued on next page]

WO 2019/237042 A1

Published:

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

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

TARGETING JAML-CAR INTERACTIONS FOR TUMOR IMMUNOTHERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The subject patent application claims the benefit of priority to U.S. Provisional Patent Application Number 62/682,698 (filed June 8, 2018; now pending). The full disclosure of the priority application is incorporated herein by reference in its entirety and for all purposes.

STATEMENT CONCERNING GOVERNMENT SUPPORT

[0002] This invention was made with government support under grant number AI064811 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] The various types of cancers represent one of the major health problems that are prevalent around the world. These include malignant tumors involving the epithelial tissues, e.g., skin cancers and cancers involving the intestine. Over the past decade or so, there have been substantial progresses in the development of immunotherapies in the treatment of cancer. Cancer immunotherapies trigger the body's own immune system to find and destroy neoplastic cells. Natural killer T cells (NKT) and γδ T cells have been identified as critical components in cancer immune surveillance. The initial success of preclinical trials in the last decades has evoked NKT or γδ T cells based immunotherapeutic approaches for the treatment of cancer. However, a significant proportion of patients do not have a sufficient number of NKT or γδ T cells and/or lack sufficient cells with normal function.

[0004] There is an unmet need in the art for better and more efficacious immunotherapies for treating various types of cancers. The present invention addresses this and other unfulfilled needs in the art.

SUMMARY OF THE INVENTION

[0005] In one aspect, the invention provides methods for promoting T cell tumor infiltration in a subject. The methods entail administering to the subject a pharmaceutical composition that contains a therapeutically effective amount of an agent that enhances the specific binding between junctional adhesion molecule (JAML) on T cells and Coxsackie and adenovirus receptor (CAR) on tumor cells. This will lead to enhanced T cell tumor infiltration in the subject. In some methods, the subject is also treated with an immune checkpoint inhibitor. The checkpoint inhibitor can be administered to the subject prior to, simultaneously with, or subsequent to treatment with the agent that enhances JAML-CAR interaction. In some embodiments, the employed checkpoint inhibitor is a CTLA inhibitor, a PD-1 inhibitor or a PD-L1 inhibitor.

[0006] In some embodiments, the employed agent for enhancing JAML-CAR interaction is a monoclonal antibody targeting JAML. In some preferred embodiments, the employed monoclonal antibody targeting JAML does not block or interfere with JAML binding to CAR. In some methods, the monoclonal antibody targeting JAML has a binding specificity that is the same as that of an antibody that has HCDR1-3 and LCDR1-3 sequences respectively shown in SEQ ID NOs:1-6. In some embodiments, the employed monoclonal antibody targeting JAML has HCDR1-3 and LCDR1-3 sequences respectively shown in SEQ ID NOs:1-6. In some embodiments, the employed monoclonal antibody targeting JAML is Fab antibody HL4E10. In some embodiments, the employed monoclonal antibody targeting JAML is a humanized antibody or a chimeric antibody. For example, the methods can employ a humanized antibody that is derived from Fab antibody HL4E10. In some of these embodiments, the humanized antibody contains at least one CDR as shown in SEQ ID NOs:1-6. In some embodiments, the humanized antibody contains heavy chain CDR1-3 as shown in SEQ ID NOs:1-3, respectively. In some embodiments, the humanized antibody contains heavy chain CDRs 1-3 and light chain CDRs 1-3 as shown in SEQ ID NOs:1-6, respectively.

[0007] In some methods of the invention, the employed agent that enhances JAML-CAR interaction contains a soluble CAR molecule. In some of these embodiments, the soluble CAR molecule is a CAR-Fc fusion. In some embodiments, the employed soluble CAR molecule is the extracellular domain of CAR. In various embodiments, the tumor afflicted

by the subject in need of treatment is a skin tumor, a colorectal tumor, a lung tumor, or an ovarian tumor.

[0008] In a related aspect, the invention provides methods for stimulating T cell killing of a tumor in a subject. These methods involve administering to a subject afflicted with a tumor a pharmaceutical composition that contains a therapeutically effective amount of an agent that enhances the specific interaction between junctional adhesion molecule (JAML) on T cells and Coxsackie and adenovirus receptor (CAR) on tumor cells. This will result in stimulated T cell killing of the tumor in the subject. In some of these methods, the subject is also treated with an immune checkpoint inhibitor. In various embodiments, the checkpoint inhibitor can be administered to the subject prior to, simultaneously with, or subsequent to treatment with the agent that enhances JAML-CAR interaction. In some embodiments, the employed agent that enhances JAML-CAR interaction is a monoclonal antibody targeting JAML. In some of these embodiments, the employed monoclonal antibody targeting JAML has a binding specificity that is the same as that of an antibody comprising HCDR1-3 and LCDR1-3 sequences respectively shown in SEQ ID NOs:1-6. In some embodiments, the employed monoclonal antibody targeting JAML is a humanized antibody that is derived from Fab antibody HL4E10. In some embodiments, the agent targeting JAML-CAR interaction contains a soluble CAR molecule. In some of these embodiments, the employed soluble CAR molecule is a CAR-Fc fusion. In various embodiments, the tumor afflicted by the subject is a skin tumor, a colorectal tumor, a lung tumor, or an ovarian tumor.

[0009] In another aspect, the invention provides humanized antibodies that are derived from Fab antibody HL4E10. The humanized antibodies contain one or more CDR sequence as shown in SEQ ID NOs:1-6. Typically, the humanized antibodies of the invention can specifically recognize junctional adhesion molecule (JAML) but do not block JAML interaction with Coxsackie and adenovirus receptor (CAR). In some embodiments, the humanized antibodies of the invention additionally contain one or more amino acid substitutions in its framework region relative to that of Fab antibody HL4E10. In some embodiments, the humanized antibody contains heavy chain CDR1-3 as shown in SEQ ID NOs:1-3, respectively. In some embodiments, the humanized antibody contains heavy chain CDRs 1-3 and light chain CDRs 1-3 as shown in SEQ ID NOs:1-6, respectively.

[0010] In another aspect, the invention provides soluble polypeptide fragments derived from Coxsackie and adenovirus receptor (CAR) that are capable of binding to the junctional

adhesion molecule (JAML) on T cells. In some embodiments, these soluble polypeptide fragments are protein fusions with a Fc domain.

[0011] A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification and claims.

DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 shows that expression of JAML and CAR is associated with improved survival in many epithelial cancers.

[0013] Figure 2 shows that expression of JAML is a favorable prognostic factor in many epithelial cancers.

[0014] Figure 3 shows that high expression of JAML in tumors is associated with improved survival in melanoma and head and neck squamous cell carcinoma (TCGA).

[0015] Figure 4 shows that JAML-CAR interaction is a novel mechanism of T cell tumor infiltration and is required for antitumor immunity.

[0016] Figure 5 shows that JAML is upregulated on activated T cells and is highly expressed on tumor-infiltrating lymphocytes (TILs).

[0017] Figure 6 shows that increased expression of CAR turns “cold” tumors “hot” and improves antitumor immune response.

[0018] Figure 7 shows that human melanoma tissues with high CAR expression have increased T cell infiltration.

[0019] Figure 8 shows in vivo anti-JAML stimulation to induce T cell tumor infiltration.

[0020] Figure 9 is a schematic illustration of stimulating T cell tumor infiltration via intratumoral hydrogel injection of agents targeting JAML-CAR interaction.

[0021] Figure 10 a schematic illustration of stimulating T cell tumor infiltration via delivery of soluble CAR via hydrogel.

DETAILED DESCRIPTION

[0022] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains. The following references provide one of skill with a general definition of many of the terms used in this invention: *Academic Press Dictionary of Science and Technology*, Morris (Ed.), Academic Press (1st ed., 1992); *Illustrated Dictionary of*

Immunology, Cruse (Ed.), CRC Pr I LIc (2nd ed., 2002); *Oxford Dictionary of Biochemistry and Molecular Biology*, Smith et al. (Eds.), Oxford University Press (revised ed., 2000); *Encyclopaedic Dictionary of Chemistry*, Kumar (Ed.), Anmol Publications Pvt. Ltd. (2002); *Dictionary of Microbiology and Molecular Biology*, Singleton et al. (Eds.), John Wiley & Sons (3rd ed., 2002); *Dictionary of Chemistry*, Hunt (Ed.), Routledge (1st ed., 1999); *Dictionary of Pharmaceutical Medicine*, Nahler (Ed.), Springer-Verlag Telos (1994); *Dictionary of Organic Chemistry*, Kumar and Anandand (Eds.), Anmol Publications Pvt. Ltd. (2002); and *A Dictionary of Biology (Oxford Paperback Reference)*, Martin and Hine (Eds.), Oxford University Press (4th ed., 2000). In addition, the following definitions are provided to assist the reader in the practice of the invention.

[0023] The term "agent" or "test agent" includes any substance, molecule, element, compound, entity, or a combination thereof. It includes, but is not limited to, e.g., protein, polypeptide, peptide or mimetic, small organic molecule, polysaccharide, polynucleotide, and the like. It can be a natural product, a synthetic compound, or a chemical compound, or a combination of two or more substances. Unless otherwise specified, the terms "agent", "substance", and "compound" are used interchangeably herein. In some screening methods of the invention, the employed test agents or candidate compounds are small organic molecules.

[0024] As used herein, a checkpoint inhibitor or immune checkpoint inhibitor refers to a drug or compound that can block proteins that stop the immune system from attacking harmful cells (e.g., cancer cells). By eliciting or inducing an immune system attack on cancer cells, checkpoint inhibitors can overcome one of cancer's main defenses against an immune system attack. Exemplary checkpoint inhibitors include CTLA-4 inhibitors, PD-1 inhibitors and PD-L1 inhibitors.

[0025] The term "analog" or "derivative" is used herein to refer to a molecule that structurally resembles a reference molecule (e.g., an JAML-targeting antibody exemplified herein) but which has been modified in a targeted and controlled manner, by replacing a specific substituent of the reference molecule with an alternate substituent. Compared to the reference molecule, an analog would be expected, by one skilled in the art, to exhibit the same, similar, or improved utility. Synthesis and screening of analogs to identify variants of known compounds having improved traits (such as higher binding affinity for a target molecule) is an approach that is well known in pharmaceutical chemistry.

[0026] Administration "in combination with" one or more other therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

[0027] The term “contacting” has its normal meaning and refers to combining two or more agents (e.g., polypeptides or small molecule compounds) or combining agents and cells. Contacting can occur in vitro, e.g., combining two or more agents or combining an agent and a cell or a cell lysate in a test tube or other container. Contacting can also occur in a cell or in situ, e.g., contacting two polypeptides in a cell by coexpression in the cell of recombinant polynucleotides encoding the two polypeptides, or in a cell lysate. Contacting can also occur inside the body of a subject, e.g., by administering to the subject an agent which then interacts with the intended target (e.g., a tissue or a cell).

[0028] The Fc fragment or Fc domain of an immunoglobulin comprises the carboxy-terminal portions of both H chains held together by disulfides. The effector functions of immunoglobulins are determined by sequences in the Fc region, which region is also the part recognized by Fc receptors (FcR) found on certain types of cells.

[0029] Junctional adhesion molecule-like protein (JAML) is expressed on the surface of several immune cells, including resting epithelial γδ T cells, activated epithelial and lymphoid γδ T cells, activated CD8 T cells, and monocytes and neutrophils. Coxsackie and adenovirus receptor (CAR) is widely expressed in epithelial tissues, including skin, lung, small intestine, colon, liver, pancreas, bladder and prostate. Its expression is dysregulated in many human cancers. Cancer immunotherapies are dependent on immune cell infiltration into tumors.

[0030] The terms “subject” and "patient" are used interchangeably and refer to mammals such as human patients and non-human primates, as well as experimental animals such as rabbits, rats, and mice, and other animals. Animals include all vertebrates, e.g., mammals and non-mammals, such as dogs, cats, sheeps, cows, pigs, rabbits, chickens, and etc. Preferred subjects for practicing the therapeutic methods of the present invention are human.

[0031] The term "inhibiting" or "inhibition," in the context of tumor growth or tumor cell growth, refers to delayed appearance of primary or secondary tumors, slowed development of primary or secondary tumors, decreased occurrence of primary or secondary tumors, slowed or decreased severity of secondary effects of disease, or arrested tumor growth and regression of tumors. The term “prevent” or “prevention” refers to a complete or partial

inhibition of development of primary or secondary tumors or any secondary effects of disease.

[0032] The term "treat" or "treatment" refers to arrested tumor growth, and to partial or complete regression of tumors. The term "treating" includes the administration of compounds or agents to prevent or delay the onset of the symptoms, complications, or biochemical indicia of a disease (e.g., a cancer), alleviating the symptoms or arresting or inhibiting further development of the disease, condition, or disorder. Treatment may be prophylactic (to prevent or delay the onset of the disease, or to prevent the manifestation of clinical or subclinical symptoms thereof) or therapeutic suppression or alleviation of symptoms after the manifestation of the disease.

[0033] A "variant" of a reference molecule refers to a molecule substantially similar in structure and biological activity to either the entire reference molecule, or to a fragment thereof. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the sequence of amino acid residues is not identical.

[0034] The present invention is directed to promoting immune cell (e.g., T cells) infiltration into tumors, which is important for cancer immune therapies. For example, checkpoint blockade therapies are more successful in patients with pre-established T cell response within tumors ("hot" tumors) whereas tumors lacking immune cell infiltration ("cold" tumors) are unresponsive. Furthermore, all cancer immunotherapies including tumor vaccines, CAR T cells, etc. are dependent on immune cell infiltration into tumors. There are cancers that unresponsive to checkpoint blockade, e.g., colorectal cancer, lung cancer, ovarian cancer and melanoma, indicating a need for additional therapeutic interventions. Accordingly, the invention provides methods of promoting the interaction between JAML on T cells and CAR on various tumor cells (e.g., epithelial tumor cells). As demonstrated herein, methods of the invention provides a novel strategy to turn cold tumors into hot tumors. In some related embodiments, the present invention provides methods of stimulating T cell killing of tumor cells via promoting the JAML-CAR interaction. The methods of the invention use agents that can specifically target the JAML-CAR interaction and induce T cell migration into tumor cells. Any of the antibody agents targeting JAML or CAR-derived polypeptide agents detailed herein can be used in the methods of the invention.

In some embodiments, the agent used in the methods of the invention is an antibody that can specifically bind to JAML on T cells in enhancing JAML-CAR interaction. Preferably, while directing JAML-expressing T cells (e.g., γδ T cells) to tumors by binding to JAML, the antibodies utilized in the methods of the invention do not block or substantially affect JAML interaction with CAR. Antibodies that specifically recognize JAML are known in the art. These include Fab antibody HL4E10 as described in Verdino et al., PLoS One 6: e19828, 2011, and various other JAML antibodies that are commercially available. The latter include antibodies ab67843 and ab183714 from Abcam (Cambridge, MA), monoclonal antibody MAB34491 from R&D Systems, Inc. (Minneapolis, MN), and monoclonal antibody MAB34491 from LifeSpan BioSciences, Inc. Impact of binding of any of these antibodies on JAML interaction with CAR can be readily assessed with routinely practiced biochemical or immunological techniques, e.g., competitive ELISA.

[0035] In some methods of the invention, the agent that specifically targets the JAML-CAR interaction can be a soluble CAR agent that can specifically bind to JAML. In some of these embodiments, the soluble CAR agent can be directly delivered into tumors to promote T cell migration into tumors. The soluble CAR agent can be a fragment or domain of CAR that is capable of binding to JAML (e.g., the extracellular domain of CAR). It can also be a fusion protein that contains such a fragment that is fused to a heterologous protein domain, e.g., an Fc domain. By fusing to an Fc domain, the delivered polypeptide agent will have a markedly improved plasma half-life and slowed renal clearance, which prolongs therapeutic activity. In addition, the attached Fc domain also enables the agents to interact with Fc-receptors (FcRs) found on immune cells, which is particularly important for their use in oncological therapies. In some other embodiments, a soluble CAR polypeptide can be coupled to a suitable carrier such as degradable hydrogels to facilitate its delivery into tumors. See, e.g., Kislukhin et al., J. Am. Chem. Soc., 134: 6491–6497, 2012; and Higginson et al., J. Am. Chem. Soc. 137: 4984–4987, 2015. By administering such agents directly to tumors, enhanced T cell migration to and interaction with tumor cells can be induced. Production of soluble CAR polypeptides capable of binding to JAML and their conjugation to carriers can be readily carried out in accordance with methods that are routinely practiced in the art. See, e.g., Witherden et al., Science 329:1205, 2010; Verdino et al., Structure 19:80-89, 2011; Verdino et al., Science 329:1210, 2010; Verdino et al., PloS ONE 6: e19828, 2011; Pinkert et al., J. Virol. 90:5601-5610, 2016; Houri et al., PLoS One.

8: e73296, 2013; Patzke et al., J. Neurosci. 30:2897-2910, 2010; and Freimuth et al., J. Virol. 73:1392-1398, 1999.

[0036] In addition to agents that promote JAML-CAR interactions, the therapeutic methods of the invention can additionally include a therapy based on immune checkpoint inhibitors. Immune checkpoints are molecules on certain immune cells that need to be activated (or inactivated) to start an immune response. Tumor cells can sometimes find ways to use these checkpoints to avoid being attacked by the immune system. Immune checkpoint inhibitors are drugs that target these checkpoints in the treatment of tumors. In the combination therapies of the invention, the agent promoting JAML-CAR interactions and the checkpoint inhibitor can be administered to the subject simultaneously. Alternatively, they can be administered to the subject sequentially. Thus, the subject can be treated with the agent promoting JAML-CAR interaction prior to or subsequent to treatment with the checkpoint inhibitor. Any known immune checkpoint inhibitors can be employed in these methods of the invention. These include, e.g., drugs that target PD-1 or PD-L1. PD-1 is a checkpoint protein on T cells. It normally acts as a type of "off switch" that helps keep the T cells from attacking other cells in the body. This is achieved when PD-1 attaches to PD-L1, a protein present on some normal cells and cancer cells. Some cancer cells have large amounts of PD-L1, which helps them evade immune attack. Suitable immune checkpoint inhibitors also include drugs also include that target CTLA-4. CTLA-4 is another protein on some T cells that acts as a type of "off switch" to keep the immune system in check.

[0037] Methods of the invention can employ any of the immune checkpoint inhibitors known in the art. For example, monoclonal antibodies that target either PD-1 or PD-L1 can block this binding and boost the immune response against cancer cells. These drugs have shown a great deal of promise in treating certain cancers. Specific examples of drugs that target PD-1 include: Pembrolizumab (Keytruda) and Nivolumab (Opdivo). These drugs have been shown to be helpful in treating several types of cancer, including melanoma of the skin, non-small cell lung cancer, kidney cancer, bladder cancer, head and neck cancers, and Hodgkin lymphoma. They are also being studied for use against many other types of cancer. Specific examples of drugs that target PD-L1 include Atezolizumab (Tecentriq), Avelumab (Bavencio) and Durvalumab (Imfinzi). These drugs have also been shown to be helpful in treating different types of cancer, including bladder cancer, non-small cell lung cancer, and

Merkel cell skin cancer (Merkel cell carcinoma). They are also being studied for use against other types of cancer. Specific examples of immune checkpoint inhibitors targeting CTLA-4 includes Ipilimumab (Yervoy). This is a monoclonal antibody that attaches to CTLA-4 and stops it from working. This can boost the body's immune response against cancer cells. Other than these known immune checkpoint inhibitors, the combination therapies of the invention can further utilize many other immune checkpoint inhibitors or other drugs that are currently being evaluated in various clinical trials. See, e.g., "What's new in cancer immunotherapy research?" at https://www.cancer.org/treatment.

[0038] In a related aspect, the invention provides specific agents that are capable of promoting the specific binding between JAML on T cells and CAR on tumor cells. These include monoclonal antibodies that target JAML, including antibodies that can be derived from any of the known JAML-targeting antibodies noted above. Preferably, such antibody agents do not block or substantially interfere with JAML binding with CAR. Antibody agents of the invention include a full-length antibody or an antibody fragment that specifically recognizes or binds to the extracellular domain of CAR, e.g., human CAR. For example, the antibody, antibody fragment or antibody-based binding protein can be polyclonal, monoclonal, recombinant, chimeric, or humanized. Furthermore, the antibody can be of any isotype including without limitation IgA, IgD, IgE, IgG, or IgM. Thus, for example, the antibody can be any IgA such as IgAl or IgA2, or any IgG such as IgGl , IgG2, IgG3, IgG4, or synthetic IgG. The antibody can also be any antibody fragment or antibody-based binding protein having specificity for the extracellular domain of CAR, such as F(ab)2, Fv, scFv, IgGACH2, F(ab')2, scFv2CH3, Fab, VL, VH, scFv4, scFv3, scFv2, dsFv, Fv, scFv-Fc, (scFv)2, a diabody, and a bivalent antibody. The antibody can be any modified or synthetic antibody, including, but not limited to, non-depleting IgG antibodies, or other Fc or Fab variants of antibodies.

[0039] In some embodiments, the antibody agents of the invention are derived from antibody HL4E10. Antibody HL4E10 is a hamster Fab targeting JAML and is strongly costimulatory for γδ T cells. See, e.g., Verdino et al., PLoS ONE 6: e19828, 2011. Its heavy and light chain CDR sequences are shown in SEQ ID NOs:1-6, respectively. Some antibody agents of the invention are humanized or chimeric antibodies derived from Fab antibody HL4E10. For example, human chimeric antibodies are those in which at least one region of the antibody is from a human immunoglobulin. A human chimeric antibody

typically contain variable regions from a non-human animal, e.g. a rodent, with the constant regions from a human. In contrast, a humanized antibody uses CDRs from the non-human antibody with most or all of the variable framework regions from and all the constant regions from a human immunoglobulin. In some embodiments, a "humanized antibody" refers to an antibody or antibody fragment, antigen-binding fragment, or antibody-based binding protein that contains antibody V_H or V_L domains with a homology to human V_H or V_L antibody framework sequences having a T20 score of greater than 80, as defined by defined by Gao et al. (2013) BMC Biotechnol. 13, pp. 55.

[0040] In some embodiments, the antibodies of the invention contain one or more CDR sequences that are identical to that of antibody HLE410. Relative to antibody HLE410, the antibodies of the invention may additionally contain various modifications in the CDR regions as well as the framework regions. For example, the antibodies can contain modifications (e.g., amino acid deletions as well as conservative or non-conservation substitutions) in one or more of the heavy chain CDRs and light chain CDRs. The antibodies can additionally contain one or more modifications in its framework regions relative to that of Fab antibody HL4E10. Similar to HL4E10, the humanized or chimeric antibody agents of the invention are all capable of enhancing interaction between junctional adhesion molecule (JAML) and the Coxsackie and adenovirus receptor (CAR). In some preferred embodiments, the antibody agents of the invention do not block or interfere with JAML binding to CAR. In some embodiments, the antibody agents of the invention contain one, two or all three of its heavy chain CDRs1-3 that are identical to DYGVH (SEQ ID NO:1), IIGHAGGTDYNSNLKS (SEQ ID NO:2), and HFYTYFDV (SEQ ID NO:3), respectively. Alternatively or additionally, these antibody agents of the invention may contain one, two or all three of its light chain CDRs 1-3 respectively shown in SGDKLSDVYVH (SEQ ID NO:4), EDNRRPS (SEQ ID NO:5) and QSWDGTNSAWV (SEQ ID NO:6). In some embodiments, the antibodies contain heavy chain CDRs1-3 and light chain CDRs1-3 respectively shown in SEQ ID NOs:1-6. In any of these embodiments, the antibodies may further contain various modifications in the framework regions. Relative to antibody HL4E10, the humanized or chimeric antibodies of the invention are expected to have similar or better activities and/or immunogenic properties. For example, these antibodies should have similar or better binding specificity for human JAML, substantively reduced immunogenicity in human subjects, and equal or better properties in not interfering

with JAML binding to CAR. Such characteristics of the antibodies of the invention can be readily examined and confirmed via standard techniques of immunology that have been routinely practiced in the art. In various embodiments, properties of the antibody agents can be examined in animal models. For example, mouse models can be used to assess in vivo effect of the antibodies on T cell tumor infiltration, tumor-infiltrating lymphocyte (TIL) survival, and cytokine production.

[0041] The various antibodies and antibody fragments (or "antigen-binding fragments") thereof described herein can be produced by enzymatic or chemical modification of the intact antibodies, or synthesized de novo using recombinant DNA methodologies, or identified using phage display libraries. Methods for generating these antibodies, antibody-based binding proteins, and antibody fragments thereof are all well known in the art. For example, chimeric and humanized antibodies may be prepared by methods well known in the art including CDR grafting approaches (see, e.g., U.S. Patent Nos. 5,843,708; 6,180,370; 5,693,762; 5,585,089; 5,530,101), chain shuffling strategies (see e.g., U.S. Patent No. 5,565,332; Rader et al., Proc. Natl. Acad. Sci. USA (1998) 95:8910-8915), molecular modeling strategies (U.S. Patent No. 5,639,641), and the like. In some embodiments, anti-human JAML antibody can be generated via by directed mutagenesis of a known JAML antibody described herein, e.g., the HL4E10 antibody. Similarly, single chain antibodies can be obtained with routinely practiced immunological methods, e.g., phage display libraries or ribosome display libraries, gene shuffled libraries (see, e.g., McCafferty et al., Nature 348:552-554, 1990; and U.S. Pat. No. 4,946,778). In particular, scFv antibodies can be obtained using methods described in, e.g., Bird et al., Science 242:423-426, 1988; and Huston et al., Proc. Natl. Acad. Sci. USA 85:5879-5883, 1988. Fv antibody fragments can be generated as described in Skerra and Plückthun, Science 240:1038-41, 1988. Disulfide-stabilized Fv fragments (dsFvs) can be made using methods described in, e.g., Reiter et al., Int. J. Cancer 67:113-23, 1996. Similarly, single domain antibodies (dAbs) can be produced by a variety of methods described in, e.g., Ward et al., Nature 341:544-546, 1989; and Cai and Garen, Proc. Natl. Acad. Sci. USA 93:6280-85, 1996. Camelid single domain antibodies can be produced using methods well known in the art, e.g., Dumoulin et al., Nat. Struct. Biol. 11:500–515, 2002; Ghahroudi et al., FEBS Letters 414:521–526, 1997; and Bond et al., J. Mol. Biol. 332:643-55, 2003. Other types of antigen-binding fragments (e.g., Fab, $F(ab')_2$ or Fd fragments) can also be readily produced with routinely practiced

immunology methods. See, e.g., Harlow & Lane, *Using Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1998; Kohler & Milstein, *Nature* 256:495-497, 1975; Kozbor et al., Immunology Today 4:72, 1983; and Cole et al., pp. 77-96 in Monoclonal Antibodies and Cancer Therapy, 1985.

[0042] In some other embodiments, the invention provides soluble CAR derived polypeptide agents that are capable of promoting the specific binding between JAML on T cells and CAR on tumor cells. These include, e.g., the extracellular domain of CAR or any JAML-binding fragments thereof. As described above, such polypeptide agents can be readily obtained via methods well known in the art. In some of these embodiments, the soluble CAR fragment can be further linked to a fusion partner or carrier protein. The fusion partner can be any molecule or moiety that can improve or enhance the biological, pharmacokinetics, or pharmacodynamics properties of the soluble CAR polypeptide. In some of these embodiments, the soluble CAR polypeptide such as the extracellular domain of CAR or a JAML-binding fragment thereof can be fused to the Fc domain of an IgG. Fusion with an Fc-domain provides a number of beneficial biological and pharmacological properties. For example, the presence of the Fc domain can markedly increase the plasma half-life of the hybrid molecule, which prolongs therapeutic activity, owing to its interaction with the salvage neonatal Fc-receptor, as well as to the slower renal clearance for larger sized molecules. The attached Fc domain also enables the molecules to interact with Fc-receptors (FcRs) found on immune cells, a feature that is particularly important for their use in oncological therapies and vaccines. As the Fc domain folds independently, it can improve the solubility and stability of the fused CAR polypeptide both *in vitro* and *in vivo*. Further, the Fc region allows for easy cost-effective purification by protein-G/A affinity chromatography during manufacture. The soluble CAR polypeptide can be fused with an Fc domain at either its N-terminus or C-terminus. Hybrid or fusion molecules containing a soluble CAR polypeptide of the invention and a fusion partner such as Fc domain can be readily generated in accordance with standard recombination techniques, routinely practiced protein synthesis methods or the protocols described herein.

[0043] The methods for promoting JAML-CAR interaction and the related agents as described herein can be employed in various therapeutic or prophylactic applications. In particle, they are suitable for treating cancers or preventing the development of tumors, esp. tumors that have CAR expression in their development. The cancers and tumors suitable for

treatment with compositions and methods of the present invention can be those present in a variety of tissues and organs. They also include cancer cells, tumor cells, which include malignant tumor cells, and the like that are found in the component cells of these tissues and/or organs. Examples include brain tumors (glioblastoma multiforme and the like), spinal tumors, maxillary sinus cancer, cancer of the pancreatic gland, gum cancer, tongue cancer, lip cancer, nasopharyngeal cancer, mesopharyngeal cancer, hypopharyngeal cancer, laryngeal cancer, thyroid cancer, parathyroid cancer, lung cancer, pleural tumors, cancerous peritonitis, cancerous pleuritis, esophageal cancer, stomach cancer, colon cancer, bile duct cancer, gallbladder cancer, pancreatic cancer, hepatic cancer, kidney cancer, bladder cancer, prostate cancer, penile cancer, testicular tumors, cancer of the adrenal glands, uterocervical cancer, endometrial cancer, vaginal cancer, vulvar cancer, ovarian cancer, ciliated epithelial cancer, malignant bone tumors, soft-tissue sarcomas, breast cancer, skin cancer, malignant melanomas, basal cell tumors, leukemia, myelofibrosis with myeloid metaplasia, malignant lymphoma tumors, Hodgkin's disease, plasmacytomas, and gliomas.

[0044] In some preferred embodiments, therapeutic methods of the invention are directed to treating tumors which contain high or upregulated CAR expressions. For example, CAR upregulation was observed in cancers of the endometrium, ovary, cervix, breast and lung, as well as neuroblastomas and medulloblastomas. See, e.g., Martino et al, Virol. 271: 99-108, 2000; Martin et al, Clin. Exp. Med. 5:122-128, 2005; Persson et al, J. Neurooncol. 78:1-6, 2006; Wang et al, J. Mol. Histol. 37:153-160, 2006; Reimer et al, Int. J. Cancer. 120: 2568-2575, 2007; Giaginis et al, World J. Surg. Oncol. 6: 59, 2008; Wu et al., Cell Death & Disease 1: e70, 2010; and Dietel et al, J. Mol. Med. 89:621-630, 2011. It was also reported that, in breast and lung cancer types, high CAR expression has been linked to poor overall survival and shorter disease-free survival, respectively. See, e.g., Martin et al, Clin. Exp. Med. 5:122-128, 2005; and Wunder et al, Cancer Gene Ther. 20: 25-32, 2012. In some specific embodiments, methods of the invention can be employed for treating any of these tumors, as well as other tumors involving CAR expression in the epithelium or skin (e.g., melanoma).

[0045] Generally, the treatment should affect a subject, tissue or cell to obtain a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or sign or symptom thereof. It can also be therapeutic in terms of a partial or complete cure for a disease or disorder (e.g., tumor

growth) that is associated with or mediated by CAR expression or biochemical activities, or amelioration of adverse effect that is attributable to the disorder. Suitable subjects include an invertebrate, a vertebrate, a mammal, particularly a human. The therapies described in the present invention can be used alone or in combination with any of various drugs, including known antitumor drugs (antineoplastic drugs), tumor metastasis-inhibitors, analgesics, anti-inflammatory drugs, immunoregulators (or immunomodulators) and/or immunosuppressants, which can be employed as not being restricted to particular species as long as they serve effectively or advantageously. In some embodiments, the therapies can be employed in combination with treatments based on immune checkpoint inhibitors, as noted above.

[0046] The JAML-CAR targeting compounds can be administered alone to a subject in need of treatment. More preferably, they are administered in the form of a pharmaceutical composition or preparation in admixture with any of various pharmacologically-acceptable additives. For example, the compounds may be administered in the form of a convenient pharmaceutical composition or formulation suitable for oral, topical, parenteral application, or the like. Pharmaceutical compositions of the invention can be prepared in accordance with methods well known and routinely practiced in the art. See, e.g., Remington: *The Science and Practice of Pharmacy*, Mack Publishing Co., 20th ed., 2000; and *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978. Pharmaceutical compositions are preferably manufactured under GMP conditions. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for molecules of the invention include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, e.g., polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

[0047] Pharmaceutical composition containing a JAML-CAR targeting compound can be administered locally or systemically in a therapeutically effective amount or dose. They

can be administered parenterally, enterically, by injection, rapid infusion, nasopharyngeal absorption, dermal absorption, rectally and orally. The JAML-CAR targeting compound for use in the methods of the invention should be administered to a subject in an amount that is sufficient to achieve the desired therapeutic effect (e.g., eliminating or ameliorating symptoms associated with tumor development and growth) in a subject in need thereof. Typically, a therapeutically effective amount or efficacious dose of the compound employed in the pharmaceutical compositions of the invention should inhibit, slow or suppress tumor growth in a subject. As noted below, actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response without being toxic to the subject.

[0048] The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, the route of administration, the time of administration, and the rate of excretion of the particular compound being employed. It also depends on the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, gender, weight, condition, general health and prior medical history of the subject being treated, and like factors. Methods for determining optimal dosages are described in the art, e.g., Remington: *The Science and Practice of Pharmacy*, Mack Publishing Co., 20th ed., 2000. For a given JAML-CAR targeting compound, one skilled in the art can easily identify the effective amount by using routinely practiced pharmaceutical methods. Dosages used *in vitro* or *in situ* studies may provide useful guidance in the amounts useful for *in vivo* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Typically, a pharmaceutically effective dosage would be between about 0.001 and 100 mg/kg body weight of the subject to be treated.

[0049] The JAML-CAR targeting compounds and other therapeutic regimens described herein are usually administered to the subjects on multiple occasions. Intervals between single dosages can be daily, weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of the compounds and the other therapeutic agents used in the subject. In some methods, dosage is adjusted to achieve a plasma compound concentration of 1–1000 μg/ml, and in some methods 25-300 μg/ml or 10-100 μg/ml.

Alternatively, the therapeutic agents can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the JAML-CAR targeting compound and the other drugs in the subject. The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, a relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some subjects may continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the subject shows partial or complete amelioration of symptoms of disease. Thereafter, the subject can be administered a prophylactic regime.

EXAMPLES

[0050] The following examples are offered to illustrate, but not to limit the present invention.

Example 1. Targeting JAML-CAR binding to promote tumor infiltration of T cells

[0051] A number of studies that were performed and that can be performed as described herein are able to show the effect of JAML and CAR expression on T cell tumor infiltration, as well as feasibility of targeting JAML-CAR in the treatment of cancer. Detailed description of these studies is provided in Figures 1-10. The studies also include testing safety of anti-JAML stimulation, with or co-administration of immune checkpoint inhibitors, by quantifying aberrant T cell infiltration into non-tumor tissues (e.g. skin, gut, lung) compared to tumor tissues. Such safety test can be performed with, e.g., flow-cytometry and histology analysis. The test can also include assessment of inflammatory cytokines in serum.

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[0052] It is understood that the examples and embodiments described herein are for illustrative purposes only, and that various modifications or changes in light thereof will be apparent to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

[0053] All publications, GenBank sequences, ATCC deposits, patents and patent applications cited herein are hereby expressly incorporated by reference in their entirety and for all purposes as if each is individually so denoted.

WE CLAIM:

1. A method for promoting T cell tumor infiltration in a subject, comprising administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of an agent that enhances the specific binding between junctional adhesion molecule (JAML) on T cells and Coxsackie and adenovirus receptor (CAR) on tumor cells; thereby promoting T cell tumor infiltration in the subject.

2. The method of claim 1, wherein the subject is further administered with a checkpoint inhibitor.

3. The method of claim 2, wherein the checkpoint inhibitor is administered to the subject prior to, simultaneously with, or subsequent to treatment with the agent that enhances JAML-CAR interaction.

4. The method of claim 2, wherein the checkpoint inhibitor is a CTLA inhibitor, a PD-1 inhibitor or a PD-L1 inhibitor.

5. The method of claim 1, wherein the agent that enhances JAML-CAR interaction is a monoclonal antibody targeting JAML.

6. The method of claim 5, wherein the monoclonal antibody targeting JAML does not block or interfere with JAML binding to CAR.

7. The method of claim 5, wherein the monoclonal antibody targeting JAML has a binding specificity that is the same as that of an antibody comprising HCDR1-3 and LCDR1-3 sequences respectively shown in SEQ ID NOs:1-6.

8. The method of claim 5, wherein the monoclonal antibody targeting JAML comprises HCDR1-3 and LCDR1-3 sequences respectively shown in SEQ ID NOs:1-6.

9. The method of claim 5, wherein the monoclonal antibody targeting JAML is Fab antibody HL4E10.

10. The method of claim 5, wherein the monoclonal antibody targeting JAML is a humanized antibody or a chimeric antibody.

11. The method of claim 10, wherein the humanized antibody is derived from Fab antibody HL4E10.

12. The method of claim 10, wherein the humanized antibody comprises at least one CDR as shown in SEQ ID NOs:1-6.

13. The method of claim 10, wherein the humanized antibody comprises heavy chain CDR1-3 as shown in SEQ ID NOs:1-3, respectively.

14. The method of claim 10, wherein the humanized antibody comprises heavy chain CDRs 1-3 and light chain CDRs 1-3 as shown in SEQ ID NOs:1-6, respectively.

15. The method of claim 1, wherein the agent that enhances JAML-CAR interaction comprises a soluble CAR molecule.

16. The method of claim 15, wherein the soluble CAR molecule is a CAR-Fc fusion.

17. The method of claim 15, wherein the soluble CAR molecule is the extracellular domain of CAR.

18. The method of claim 1, wherein the tumor is a skin tumor, a colorectal tumor, a lung tumor, or an ovarian tumor.

19. A method for stimulating T cell killing of a tumor in a subject, comprising administering to a subject afflicted with a tumor a pharmaceutical composition comprising a therapeutically effective amount of an agent that enhances the specific interaction between junctional adhesion molecule (JAML) on T cells and Coxsackie and adenovirus receptor (CAR) on tumor cells; thereby stimulating T cell killing of the tumor in the subject.

20. The method of claim 19, wherein the subject is further administered with a checkpoint inhibitor.

21. The method of claim 20, wherein the checkpoint inhibitor is administered to the subject prior to, simultaneously with, or subsequent to treatment with the agent that enhances JAML-CAR interaction.

22. The method of claim 19, wherein the agent that enhances JAML-CAR interaction is a monoclonal antibody targeting JAML.

23. The method of claim 22, wherein the monoclonal antibody targeting JAML has a binding specificity that is the same as that of an antibody comprising HCDR1-3 and LCDR1-3 sequences respectively shown in SEQ ID NOs:1-6.

24. The method of claim 22, wherein the monoclonal antibody targeting JAML is a humanized antibody that is derived from Fab antibody HL4E10.

25. The method of claim 19, wherein the agent that enhances JAML-CAR interaction comprises a soluble CAR molecule.

26. The method of claim 25, wherein the soluble CAR molecule is a CAR-Fc fusion.

27. The method of claim 19, wherein the tumor is a skin tumor, a colorectal tumor, a lung tumor, or an ovarian tumor.

28. A humanized antibody derived from Fab antibody HL4E10, comprising one or more CDR sequence as shown in SEQ ID NOs:1-6, wherein the humanized antibody specifically recognizes junctional adhesion molecule (JAML) but does not block JAML interaction with Coxsackie and adenovirus receptor (CAR).

29. The humanized antibody of claim 28, further comprising one or more amino acid substitutions in its framework region relative to that of Fab antibody HL4E10.

30. The humanized antibody of claim 28, comprising heavy chain CDR1-3 as shown in SEQ ID NOs:1-3, respectively.

31. The humanized antibody of claim 28, comprising heavy chain CDRs 1-3 and light chain CDRs 1-3 as shown in SEQ ID NOs:1-6, respectively.

32. A soluble polypeptide fragment derived from Coxsackie and adenovirus receptor (CAR) that is capable of binding to the junctional adhesion molecule (JAML) on T cells.

33. The soluble polypeptide fragment of claim 32, which is a fusion with a Fc domain.

Expression of JAML and CAR is associated with improved survival in many epithelial cancers (The Human Protein Atlas Database)

JAML Expression

5-year Survival Rates Based on JAML Expression (The Human Protein Atlas)

Cancer Type	Colorectal	Head and Neck	Lung	Cervical	Uterine	Melanoma*
High Expression	74%	48%	47%	89%	80%	40%
Low Expression	54%	36%	33%	59%	66%	0%

CAR Expression

5-year Survival Rates Based on CAR Expression (The Human Protein Atlas)

Cancer Type	Colorectal	Head and Neck	Lung	Urothelial	Stomach	Melanoma*
High Expression	65%	51%	51%	49%	40%	38%
Low Expression	50%	39%	38%	35%	25%	0%

* 3-year survival rate

FIG. 1

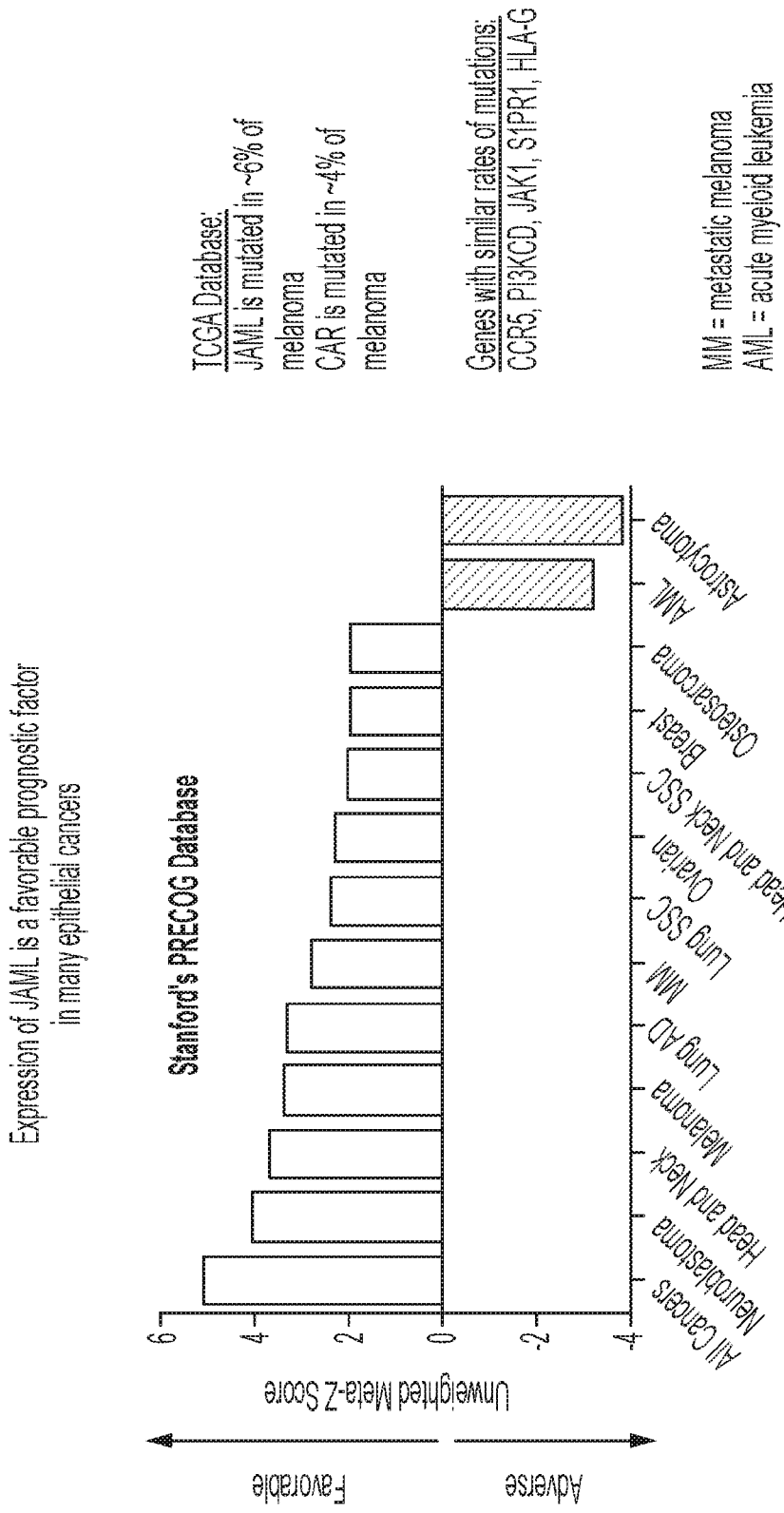
Expression of JAML is a favorable prognostic factor in many epithelial cancers
Stanford's PRECOG Database
Unweighted Meta-Z Score
6
4
2
0
-2
-4
Favorable
Adverse
All Cancers
Neuroblastoma
Head and Neck
Melanoma
Lung AD
MM
Lung SSC
Ovarian
Head and Neck SSC
Breast
Osteosarcoma
AML
Astrocytoma
TCGA Database:
JAML is mutated in ~6% of melanoma
CAR is mutated in ~4% of melanoma
Genes with similar rates of mutations:
CCR5, PI3KCD, JAK1, S1PR1, HLA-G
MM = metastatic melanoma
AML = acute myeloid leukemia

FIG. 2

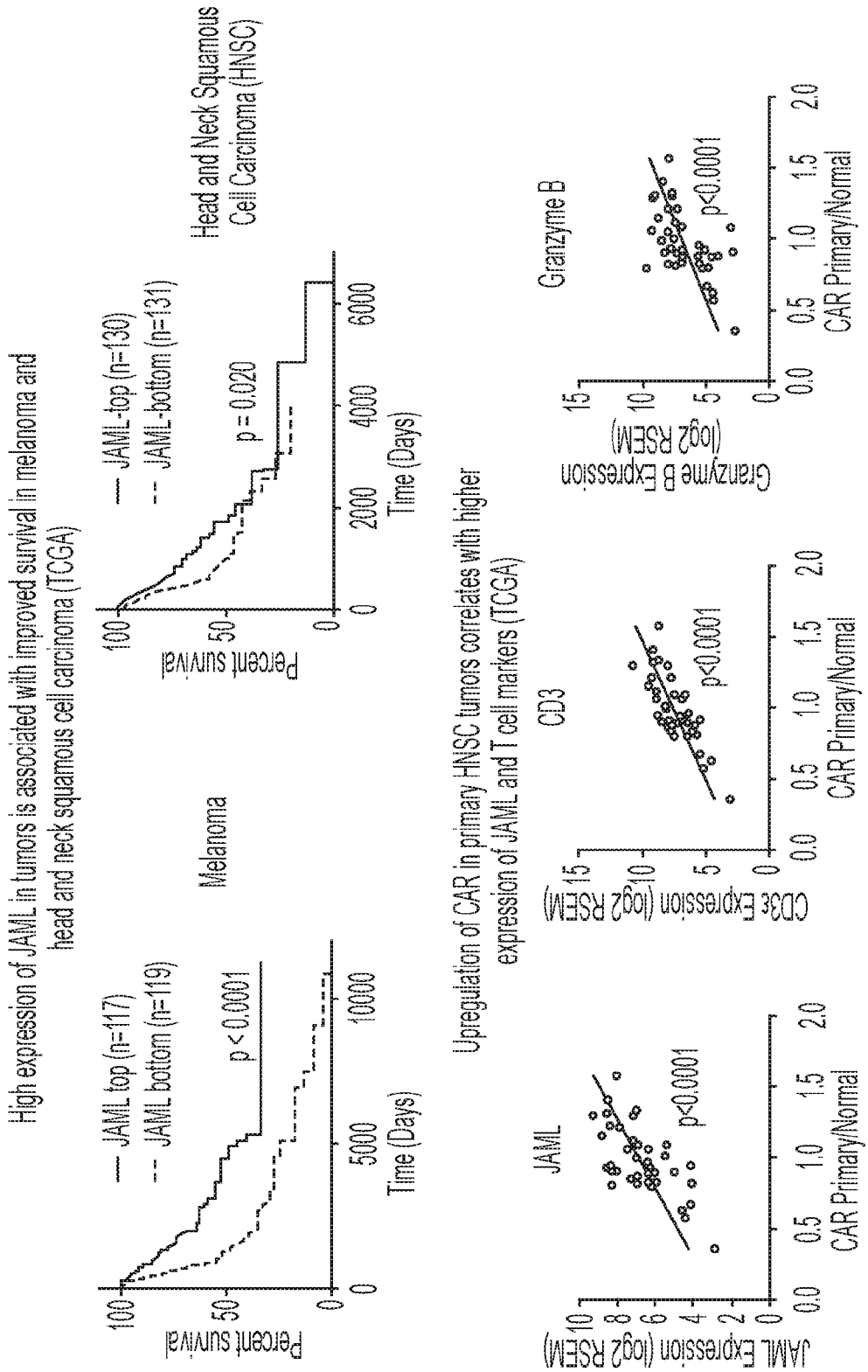
High expression of JAML in tumors is associated with improved survival in melanoma and head and neck squamous cell carcinoma (TCGA)
JAML top (n=117)
JAML bottom (n=119)
p < 0.0001
Melanoma
Percent survival
Time (Days)
JAML-top (n=130)
JAML-bottom (n=131)
p = 0.020
Head and Neck Squamous Cell Carcinoma (HNSC)
Upregulation of CAR in primary HNSC tumors correlates with higher expression of JAML and T cell markers (TCGA)
JAML
p<0.0001
JAML Expression (log2 RSEM)
CAR Primary/Normal
CD3
CD3ε Expression (log2 RSEM)
Granzyme B
Granzyme B Expression (log2 RSEM)

FIG. 3

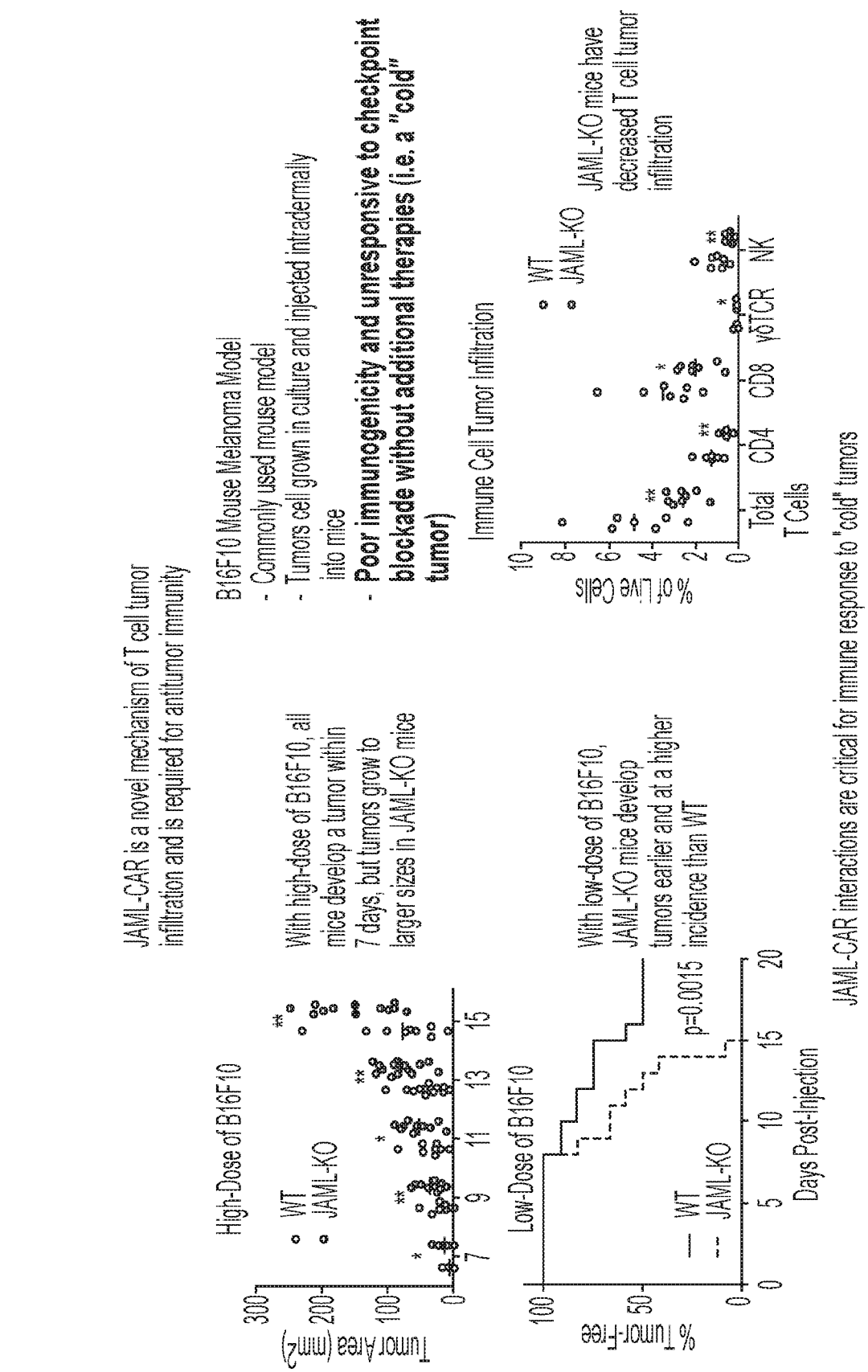
JAML-CAR is a novel mechanism of T cell tumor infiltration and is required for antitumor immunity
B16F10 Mouse Melanoma Model
- Commonly used mouse model
- Tumors cell grown in culture and injected intradermally into mice
- Poor immunogenicity and unresponsive to checkpoint blockade without additional therapies (i.e. a "cold" tumor)
Immune Cell Tumor Infiltration
WT
JAML-KO
% of Live Cells
Total CD4 CD8 γδTCR NK
T Cells
JAML-KO mice have decreased T cell tumor infiltration
High-Dose of B16F10
WT
JAML-KO
Tumor Area (mm²)
With high-dose of B16F10, all mice develop a tumor within 7 days, but tumors grow to larger sizes in JAML-KO mice
Low-Dose of B16F10
With low-dose of B16F10, JAML-KO mice develop tumors earlier and at a higher incidence than WT
p=0.0015
% Tumor-Free
Days Post-Injection
JAML-CAR interactions are critical for immune response to "cold" tumors

FIG. 4

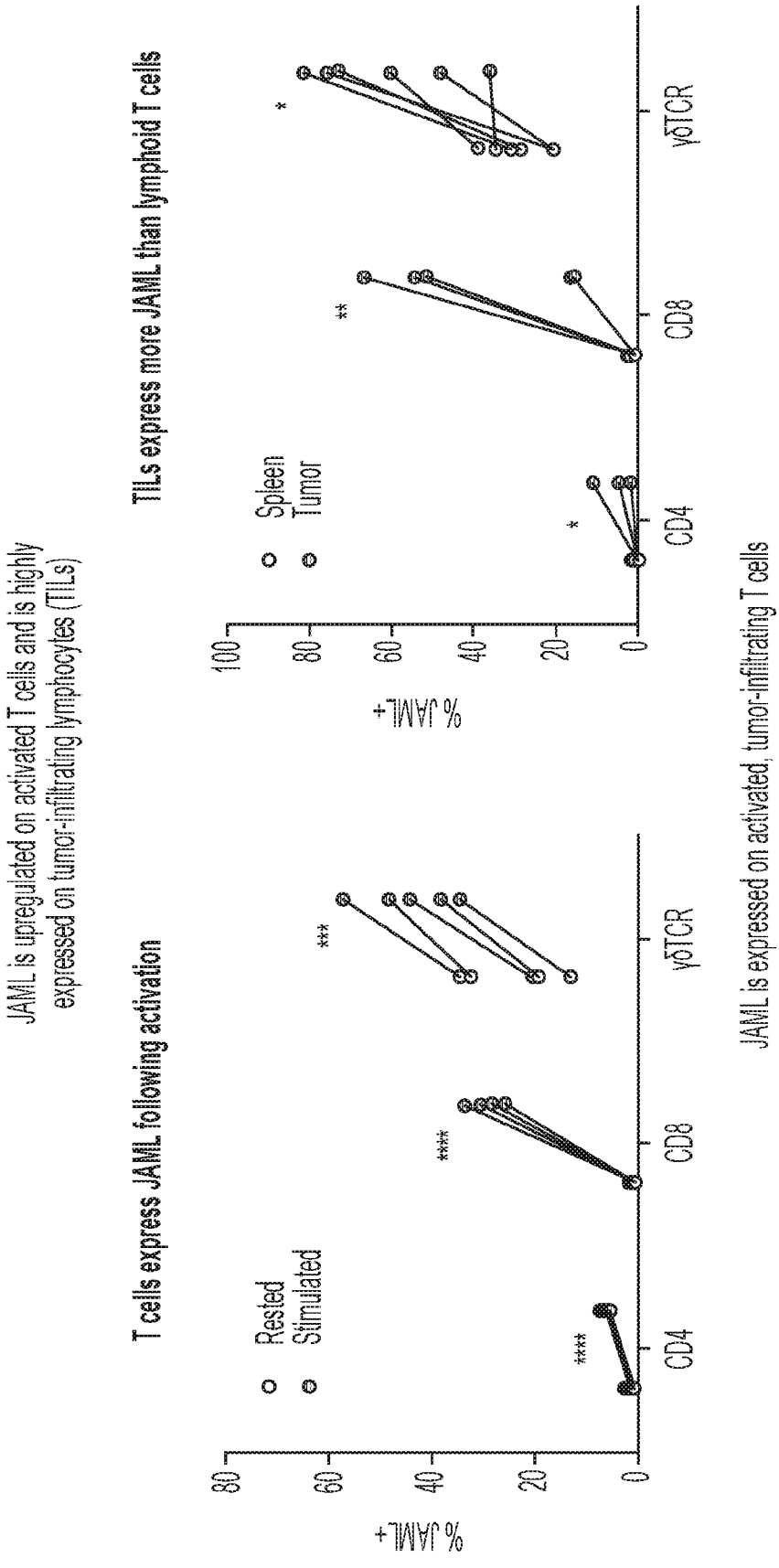
JAML is upregulated on activated T cells and is highly expressed on tumor-infiltrating lymphocytes (TILs)
T cells express JAML following activation
Rested
Stimulated
% JAML+
CD4
CD8
γδTCR
TILs express more JAML than lymphoid T cells
Spleen
Tumor
JAML is expressed on activated, tumor-infiltrating T cells

FIG. 5

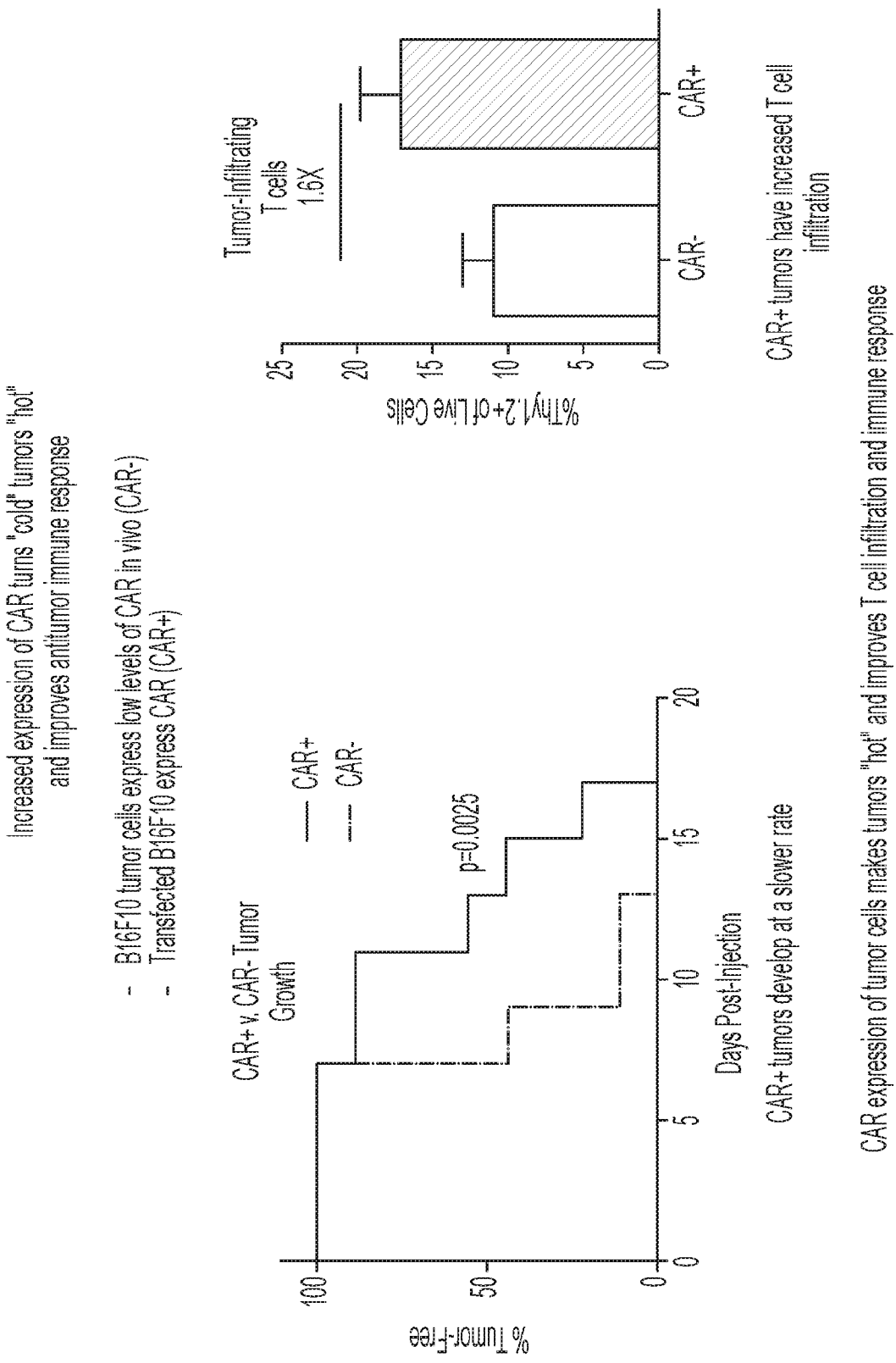
Increased expression of CAR turns "cold" tumors "hot"
and improves antitumor immune response
- B16F10 tumor cells express low levels of CAR in vivo (CAR-)
- Transfected B16F10 express CAR (CAR+)
CAR+ v. CAR- Tumor Growth
CAR+
CAR-
p=0.0025
% Tumor-Free
Days Post-Injection
CAR+ tumors develop at a slower rate
Tumor-Infiltrating T cells 1.6X
%Thy1.2+of Live Cells
CAR+ tumors have increased T cell infiltration
CAR expression of tumor cells makes tumors "hot" and improves T cell infiltration and immune response

FIG. 6

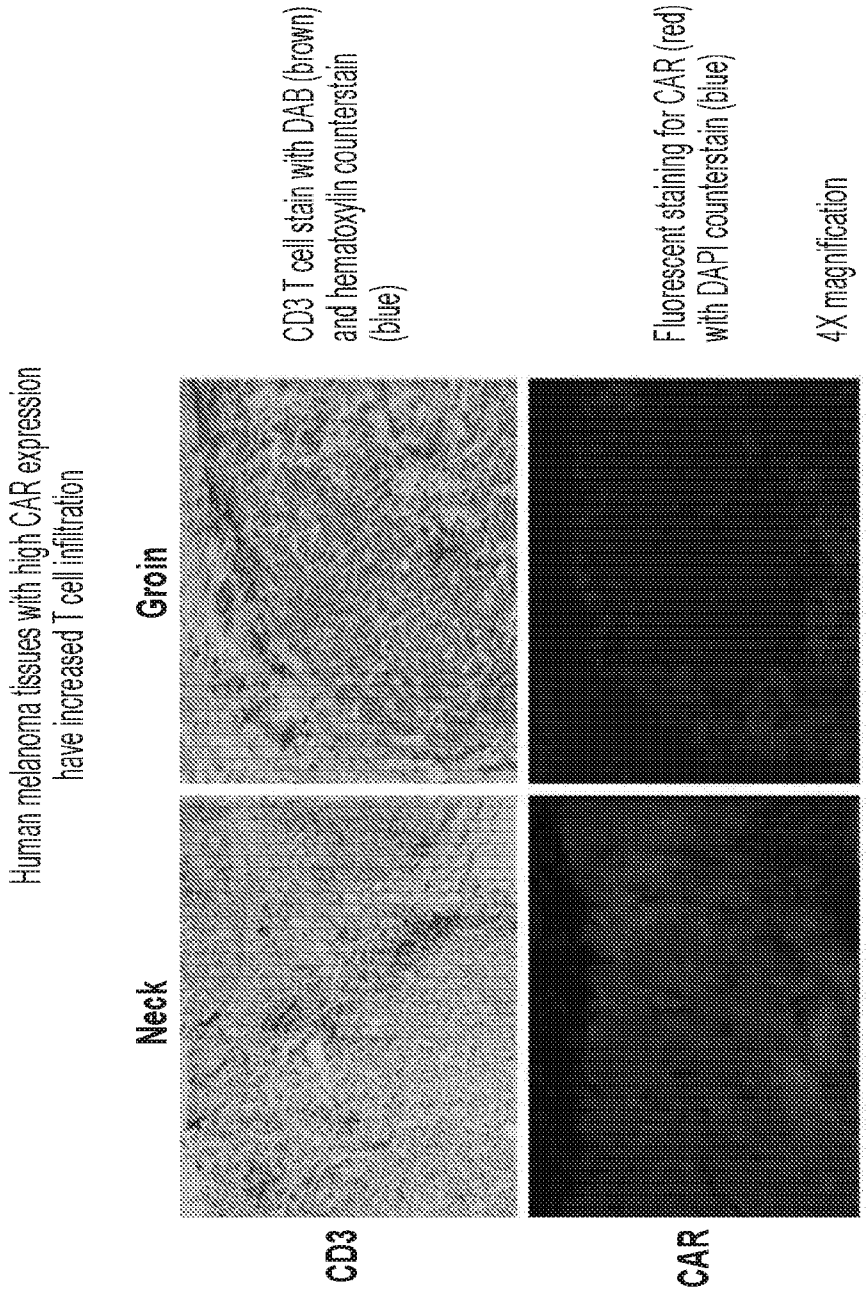
Human melanoma tissues with high CAR expression have increased T cell infiltration
Neck
Groin
CD3 T cell stain with DAB (brown) and hematoxylin counterstain (blue)
Fluorescent staining for CAR (red) with DAPI counterstain (blue)
4X magnification
CD3
CAR

FIG. 7

In vivo anti-JAML stimulation to induce T cell tumor infiltration
Work Flow
Challenge WT mice with 5x10^5 B16F10 intradermally
Administer antibody treatments on days 7, 10, 13 post-tumor challenge (4 groups-isotype, anti-JAML, anti-PD-1, combo)
Track tumor growth / rejection (measure tumors every other day)
Quantify T cell infiltration and activation at day 17 post-tumor challenge by flow-cytometry and histology
Assess if increased T cell infiltration is associated with efficacy of anti-JAML with or without anti-PD-1
Expected Results
Relative Tumor Size
30
20
10
0
isotype
anti-JAML
anti-PD-1
anti-JAML+PD-1
0
5
10
15
20
Days Post-Injection
i.p. antibody treatment
Treatment
T cell infiltration
Antitumor Response
Isotype
-
-
Anti-JAML
++
++
Anti-PD-1
+
++
Anti-JAML + anti-PD-1
+++
+++
- anti-JAML stimulation will induce tumor infiltration
- will therefore also improve efficacy of chekpoint blockade

FIG. 8

Testing Intratumoral Hydrogel Injections

Hydrogel Formulations

- Polyethylene glycol (PEG)-based gels with oxanobornadiene (OND)-thiol linkers
- OND-thiol linkers spontaneously degrade via retro-Diels-Alder reaction
- Modifications to OND linkers allow for tunable rates of degradation (from minutes to months)
- Sustained release of cargo protein, covalently attached to OND-thiol linkers, as gel degrades

Higginson *et al. JACS* (2015).

Hydrogels are non-toxic and retain protein functionality

- Hydrogel formulations are safe to use topically on mouse skin wounds
- CAR-FC protein remains functional (i.e. binds JAML) following release from hydrogel

Work flow for optimizing intratumoral hydrogel injection

Challenge WT mice with $5x10^5$ B16F10 intradermally

Prepare hydrogel formulations that degrade within 24, 48, and 96 hours

Administer hydrogel formulations containing fluorescently-labeled BSA at day 10 post-tumor challenge

Harvest tumors 2, 4, 6 days post-hydrogel injection

Assess retention and diffusion of fluorescent BSA in tumors tissue by histology

Assess safety by looking at serum cytokines

Initial testing with fluorescently-labeled BSA is to determine best hydrogel formulation for intratumoral injection

FIG. 9

Testing Delivery of CAR via Hydrogel

Work Flow for Delivery of CAR using Optimized Hydrogel Formulation

Challenge WT mice with $5x10^5$ B16F10 intradermally

Prepare hydrogel formulations containing CAR-Fc or CAR-D2 domain (does not bind JAML) as negative control

Administer hydrogel formulations at day 10 post-tumor challenge

Track tumor growth / rejection (measure tumors every other day)

Quantify T cell infiltration and activation at day 17 post-tumor challenge by flow-cytometry and histology

Advantages of Hydrogel Injections

- Tunable release (can be modified depending on cancer type / stage)
- Can be used to deliver any therapeutic
- Intratumoral delivery can reduce systemic off-target effects

Expected Results

- Sustained delivery of CAR will improve T cell tumor infiltration and reset tumor microenvironment
- Delivery of CAR will slow tumor progression

Combination therapy approaches

- Following intratumoral delivery of CAR, treat with systemic anti-JAML and/or anti-PD-1
- Add anti-JAML and/or PD-1 to hydrogel formulation

Treatment	T cell infiltration	Antitumor Response
CAR-D2 (no JAML binding)	-	-
CAR-Fc (binds JAML)	++	++
CAR-Fc + anti-PD-1	+++	+++

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/36121

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/02, 39/395; C07K 16/28 (2019.01)

CPC - A61K 38/02, 39/39533; C07K 16/28

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	(VERDINO, P et al.) cDNA Sequence and Fab Crystal Structure of HL4E10, a Hamster IgG Lambda Light Chain Antibody Stimulatory for [gamma-delta] T Cells. PLoS One. 2011, Epub 24 May 2011, Vol. 6, No. 5, e19828; pages 1-10; abstract; page 2, 2nd column, 4th paragraph; page 3, 2nd column, 1st paragraph–page 4, 1st column, 1st paragraph; page 9, 2nd column, 1st paragraph; Figures 1-2; DOI: 10.1371/journal.pone.0019828	1, 5-14, 19, 22-24, 28-31 -- 2-4, 15-18, 20-21, 25-27
X -- Y	(WITHERDEN, DA et al.) The adhesion molecule JAML is a costimulatory receptor for epithelial gamma-delta T cell activation. Science. 3 September 2010, Vol. 329, No. 5996; pages 1205-1210; page 3, 4th paragraph; Figures 3-4; DOI: 10.1126/science.1192698	32-33 -- 15-17, 25-26, 28, 30-31
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