COMBINATION OF ANTIBODIES TARGETING DLL4/VEGF AND CD137 AGONISTS OR ANTI-PD1/ANTI-PD-L1 ANTIBODIES FOR THE TREATMENT OF CANCER

TECHNICAL FIELD

[0001] The present disclosure relates to methods of using a bispecific antibody targeting VEGF-A and DLL4 in combination with CD137 agonism or PD-1/PD-L1 blockade to improve anti-tumor efficacy of the individual treatments alone in multiple syngeneic models of cancer.

BACKGROUND

[0002] An increasing body of evidence suggests that the immune system operates as a significant barrier to tumor formation and progression. The principle that naturally occurring T cells with anti-tumor potential or activity exist in a patient with cancer has rationalized the development of immunotherapeutic approaches in oncology. Immune cells, such as T cells, macrophages, and natural killer cells, can exhibit anti-tumor activity and effectively control the occurrence and growth of malignant tumors. Tumor-specific or -associated antigens can induce immune cells to recognize and eliminate malignancies (Chen & Mellman, (2013) *Immunity* 39(1):1-10). Despite the existence of tumor-specific immune responses, malignant tumors often evade or avoid immune attack through a variety of immunomodulatory mechanisms resulting in the failure to control tumor occurrence and progression (Motz & Coukos, (2013) *Immunity* 39(1):61-730). Indeed, an emerging hallmark of cancer is the exploitation of these immunomodulatory mechanisms and the disablement of anti-tumor immune responses, resulting in tumor evasion and escape from immunological killing (Hanahan and Weinberg (2011) *Cell* 144(5):646-674).

[0003] Traditionally, monoclonal antibodies (mAbs) blocking immune checkpoints have been used as monotherapy. Antibodies that block immune escape and that block angiogenesis have been used with success. Because the interaction of PD-L1 and PD-1 can inhibit the T-cell response, mAbs targeting programmed cell death protein 1 (PD-1) or programmed cell death-ligand 1 (PD-L1) have been shown to reactivate suppressed T-cells to block the cancer-immune escape.

[0004] Combinatorial strategies, such as targeting different immune modulatory receptors, hold promise to increase the breadth and duration of response to cancer therapy. Delta-like ligand 4 ("DLL4") is a delta-class ligand that binds to Notch proteins which are overexpressed in vascular endothelial cells. DLL4 is also known as a major factor that

regulates angiogenesis. Angiogenesis refers to the mechanism by which new blood vessels are formed from the pre-existing blood vessels. Specifically, DLL4 binds to the Notch 1 or Notch 4 receptor which is overexpressed in vascular endothelial cells. Although it is also expressed in normal blood vessels, DLL4 is highly overexpressed in cancer blood vessels. In tumors, angiogenesis is caused by angiogenic factors such as vascular endothelial growth factor ("VEGF") to supply oxygen and nutrients to the cancer tissue. Angiogenesis plays an important role not only in the growth of the tumor, but also in the metastasis of the tumor. When Notch signaling by DLL4 in tumors is blocked, angiogenesis cannot be easily controlled. Inhibition of DLL4 signaling can inhibit tumor growth in some tumor models. In addition, when Notch signaling by DLL4 is inhibited, autoimmune disease can be treated by increasing the number of regulatory T cells (Treg) (US Patent Publication No. 2011-0189200). For these reasons, DLL4 is a target in the treatment of cancers and autoimmune diseases.

[0005] Tumor angiogenesis, the formation of new blood vessels in solid tumors, plays an important role in tumor cell survival, growth, and metastasis. A major driving force of tumor angiogenesis is the signaling pathway involving VEGF and its receptors ("VEGFR"). Several angiogenesis inhibitors, including antibodies and small molecule compounds targeting the VEGF/VEGFR signaling pathway, have been approved by the Food and Drug Administration ("FDA"), and are used for the treatment of many different types of cancers. Besides cancer treatment, VEGF/VEGFR inhibitors, including antibody fragments, aptamers, and VEGF-Traps are also approved and used for the treatment of ocular diseases caused by pathological angiogenesis. Blocking VEGF/VEGFR can inhibit VEGF-driven tumor angiogenesis. Moreover, the regression of tumor vessels is dependent on the VEGF signaling pathway. VEGF inhibitors alone, however, are not capable of destroying all tumor blood vessels. Further, preclinical studies indicate that VEGF inhibitors alone may result in an increasingly aggressive and invasive pattern of tumors. In addition, some cancer patients may eventually experience resistance to anti-VEGF therapy.

[0006] The largely successful anticancer therapeutic antibody drug Avastin® (Genentech/Roche) was approved by the FDA in 2004. Avastin targets VEGF and inhibits angiogenesis. Recent clinical models and preclinical animal model studies have indicated, however, that not all solid tumors respond to VEGF inhibitors. In addition, several reported cases indicate that some tumors treated with VEGF inhibitors in the initial stage show resistance after a certain time. Some studies show that the administration of VEGF inhibitors may make cancer cells more aggressive and result in easier metastasis. Such reports have

propelled research and development of novel anticancer targets that overcome Avastin resistance or that have efficacy superior to that of Avastin. Both the DLL4/Notch and VEGF/VEGF-receptor signaling pathways help orchestrate vascular sprouting and migration of the endothelial tip cells, and are functionally interconnected (*See, e.g.*, Akil, A., et al., *Notch Signaling in Vascular Endothelial Cells, Angiogenesis, and Tumor Progression: An Update and Prospective.* Front Cell Dev Biol, 2021. **9**: p. 642352.). Typically, angiogenesis occurs in physiological processes, such as wound healing, and angiogenesis is also required for tumors to grow beyond a minimal size (*See* Dvorak, H.F., *Tumors: wounds that do not heal-redux.* Cancer Immunol Res, 2015. **3**(1): p. 1-11; and Nagy, J.A., A.M. Dvorak, and H.F. Dvorak, *Vascular hyperpermeability, angiogenesis, and stroma generation.* Cold Spring Harb Perspect Med, 2012. **2**(2): p. a006544.). Because of this, pharmacological interference with this process has been postulated to serve as an effective anticancer strategy (*See* Folkman, J., *Tumor angiogenesis: therapeutic implications.* N Engl J Med, 1971. **285**(21): p. 1182-6.). Indeed, neutralizing VEGF or its primary angiogenic receptor, KDR (VEGFR-2) with biologics or small molecule kinase inhibitors respectively, can prevent the growth of many rapidly growing mouse tumors in the clinic (*See, e.g.*, Ferrara, N., *Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications.* Semin Oncol, 2002. **29**(6 Suppl 16): p. 10-4; Dvorak, H.F., *Rous-Whipple Award Lecture. How tumors make bad blood vessels and stroma.* Am J Pathol, 2003. **162**(6): p. 1747-57; Jain, R.K., et al., *Lessons from phase III clinical trials on anti-VEGF therapy for cancer.* Nat Clin Pract Oncol, 2006. **3**(1): p. 24-40; and Hurwitz, H., et al., *Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer.* N Engl J Med, 2004. **350**(23): p. 2335-42.).

[0007] Further, the immunological effects of inhibition of angiogenesis may synergistically enhance the efficacy of immune checkpoint blockers. Some studies have shown that Avastin® reduced the density of angiopoietin-2-positive vessels and improved infiltration of CD4+ T and CD8+ T cells, as well as mature dendritic cells, suggesting that VEGF blockade could enhance the efficacy of immunotherapy through improving the infiltration of immune cells (*See* Boucher, Y., Kumar A.S., Posada J.M., Gjini E., Pfaff K., Lipschitz M., Lako A., Duda D.G., Rodig S.J., Hodi F.S., and Jain R.K. *Bevacizumab improves tumor infiltration of mature dendritic cells and effector T-cells in triple-negative breast cancer patients*. NPJ Precis Oncol. 2021. 5(1):62.).

[0008] Analogously, DLL4 blockade with monoclonal antibodies leads to tumor growth inhibition in preclinical animal models, and the effect is enhanced by VEGF blockade

or chemotherapy (*See, e.g.*, Miles, K.M., et al., *Dll4 blockade potentiates the anti-tumor effects of VEGF inhibition in renal cell carcinoma patient-derived xenografts.* PLoS One, 2014. **9**(11): p. e112371; Ridgway, J., et al., *Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis.* Nature, 2006. **444**(7122): p. 1083-7; Fischer, M., et al., *Anti-DLL4 inhibits growth and reduces tumor-initiating cell frequency in colorectal tumors with oncogenic KRAS mutations.* Cancer Res, 2011. **71**(5): p. 1520-5; and Hoey, T., et al., *DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency.* Cell Stem Cell, 2009. **5**(2): p. 168-77.).

[0009] CD137 (alternatively known as "tumor necrosis factor receptor superfamily member 9" (TNFRSF9), 4-1BB, and "induced by lymphocyte activation" (ILA)) is a transmembrane co-stimulatory receptor protein belonging to the tumor necrosis factor superfamily. CD137 is a T-cell co-stimulatory receptor induced upon T-cell receptor (TCR) activation (Nam et al., (2005) Curr Cancer Drug Targets 5:357-363; Watts et al., (2005) Annu Rev Immunol 23:23-68). In addition to its expression on activated CD4+ and CD8+ T-cells, CD137 is also expressed on CD4+CD25+ regulatory T-cells, activated natural killer (NK) and NK-T-cells, monocytes, neutrophils, and dendritic cells.

[0010] Under physiological conditions, CD137 is ligated by CD137 ligand (CD137L), an agonist membrane molecule present on antigen-presenting cells including B cells, monocytes, macrophages, and dendritic cells (Watts et al., (2005) Annu Rev Immunol 23:23-68). Upon interaction with its ligand, CD137 leads to increased TCR-induced T-cell proliferation, cytokine production, functional maturation, and prolonged CD8+ T-cell survival. The potential of CD137 co-stimulation using various agonists (e.g. agonistic antibodies, recombinant CD137L protein, and CD137-specific aptamers) to enable the immune system to attack tumors has been documented in numerous models (Dharmadhikari et al., (2016) Oncoimmunology 5(4):e1113367).

SUMMARY OF THE DISCLOSURE

[0011] The present disclosure is based, at least in part, on the discovery that combinatorial strategies, such as targeting different immune modulatory receptors, hold promise to increase the breadth and duration of response to cancer therapy. As such, disclosed herein is a method of treating cancer comprising administering a bispecific antibody which targets VEGF-A and DLL4, in combination with an agonistic CD137 antibody, or monoclonal antibodies that disrupt the PD-1/PD-L1 signaling axis. Coupling

VEGF-A/DLL4 targeting with either CD137 agonism or PD-1/PD-L1 blockade increased the anti-tumor efficacy of the individual treatments alone in the CT26 and LLC1 syngeneic mouse tumor models. Notably, the combination of the VEGF-A/DLL4 targeting bispecific antibody and the CD137 agonist or PD-1/PD-L1 blocker retained anti-tumor activity even in tumor lines made MHC-I deficient or negative. In fact, the combination of the VEGF-A/DLL4 targeting bispecific antibody and the CD137 agonist or PD-1/PD-L1 blocker overcame reduced efficacy of targeting CD137 or PDL1 alone due to beta-2-microglobulin (B2M), an essential component of MHC class I antigen presentation, loss (MHC-negative). Moreover, the combination was unexpectedly effective in both the wild type models and the B2M knock-out (KO) model. As described herein, the findings suggest that the bispecific antibody may provide enhanced clinical benefits when employed alongside immuno-modulating agents, such as agonistic anti-CD137 antibodies or PD-1/PD-L1 blockers.

[0012] As described above, bispecific antibodies targeting VEGF-A and DLL4 are undergoing clinical investigation in multiple cancer types, including biliary and colon cancers. Given the important roles of both targets in the function of multiple immune cell populations and their common presence within tumors, the disclosure provides methods that combine the anti-tumor efficacy of the bispecific antibody targeting the DLL4 and VEGF pathways and several immunotherapies. Furthermore, the murine equivalent of the bispecific antibody targeting the DLL4 and VEGF pathways administered together with either CD137 agonism or PD-1/PD-L1 blockade markedly increased the anti-tumor efficacy of the individual treatments alone in multiple syngeneic mouse models of cancer. In addition, the combination of the bispecific antibody targeting DLL4 and VEGF pathways together with the CD137 agonist retained potent anti-tumor activity even in tumor lines made MHC-I deficient or fully negative through the deletion of the *B2M* gene. Thus, presented herein are methods of enhancing clinical benefits by administering a bispecific antibody targeting the DLL4 and VEGF pathways alongside immuno-modulating agents, such as agonistic anti-CD137 antibodies or PD-1/PD-L1 blockers.

[0013] In view of the foregoing, provided herein are methods of oncological therapeutic treatment comprising administering a recombinant bispecific antibody of the human IgG1 isotype containing at least one single chain variable fragments (scFvs) that binds to DLL4 linked to an antibody that binds and neutralizes the activity of human VEGF-A in combination with immuno-modulating agents. In some instances, the immuno-modulating agents target complementary mechanisms of action. For example, the immuno-modulating agents may include agonistic anti-CD137 antibodies or PD-1/PD-L1 blockers.

[0014] Also provided herein, are methods for increasing anti-tumor efficacy comprising administering a bispecific antibody targeting VEGF-A and DLL4 together with an agonistic anti-CD137 antibody, and/or monoclonal antibodies that disrupt the PD-1/PD-L1 signaling axis. In some instances, the tumor may arise from biliary tract cancer (BTC), colorectal cancer (CRC), gastric cancer, cholangiocarcinoma cancer, pancreatic cancer, non-small cell lung cancer (NSCLC), and/or in patients who have been previously treated with one or more checkpoint inhibitors (CPIs). Further, the previously treated tumor or cancer may have evaded treatment.

[0015] Provided herein are methods for increasing the anti-tumor efficacy of individual treatments alone comprising simultaneously administering a combination of a bispecific antibody targeting VEGF-A and DLL4 and an agonistic anti-CD137 antibody, or one or more monoclonal antibodies that disrupt the PD-1/PD-L1 signaling axis.

[0016] Also provided herein are methods for increasing anti-tumor activity in MHC-I deficient tumor cell lines comprising administering a combination of a bispecific antibody targeting VEGF-A and DLL4 and an agonistic anti-CD137 antibody, or one or more monoclonal antibodies that disrupt the PD-1/PD-L1 signaling axis. In some examples, the subject may be post-CPI (i.e., the subject may have been previously treated with a CPI) and/or the tumor to be treated may exhibit CPI resistance. For example, the tumor to be treated may exhibit loss of antigen presentation following progression on checkpoint blockade therapy.

[0017] Also provided herein are methods for increasing the anti-tumor efficacy of the individual treatments alone comprising coupling VEGF-A/DLL4 targeting with either CD137 agonism or PD-1/PD-L1 blockade.

[0018] Provided herein are methods of enhancing clinical benefits of cancer treatment using a bispecific antibody targeting VEGF-A and DLL4 comprising administering an agonistic anti-CD137 antibody or PD-1/PD-L1 blockade in combination with the bispecific antibody targeting VEGF-A and DLL4. In some examples, the cancer may be BTC, CRC, gastric cancer, cholangiocarcinoma cancer, pancreatic cancer, and/or NSCLC, or any other cancer, and in particular may include any tumor or cancer that has evaded treatment after patients suffering from the cancer have been previously treated with one or more CPIs.

[0019] Provided herein are methods of treating cancer comprising administering a dual-targeting protein to a subject, wherein the dual-targeting protein comprises a protein that specifically binds to DLL4 and VEGF thereby blocking DLL4 and VEGF signaling, in combination with agonistic anti-CD137 antibody or PD-1/PD-L1 blocker.

[0020] Provided herein are methods of treating cancer comprising administering a dual-targeting protein to a subject, wherein the dual-targeting protein comprises a protein that simultaneously blocks DLL4 and VEGF signaling pathways in combination with one or more agonistic anti-CD137 antibodies and/or one or more PD-1/PD-L1 blockers. In some examples, multiple anti-CD137 antibodies or multiple PD-1/PD-L1 blockers may be administered in conjunction with the dual-targeting protein. In addition, the cancer may be BTC, CRC, gastric cancer, cholangiocarcinoma cancer, pancreatic cancer, and/or NSCLC, or any other cancer or a cancer that has evaded treatment after patients have been previously treated with one or more CPIs.

[0021] Also provided herein are methods for first line treatment of cancer comprising administering a combination of a bispecific antibody targeting VEGF-A and DLL4 and an agonistic anti-CD137 antibody, or monoclonal antibodies that disrupt the PD-1/PD-L1 signaling axis. In some examples, multiple anti-CD137 antibodies and/or multiple PD-1/PD-L1 blockers may be administered.

[0022] Provided herein are methods of increasing potency of anti-tumor activity comprising administering a combination of a bispecific antibody targeting VEGF-A and DLL4 and an agonistic anti-CD137 antibody, or a monoclonal antibody that blocks PD-1/PD-L1 signaling axis. In some examples, multiple anti-CD137 antibodies or multiple PD-1/PD-L1 blockers may be administered.

[0023] Also disclosed herein are methods of oncological therapeutic treatment comprising administering a bispecific antibody that blocks both the DLL4/Notch and VEGF/VEGF-receptor signaling pathways in combination with a molecule that blocks PD-1/PD-L1 pathway or an CD137 agonist. In some examples, the oncological therapeutic treatment may be directed at BTC, CRC, gastric cancer, cholangiocarcinoma cancer, pancreatic cancer, and/or NSCLC.

[0024] Provided herein are methods of treating BTC comprising administering a bispecific antibody that blocks DLL4/Notch and VEGF/VEGF-receptor signaling in combination with an anti-PD-1/PD-L1 antibody and/or an CD137 agonist.

[0025] Provided herein are methods of treating CRC comprising administering a bispecific antibody that blocks DLL4/Notch and VEGF/VEGF-receptor signaling in combination with an anti-PD-1/PD-L1 antibody and/or an CD137 agonist.

[0026] Provided herein are methods of treating cancers that have evaded treatment after previously being treated with one or more CPIs comprising administering a bispecific

antibody that blocks DLL4/Notch and VEGF/VEGF-receptor signaling in combination with an anti-PD-1/PD-L1 antibody and/or an CD137 agonist.

[0027] Provided herein are methods of enhancing the efficacy of treating BTC, CRC, gastric cancer, cholangiocarcinoma cancer, pancreatic cancer, NSCLC, and/or other cancers that have evaded treatment after patients have been previously treated with one or more CPIs, comprising administering a bispecific antibody that targets and/or blocks DLL4/Notch and VEGF/VEGF-receptor signaling in combination with an anti-PD-1/PD-L1 antibody and/or an CD137 agonist.

[0028] The protein that specifically binds to DLL4 may be in the form of a full-length antibody, Fab', F(ab')2, Fab, Fv, IgG, or scFv (Single-chain variable fragment).

[0029] The protein that specifically binds to VEGF may be in the form of a full-length antibody, Fab', F(ab')2, Fab, Fv, IgG, or scFv (single-chain variable fragment).

[0030] The dual-targeting protein may be in a form in which the protein that binds specifically to DLL4 and the IgG (immunoglobulin G)-type antibody that binds specifically to VEGF are connected to each other by a linker. The linker may be a peptidyl linker or a non-peptide linker.

[0031] In some embodiments, the dual-targeting protein specifically binding to VEGF may include Bevacizumab.

[0032] The recombinant bispecific antibody as described herein may comprise a human IgG1 isotype which may contain a scFv capable of binding to DLL4 linked to the heavy chain of an antibody that binds and neutralizes the activity of VEGF. For example, the bispecific antibody may contain a scFv binding to DLL4 linked to the heavy chain of a bevacizumab biosimilar that binds and neutralizes the activity of human VEGF. Anti-tumor activity may be facilitated in combination with chemotherapy across a range of solid tumors, including colorectal, gastric, cholangiocarcinoma, pancreatic, and NSCLC. Other cancers, including BTC, small-cell lung cancer (SCLC), melanoma, and mesothelioma may also be treated with the antibodies described herein.

[0033] In some examples, the anti-PD-L1 antibody may be Atezolizumab.

[0034] Tumors may exhibit a loss of HLA-I and evade the immune system. In some instances, subjects may have previously undergone immunotherapy (post-checkpoint inhibitor). In some embodiments, a subject may have been previously treated with one or more CPIs and in such case, the cancer may have evaded treatment. Thus, the subject's cancer may have progressed despite treatment with CPIs.

[0035] Furthermore, the present disclosure also provides the generation and use of genetically modified cell lines carrying MHC-I defects commonly seen in post-CPI patients leading to MHC-I deficiency. Specifically, two isograft models based on the CT26 and MC38 lines that exhibit total MHC loss following genetic deletion of the B2m gene ($CT26^{B2m-/-}$ and $MC38^{B2m-/-}$), one model with partial MHC-I loss by deleting both H2-K1 alleles in MC38 cells (which eliminates 1/3 of the MHC-I presentation potential), and a line with increased NK resistance ($CT26^{B2m-/-E}$) which was generated by serial passaging of the $CT26^{B2m-/-}$ cells in tumor experienced mice. By expanding and characterizing the cells after serial passage, novel models of checkpoint inhibitor resistance were developed for use in the study of checkpoint inhibition resistance mechanisms. Mouse models described herein may lack both class I MHC, individual MHC alleles as well as the tumor suppressor CDKN2A.

BRIEF DESCRIPTION OF DRAWINGS

[0036] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0037] **FIG. 1:** A mouse CTX-009 surrogate bispecific antibody binds to endothelial cells and blocks both VEGF and DLL4 activity in vitro. **FIG. 1A** shows a bispecific antibody targeting murine VEGF-A and DLL4 generated to model CTX-009 activity in isogenic tumor models (mCTX-009). The CH and CL regions of the IgG scaffold are of the murine IgG2a isotype, whereas the variable regions are human; **FIG. 1B** shows mCTX-009 bound to mouse aortic endothelial cells (mAEC); **FIG. 1C** is a schematic showing mCTX-009 delayed the VEGF-dependent reconstitution of a cellular monolayer in a mAEC scratch assay; and **FIG. 1D** is a graph showing mCTX-009 blocked DLL4-induced NOTCH1 activity in a reporter assay. Luciferase reporter expression under the control of NOTCH1-response elements activated by plastic bound DLL4 is blocked by mCTX-009 as measured by luminescence (IC50= 2.90e-8M). **FIG. 1E** shows that both mCTX-009 and mAvastin blocked to a similar extent the proliferation of mAEC grown in the presence of spent CT26 cell culture medium (which contains pro-angiogenic factors such as VEGF).

[0038] **FIG. 2:** Superior efficacy of the mCTX-009 and mCTX-471 combination in selected CPI-sensitive and CPI-refractory models. The graphs show that mCTX-009 or mCTX-471 monotherapies produced a modest response in the highly refractory LLC1 model, while their combination produced a markedly enhanced anti-tumor activity. Similarly,

combination of the two treatments produced curative responses (60% and trending lower at takedown) in the immune CPI (Checkpoint inhibitor) responsive CT26 model. Shown is the average tumor volume of 9 (LLC1) or 7 (CT26) mice per group.

[0039] **FIG. 3:** B2mKO negative cell lines. **FIG. 3A** is a schematic showing loss of HLA (human leukocyte antigen), commonly observed in patients with resistance to CPI, can be recapitulated in cell lines, albeit in an HLA-allele non-specific manner, by elimination of the B2m gene. Deletion of B2m produced an MHC-I null phenotype in both CT26 and MC38 cell lines (bottom panels). Unlike the parental lines, $CT26^{B2mKO}$ and $MC38^{B2mKO}$, IFN-γ fails to further induce the expression of MHC-I. **FIG. 3B** is a graph showing that loss of B2m did not significantly affect proliferation *in vitro*, however, proliferation in mice was severely affected.

[0040] **FIG. 4:** NK depletion restores B2mKO cell engraftment. **FIG. 4** illustrates that the formation of $CT26^{B2mKO}$ tumors was facilitated when NK-cells were depleted with an anti-Asialo GM1 anitbody. NK depletion restored engraftment to levels similar or better than seen with parental CT26 cells (not shown).

[0041] **FIG. 5:** Escape of B2mKO cells as a model of CPI progression. **FIG. 5A** is a graph that illustrates mice cured from CT26 tumors by either mCTX-471 or the combination of mCTX-471 and mCTX-009 (experienced) or age matched tumor-naïve mice challenged with either the CT26 or $CT26^{B2mKO}$ cells. Tumor experienced mice were capable of dramatically delaying or completely suppressing the growth of both cell types, whereas tumor-naïve mice were not. **FIG. 5B** is a graph showing $CT26^{B2m}$ tumors that eventually grew in experienced mice reinoculated in naive mice. A 10-fold lower inoculum was required for $CT26^{B2mKO}$ escaper ($CT26^{B2mKO\text{-}E}$) to produce tumors similar to $CT26^{B2mKO}$, suggesting the tumors acquired resistance.

[0042] **FIG. 6:** Superior efficacy of the mCTX-009 + mCTX-471 combination vs monotherapies in $MC38^{B2mKO}$. **FIG 6A** illustrates that MC38 tumors are very responsive to mCTX-471 treatment (inoculum of $1x10^5$ – $2.5x10^5$ cells/mouse, mCTX-471 @1.5 mpk, q7dx2). **FIG. 6B** depicts MC38 cells that were rendered MHC-I negative by B2M deletion respond to mCTX-471; however, the efficacy was increased by combining it with mCTX-009 (6.8 mpk, q3dx5). The combination was more potent than the monotherapies. **FIG. 6C** shows that under the same conditions, the combination of Atezolizumab (3 mpk, q3dx3) and mCTX-009 was not additive. **FIG. 6D** depicts TIL analysis by flow cytometry. mCTX-009 + mCTX-471 increased the proportion of CD8+ T cells and decreased the amount of tumor associated macrophages (TAMs) in MC38. **FIG. 6E** illustrates that the $MC38^{B2mKO}$ model

exhibits a roughly equal amount of CD4 and CD8 TILs. Treatment with the mCTX-471 and Atezo combinations increased the relative amount of CD8 T cells (left panel), more for Atezo. The CTX-471 combination was better than the Atezo combination in increasing the amount of "innate killers" of the CD8+ or non CD4/CD8 lineages (middle two panels). Both combination treatments further reduced the fraction of infiltrating TAMs.

[0043] **FIG. 7:** Rejection of $CT26^{B2mKO}$ cells by mCTX-009 + mCTX-471 cured mice is critically dependent on both CD4 and NK cells. **FIG. 7A** is a graph showing tumor-naïve mice challenged with $CT26^{B2mKO}$ cells ($1x10^6$ per mouse) in NK cells or CD4+ T cell depleted animals. **FIG. 7B** illustrates that CT26-tumor-experienced mice with transient depletion of NK and CD4 cells rendered CT26-tumor-experienced mice more vulnerable to the MHC-I negative tumor cell challenge.

[0044] **FIG. 8:** Depicts an activity table showing a summary of *in vivo* activity.

[0045] **FIG. 9:** Enhanced recognition threshold of Immuno-Oncology (IO) resistant CT26 cells by splenocytes of tumor-experienced mice. **FIG. 9A** is a schematic and graph showing splenocytes from mice previously challenged with CT26 tumors and cured via CTX-471 and CTX-009 treatment (left panel) contain precursors that react to CT26 cells, producing approximately 10 times more IFN-γ against the indicated target cell lines (x-axis) compared to splenocytes from naïve mice (right panel). **FIG. 9B** is a graph showing a similar percentage of CD4, CD8, B, Monocyte and NK cells in the spleens from naïve vs tumor experienced mice.

[0046] **FIG. 10:** Loss of CD8 T-cells and murine dendritic cells and increase in CD4 T-cells in the $CT26^{B2m-/-E}$ model. The graph depicts baseline tumor infiltrating lymphocytes (TILs) characterization in the CT26 tumor series.

[0047] **FIG. 11:** The CTX-009/CTX-471 combination is effective in the $CT26^{B2m-/-E}$ model. The graphs illustrate that the combination of CTX-471 and CTX-009 is efficacious in CT26 models where conventional IO (anti PD-L1) shows reduced activity.

[0048] **FIG. 12:** Changes in TIL composition following treatment of $CT26^{B2m-/-E}$ tumors. **FIG. 12A** is a graph showing that treatment with CTX-009 or CTX-471 leads to a reduction in total resting CD4 memory T cells and B cells, whereas their combination increased the fraction of activated memory CD4 T cells. **FIG. 12B** presents immunohistochemistry (IHC) analysis of tumors (focus on the cortical areas of the tumors).

[0049] **FIG. 13:** The CTX-009/CTX-471 combination enhances the inflammasome, cytolysis and interferon pathways. **FIG. 13A** depicts a graph with selected genes representing hypoxia (Hif1a), IFN (Isg15), EMT (Smad3), and cytotoxicity (Gzmb) plotted as fold

induction over isotype-treated mice. A selection of differentially expressed genes (DEGs) highlighting those preferentially induced in the combination treatment is shown in **FIG. 13B**.

[0050] **FIG. 14:** The combination of CTX-471 and CTX-009 is efficacious in MC38-derived models where conventional IO (anti PD-L1) shows reduced activity. FIG. 14 depicts mice bearing the indicated tumor models treated with the specified antibodies (mCTX-009: 5 mpk, q3dx3; mCTX-471: 0.1 mpk, q7dx2; mAtezo: 3 mpk, q3dx3) when tumors reached ~80 mm^3 tumor volumes (Y-axis) were recorded over time.

[0051] **FIG. 15:** Evidence of NK activation in CTX-471 treated patients. **FIG. 15A** shows an increase of circulating NK cells (primarily CD56 bright) in CD137 treated patients. **FIG. 15B** shows an increase in the fraction of CD137 positive cells in sequential slides (pre-treatment and post-treatment) with CTX-471.

DETAILED DESCRIPTION

[0052] Blocking DLL4/Notch and VEGF/VEGF-receptor signaling is an important therapeutic strategy in oncology. CTX-009 is a recombinant bispecific antibody of the human IgG1 isotype which contains single chain variable fragments (scFvs) that bind DLL4 linked to the heavy chain of an antibody that binds and neutralizes the activity of human VEGF-A. Phase 1 trials demonstrated promising activity in patients with a variety of solid tumors, both as a monotherapy and in combination with chemotherapy. A Phase 2 trial in patients with advanced BTC achieved a 37.5% overall response rate when treated with CTX-009 in combination with paclitaxel in patients treated in the second- and third-line settings. Additional Phase 2 trials in colorectal and biliary tract cancers are ongoing. Described herein are mouse syngeneic tumor models and combinations of a mouse surrogate version of CTX-009 (mCTX-009) and immunotherapy approaches, namely PD-1/PD-L1 pathway blockade or CD137 agonism. Mouse models disclosed herein exhibit total MHC loss by deleting B2m in MC38 and CT26 isografts and partial loss by deleting both H2-K1 alleles in MC38 cells (which eliminates 1/3 of the MHC-I presentation potential). To counteract potential increase susceptibility to NK killing from total MHC loss, $CT26^{B2m-/-}$ cells may be passaged in vaccinated mice and a line with increased NK resistance ($CT26^{B2m-/-E}$) may be selected.

[0053] MHC class I negative tumors are generally resistant to immunotherapy. VEGF blockade has been demonstrated to alter the immunological composition of the tumor microenvironment. Provided herein we describe the therapeutic potential of immunomodulation and neo-angiogenesis disruption in mice with MHC class I negative

tumors. As mentioned above, CTX-009 is a bispecific antibody targeting VEGF-A and DLL4. CTX-471 is a next generation CD137 agonist antibody that has been both epitope and affinity optimized for agonizing CD137. CTX-471 is further described in, for example, U.S. Patent Nos. 10,716,851, 10,279,038, 10,279,039, 10,279,040, 10,350,292, and 10434175. Both antibodies are currently being tested in human clinical trials. As presented here, the combination of CTX-009 and CTX-471 significantly enhances the anti-tumor activity of either antibody alone. Furthermore, the combination of CTX- 009 and CTX-471 maintained potent anti-tumor activity even in MHC-I deficient mouse tumor models, which model loss of antigen presentation following progression on checkpoint blockade therapy in humans. In some instances, NK cells and CD4+ T cells emerge as key mediators of tumor growth control in this context. Thus, potential for improved clinical outcomes occurs when a bispecific antibody targeting VEGF-A and DLL4 is combined with immunomodulatory agents, particularly in tumors resistant to immune checkpoint inhibitors.

[0054] For example, as a standalone treatment, mCTX-009 demonstrated significant antitumor activity in the CT26, MC38, LLC1 and 4T1 isograft models. Moreover, mCTX-009 in combination with CTX-471 increased treatment effectiveness in several isograft models, including the IO resistant LLC1 model. Notably, this antitumor activity was even seen when tumors were rendered MHC-I negative due to B2M gene deletion, which recapitulates a CPI resistance mechanism observed in post CPI patients. Further, in the $MC38^{B2mKO}$ and $CT26^{B2mKO}$ models, the combination of CTX-009 and CTX-471 maintained anti-tumor activity.

[0055] As exemplified in the working examples, CTX-009 holds promise for enhanced clinical benefits when paired with immuno-modulating agents featuring complementary mechanisms of action, particularly CD137 agonism. This approach could be considered not only as a first-line treatment option, but also as an alternative where previous immunotherapy was ineffective.

[0056] The present disclosure provides a bispecific antibody which can bind specifically to DLL4 and VEGF to effectively inhibit the DLL4/Notch and VEGF/VEGFR signaling pathways. The bispecific antibody may bind specifically to VEGF and may additionally include a region that binds specifically to DLL4. In some embodiments, the VEGF and/or DLL4 binding portions may be one or more scFvs. In addition, the DLL4 binding portion may be connected to the C-terminal region of a protein similar to IgG -type Avastin. Thus, the bispecific antibody may effectively inhibit the interaction between VEGF and the VEGF receptor as well as the interaction between DLL4 and Notch protein and

exhibit anticancer effects. The present disclosure also provides an anti-CD137 agonist and/or a PD-1/PD-L1 blocker.

[0057] In some embodiments, this disclosure provides an oncological therapeutic strategy which comprises administering a recombinant bispecific antibody of the human IgG1 isotype containing at least one scFvs that binds to DLL4 linked to an antibody that binds and neutralizes the activity of human VEGF-A (FIG. 1A) in combination with immunotherapy approaches. In some instances, the immunotherapy approaches include immuno-modulating agents with complementary mechanisms of action. For example, the immuno-modulating agents may include agonistic anti-CD137 antibodies or PD-1/PD-L1 blockers. In some aspects, the provided methods increase anti-tumor efficacy and comprise administering a bispecific antibody targeting VEGF and DLL4 together with agonistic anti-CD137 antibodies, or monoclonal antibodies that disrupt the PD-1/PD-L1 signaling axis.

DEFINITIONS

[0058] Terms used in the claims and specification are defined as set forth below unless otherwise specified.

[0059] It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0060] As used herein, "about" will be understood by persons of ordinary skill and will vary to some extent depending on the context in which it is used. If there are uses of the term which are not clear to persons of ordinary skill given the context in which it is used, "about" will mean up to plus or minus 10% of the particular value.

[0061] As used herein, the term "agonist" refers to any molecule that partially or fully promotes, induces, increases, and/or activates a biological activity of a native polypeptide disclosed herein (e.g., CD137). Suitable agonist molecules specifically include agonist antibodies or antibody fragments, fragments or amino acid sequence variants of native polypeptides, peptides, antisense oligonucleotides, small organic molecules, etc. In some embodiments, activation in the presence of the agonist is observed in a dose-dependent manner. In some embodiments, the measured signal (e.g., biological activity) is at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at

least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100% higher than the signal measured with a negative control under comparable conditions. Also disclosed herein, are methods of identifying agonists suitable for use in the methods of the disclosure. For example, these methods include, but are not limited to, binding assays such as enzyme-linked immuno-absorbent assay (ELISA), Forte Bio© systems, and radioimmunoassay (RIA). These assays determine the ability of an agonist to bind the polypeptide of interest (e.g., a receptor or ligand, e.g., CD137) and therefore indicate the ability of the agonist to promote, increase or activate the activity of the polypeptide. Efficacy of an agonist can also be determined using functional assays, such as the ability of an agonist to activate or promote the function of the polypeptide. For example, a functional assay may comprise contacting a polypeptide with a candidate agonist molecule and measuring a detectable change in one or more biological activities normally associated with the polypeptide. The potency of an agonist is usually defined by its EC_{50} value (concentration required to activate 50% of the agonist response). The lower the EC_{50} value the greater the potency of the agonist and the lower the concentration that is required to activate the maximum biological response.

[0062] As used herein, the term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., a carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

[0063] Amino acids can be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, can be referred to by their commonly accepted single-letter codes. As used here, a “polar amino acid” refers to an amino acid

comprising a side chain that prefers to reside in an aqueous environment. In some embodiments, a polar amino acid is selected from the group consisting of: arginine, asparagine, aspartic acid, glutamic acid, glutamine, histidine, lysine, serine, theronine and tyrosine. Polar amino acids can be positive, negatively or neutrally charged. As used herein, a "non-polar amino acid" refers to an amino acid selected from the group consisting of: alanine, cysteine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan and valine.

[0064] As used herein, the term "amount" or "level" refers to a detectable quantity, level or abundance of a substance (e.g., a protein). When referring to a polypeptide, such as those described herein, the terms "level of expression" or "expression level" in general are used interchangeably and generally refer to a detectable amount of a polypeptide in a biological sample (e.g., on the surface of a cell).

[0065] As used herein, the term "anti-CD137 agonist antibody" (used interchangeably with the term "anti-CD137 antibody") refers to an antibody that specifically binds to CD137 and partially or fully promotes, induces, increases, and/or activates CD137 biological activity, response, and/or downstream pathway(s) mediated by CD137 signaling or other CD137-mediated function. In some embodiments, an anti-CD137 agonist antibody binds to CD137 and allows binding of CD137L. In some embodiments, an anti-CD137 agonist antibody binds to CD137 and induces multimerization of CD137. In some embodiments, an anti-CD137 agonist antibody binds to CD137 and induces the dimerization of CD137 trimers. In some embodiments, an anti-CD137 agonist antibody binds to CD137 and induces the multimerization of CD137 trimers. Examples of anti-CD137 agonist antibodies are provided herein. Methods for detecting formation of a trimer:trimer complex are known to those of skill in the art. For example, electron microscopy has been shown to detect such complexes, *see*, e.g., Won, E. The Journal of Biological Chemistry, Vol. 285 (12): 9202-9210 (2010).

[0066] As used herein, the term "antibody" refers to a whole antibody comprising two light chain polypeptides and two heavy chain polypeptides. Whole antibodies include different antibody isotypes including IgM, IgG, IgA, IgD, and IgE antibodies. The term "antibody" includes a polyclonal antibody, a monoclonal antibody, a chimerized or chimeric antibody, a humanized antibody, a primatized antibody, a deimmunized antibody, and a fully human antibody. The antibody can be made in or derived from any of a variety of species, e.g., mammals such as humans, non-human primates (e.g., orangutan, baboons, or chimpanzees), horses, cattle, pigs, sheep, goats, dogs, cats, rabbits, guinea pigs, gerbils, hamsters, rats, and mice. The antibody can be a purified or a recombinant antibody.

[0067] As used herein, the terms "antibody fragment," "antigen-binding fragment," "antigen binding portion" or similar terms refer to a fragment of an antibody that retains the ability to bind to a target antigen (e.g., CD137) and inhibit the activity of the target antigen. Such fragments include, e.g., a single chain antibody, a single chain Fv fragment (scFv), an Fd fragment, a Fab fragment, a Fab' fragment, or an $F(ab')_2$ fragment. An scFv fragment is a single polypeptide chain that includes both the heavy and light chain variable regions of the antibody from which the scFv is derived. In addition, intrabodies, minibodies, triabodies, and diabodies are also included in the definition of antibody and are compatible for use in the methods described herein. See, e.g., Todorovska et al., (2001) *J. Immunol. Methods* 248(1):47-66; Hudson and Kortt, (1999) *J. Immunol. Methods* 231(1):177-189; Poljak, (1994) *Structure* 2(12):1121-1123; Rondon and Marasco, (1997) *Annu. Rev. Microbiol.* 51:257-283, the disclosures of each of which are incorporated herein by reference in their entirety.

[0068] As used herein, the term "antibody fragment" also includes, e.g., single domain antibodies such as camelized single domain antibodies. See, e.g., Muyldermans et al., (2001) *Trends Biochem. Sci.* 26:230-235; Nuttall et al., (2000) *Curr. Pharm. Biotech.* 1:253-263; Reichmann et al., (1999) *J. Immunol. Meth.* 231:25-38; PCT application publication nos. WO 94/04678 and WO 94/25591; and U.S. patent no. 6,005,079, all of which are incorporated herein by reference in their entireties. In some embodiments, the disclosure provides single domain antibodies comprising two VH domains with modifications such that single domain antibodies are formed.

[0069] The term "antigen presenting cell" or "APC" is a cell that displays foreign antigen complexed with MHC on its surface. T cells recognize this complex using T cell receptor (TCR). Examples of APCs include, but are not limited to, dendritic cells (DCs), peripheral blood mononuclear cells (PBMC), monocytes (such as THP-1), B lymphoblastoid cells (such as C1R.A2, 1518 B-LCL) and monocyte-derived dendritic cells (DCs). Some APCs internalize antigens either by phagocytosis or by receptor-mediated endocytosis.

[0070] The term "antigen presentation" refers to the process by which APCs capture antigens and enables their recognition by T cells, e.g., as a component of an MHC-I and/or MHC-II conjugate.

[0071] As used herein, the term "binds to immobilized CD137," refers to the ability of a human antibody of the disclosure to bind to CD137, for example, expressed on the surface of a cell or which is attached to a solid support.

[0072] As used herein, the term "bispecific" or "bifunctional antibody" refers to an artificial hybrid antibody having two different heavy/light chain pairs and two different

binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, *e.g.*, Songsivilai & Lachmann, (1990) *Clin. Exp. Immunol.* 79:315-321; Kostelny et al., (1992) *J. Immunol.* 148:1547-1553.

[0073] Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chain/light-chain pairs have different specificities (Milstein and Cuello, (1983) *Nature* 305:537-539). Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences. The fusion of the heavy chain variable region is preferably with an immunoglobulin heavy-chain constant domain, including at least part of the hinge, CH2, and CH3 regions. For further details of illustrative currently known methods for generating bispecific antibodies see, e.g., Suresh et al., (1986) *Methods Enzymol.* 121:210; PCT Publication No. WO 96/27011; Brennan et al., (1985) *Science* 229:81; Shalaby et al., *J. Exp. Med.* (1992) 175:217-225; Kostelny et al., (1992) *J. Immunol.* 148(5):1547-1553; Hollinger et al., (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Gruber et al., (1994) *J. Immunol.* 152:5368; and Tutt et al., (1991) *J. Immunol.* 147:60. Bispecific antibodies also include cross-linked or heteroconjugate antibodies. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques. Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. See, e.g., Kostelny et al. (1992) J Immunol 148(5):1547-1553. The leucine zipper peptides from the Fos and Jun proteins may be linked to the Fab′ portions of two different antibodies by gene fusion. The antibody homodimers may be reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al. (1993) Proc Natl Acad Sci USA 90:6444-6448 has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the VH and VL domains of one fragment are forced to pair with the complementary VL and VH domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (scFv) dimers has also been reported. See, e.g., Gruber et al. (1994) J

Immunol 152:5368. Alternatively, the antibodies can be “linear antibodies” as described in, e.g., Zapata et al. (1995) Protein Eng. 8(10):1057-1062. Briefly, these antibodies comprise a pair of tandem Fd segments (VH-CH1-VH-CH1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific. Antibodies with more than two valences (e.g., trispecific antibodies) are contemplated and described in, e.g., Tutt et al. (1991) J Immunol 147:60. This disclosure also embraces variant forms of multi-specific antibodies such as the dual variable domain immunoglobulin (DVD-Ig) molecules described in Wu et al. (2007) Nat Biotechnol 25(11): 1290-1297. The DVD-Ig molecules are designed such that two different light chain variable domains (VL) from two different parent antibodies are linked in tandem directly or via a short linker by recombinant DNA techniques, followed by the light chain constant domain. Similarly, the heavy chain comprises two different heavy chain variable domains (VH) linked in tandem, followed by the constant domain CH1 and Fc region. Methods for making DVD-Ig molecules from two parent antibodies are further described in, e.g., PCT Publication Nos. WO 08/024188 and WO 07/024715. In some embodiments, the bispecific antibody is a Fabs-in-Tandem immunoglobulin, in which the light chain variable region with a second specificity is fused to the heavy chain variable region of a whole antibody. Such antibodies are described in, e.g., International Patent Application Publication No. WO 2015/103072.

[0074] As used herein, "B2M knockout" refers to deletion of the B2M gene and may be interchangeably refered to as "B2M KO", ”B2MKO", "B2mKO", "B2m-/-", and includes superscript versions thereof.

[0075] As used herein, "cancer antigen" refers to (i) tumor- specific antigens, (ii) tumor- associated antigens, (iii) cells that express tumor- specific antigens, (iv) cells that express tumor- associated antigens, (v) embryonic antigens on tumors, (vi) autologous tumor cells, (vii) tumor- specific membrane antigens, (viii) tumor- associated membrane antigens, (ix) growth factor receptors, (x) growth factor ligands, and (xi) any other type of antigen or antigen-presenting cell or material that is associated with a cancer.

[0076] The term "carcinoma" is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. The anti-CD137 antibodies described herein can be used to treat patients who have, who are suspected of having, or who may be at high risk for developing any type of cancer, including renal carcinoma or melanoma. Exemplary carcinomas include those forming from tissue of the

cervix, lung, prostate, breast, head and neck, colon and ovary. The term also includes carcinosarcomas, which include malignant tumors composed of carcinomatous and sarcomatous tissues. An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures.

[0077] It will also be understood by one of ordinary skill in the art that the antibodies suitable for use in the methods disclosed herein may be altered such that they vary in sequence from the naturally occurring or native sequences from which they were derived, while retaining the desirable activity of the native sequences. For example, nucleotide or amino acid substitutions leading to conservative substitutions or changes at "non-essential" amino acid residues may be made. Mutations may be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

[0078] As used herein, the term "EC_{50}" refers to the concentration of an antibody or an antigen-binding portion thereof, which induces a response, either in an *in vitro* or an *in vivo* assay, which is 50% of the maximal response, *i.e.*, halfway between the maximal response and the baseline.

[0079] As used herein, the term "effective dose" or "effective dosage" is defined as an amount sufficient to achieve or at least partially achieve the desired effect. The term "therapeutically effective dose" is defined as an amount sufficient to cure or at least partially arrest the disease and its complications in a patient already suffering from the disease. Amounts effective for this use will depend upon the severity of the disorder being treated and the general state of the patient's own immune system.

[0080] As used herein, the term "CD137" refers to a specific member of the tumor necrosis factor receptor (TNFR) family of transmembrane proteins. Alternative names and acronyms for CD137 in the art include "tumor necrosis factor receptor superfamily member 9" (TNFRSF9), 4-1BB and "induced by lymphocyte activation" (ILA) (Alderson et al., (1994) Eur J Immunol 24(9):2219-2227; Schwarz et al., (1993) Gene 134(2):295-298).

[0081] As used herein, the term "CD137L" or "CD137 ligand" refers to a member of the tumor necrosis factor (TNF) family of transmembrane proteins. Alternative names and acronyms for CD137L in the art include "tumor necrosis factor superfamily member 9" (TNFSF9) and 4-1BB ligand (4-1BBL) (Alderson et al., (1994) Eur J Immunol 24(9):2219-2227).

[0082] As used herein, "isotype" refers to the antibody class (*e.g.*, IgM or IgGl) that is encoded by heavy chain constant region genes. In some embodiments, a human monoclonal

antibody of the disclosure is of the IgG1 isotype. In some embodiments, a human monoclonal antibody of the disclosure is of the IgG1 isotype and comprises a mutation.

[0083] As used herein, the term "KD" or "K_D" refers to the equilibrium dissociation constant of a binding reaction between an antibody and an antigen. The value of K_D is a numeric representation of the ratio of the antibody off-rate constant (kd) to the antibody on-rate constant (ka). The value of K_D is inversely related to the binding affinity of an antibody to an antigen. The smaller the K_D value the greater the affinity of the antibody for its antigen. Affinity is the strength of binding of a single molecule to its ligand and is typically measured and reported by the equilibrium dissociation constant (K_D), which is used to evaluate and rank order strengths of bimolecular interactions.

[0084] As used herein, the terms "linked," "fused", or "fusion", are used interchangeably. These terms refer to the joining together of two more elements or components or domains, by whatever means including chemical conjugation or recombinant means. Methods of chemical conjugation (e.g., using heterobifunctional crosslinking agents) are known in the art.

[0085] As used herein, "MHC molecules" refers to two types of molecules, MHC class I and MHC class II. MHC class I molecules present antigen to specific CD8+ T cells and MHC class II molecules present antigen to specific CD4+ T cells. Antigens delivered exogenously to APCs are processed primarily for association with MHC class II. In contrast, antigens delivered endogenously to APCs are processed primarily for association with MHC class I.

[0086] As used herein, the term "monoclonal antibody" refers to an antibody which displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to an antibody which displays a single binding specificity and which has variable and optional constant regions derived from human germline immunoglobulin sequences. In some embodiments, human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic non-human animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

[0087] As used herein, the term "preventing" when used in relation to a condition, refers to administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition in a subject relative to a subject which does not receive the composition.

[0088] As used herein, the term "subject" includes any human or non-human animal. For example, the methods and compositions of the present invention can be used to treat a subject with an immune disorder. The term "non-human animal" includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, *etc.*

[0089] As used herein, the term "tumor microenvironment" (alternatively "cancer microenvironment"; abbreviated TME) refers to the cellular environment or milieu in which the tumor or neoplasm exists, including surrounding blood vessels as well as non-cancerous cells including, but not limited to, immune cells, fibroblasts, bone marrow-derived inflammatory cells, and lymphocytes. Signaling molecules and the extracellular matrix also comprise the TME. The tumor and the surrounding microenvironment are closely related and interact constantly. Tumors can influence the microenvironment by releasing extracellular signals, promoting tumor angiogenesis and inducing peripheral immune tolerance, while the immune cells in the microenvironment can affect the growth and evolution of tumor cells.

[0090] The term "T cell" refers to a type of white blood cell that can be distinguished from other white blood cells by the presence of a T cell receptor on the cell surface. There are several subsets of T cells, including, but not limited to, T helper cells (a.k.a. T_H cells or $CD4^+$ T cells) and subtypes, including T_H1, T_H2, T_H3, T_H17, T_H9, and T_{FH} cells, cytotoxic T cells (*i.e.,* T_C cells, $CD8^+$ T cells, cytotoxic T lymphocytes, T-killer cells, killer T cells), memory T cells and subtypes, including central memory T cells (T_{CM} cells), effector memory T cells (T_{EM} and T_{EMRA} cells), and resident memory T cells (T_{RM} cells), regulatory T cells (a.k.a. T_{reg} cells or suppressor T cells) and subtypes, including $CD4^+$ $FOXP3^+$ T_{reg} cells, $CD4^+FOXP3^-$ T_{reg} cells, Tr1 cells, Th3 cells, and $T_{reg}17$ cells, natural killer T cells (a.k.a. NKT cells), mucosal associated invariant T cells (MAITs), and gamma delta T cells ($\gamma\delta$ T cells), including $V\gamma9/V\delta2$ T cells. Any one or more of the aforementioned or unmentioned T cells may be the target cell type for a method of use of the invention.

[0091] As used herein, the terms "therapeutically effective amount" or "therapeutically effective dose," or similar terms used herein are intended to mean an amount of an agent (e.g., an anti-CD137 antibody or an antigen-binding fragment thereof) that will elicit the desired biological or medical response (e.g., an improvement in one or more symptoms of a cancer).

[0092] The terms "treat," "treating," and "treatment," as used herein, refer to therapeutic or preventative measures described herein. The methods of "treatment" employ administration to a subject, in need of such treatment, a human antibody of the present

disclosure, for example, a subject in need of an enhanced immune response against a particular antigen or a subject who ultimately may acquire such a disorder, in order to prevent, cure, delay, reduce the severity of, or ameliorate one or more symptoms of the disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment.

[0093] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure pertains. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the presently disclosed methods and compositions. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

CD137 ANTIBODIES

[0094] In some aspects, the provided methods increase anti-tumor efficacy and comprise administering a bispecific antibody targeting VEGF and DLL4 together with agonistic anti-CD137 antibodies.

[0095] In some embodiments, the methods of the present disclosure comprise concurrently administering CTX-471, an anti-CD137 agonist monoclonal antibody exhibiting potent anti-tumor activity. CTX-471 is a fully human IgG4 anti-CD137 agonist antibody designed to optimally activate T and NK cells while minimizing toxicity typically seen in this class of therapeutics. In an ongoing Phase 1 trial (NCT03881488) in patients with advanced cancers progressing after treatment with PD-1/PD-L1 inhibitors (post-CPI patients), CTX-471 has shown good tolerability below 1.2 mg/kg, with most adverse events being mild (Grade 1-2). Notably, one SCLC patient achieved a durable complete response, while partial responses were observed in melanoma and mesothelioma patients. Of 12 treated patients with pre-treatment biopsies evaluable by NGS, 2 (17%) had loss of HLA-I alleles. CTX-471 demonstrated disease control (complete response (CR) or stable disease (SD)) in both patients with HLA defects suggesting that CTX-471 might be active in this type of tumor despite defective antigen presentation. Further, CTX-471 induced pharmacodynamic changes consistent with immune activation, including increased CD4+ and CD8+ T cells expressing CD137 and higher levels of activated NK cells.

[0096] Other anti-CD137 antibodies may be contemplated for use in the present methods. For example, anti-CD137 antibodies may include but are not limited to Urelumab (BMS-663513) and Utomilumab (PF-05082566).

VEGF/DLL4 ANTIBODIES

[0097] The methods of the present disclosure comprise administering a bispecific antibody which can bind specifically to DLL4 and VEGF to effectively inhibit the DLL4/Notch and VEGF/VEGFR signaling pathways. The bispecific antibody may bind specifically to VEGF and may additionally include a region that binds specifically to DLL4. In some aspect, the VEGF and/or DLL4 binding portions may be a scFv. In addition, the DLL4 binding portion may be connected to the C-terminal region of a protein similar to IgG - type Avastin. Thus, the bispecific antibody may effectively inhibit the interaction between VEGF and VEGF receptor as well as the interaction between DLL4 and Notch protein and exhibit anticancer effects.

[0098] In some embodiments, the methods of the present disclosure comprise administering CTX-009, a bispecific antibody targeting VEGF-A and DLL4, that may be efficacious in patients with multiple different cancers, including biliary tract and colorectal cancers. CTX-009, (also referred to as TR009, ABL001 and HD105) is a recombinant bispecific antibody of the human IgG1 isotype which contains a scFv binding to DLL4 linked to the heavy chain of an antibody that binds and neutralizes the activity of human VEGF. CTX-009 is further described in U.S. Patent No. 9,598,483. CTX-009 has demonstrated promising efficacy in solid tumors. Phase 1 trials showed activity as monotherapy and with chemotherapy, while a Phase 2 trial in biliary tract cancer reported a 37.5% response rate with paclitaxel.

[0099] The murine surrogate of CTX-009, mCTX-009, is a bispecific antibody developed by combining the sequences of two mouse/human cross-reactive templates (see FIG. 1A) and was designed to disrupt the interactions between DLL4 and Notch, as well as VEGF-A and KDR. mCTX-009 is a cross-reactive antibody that binds human DLL4 and inhibits, in a dose dependent manner, the activation of Notch-1.

[00100] Other VEGF antibodies may be contemplated for use in the present methods, for example, bevacizumab (Avastin®, Genentech/Roche), an anticancer antibody drug for inhibiting angiogenesis targeting VEGF.

PD1/PDL1 ANTIBODIES

[00101] In some aspects, the provided methods increase anti-tumor efficacy and comprise administering a bispecific antibody targeting VEGF and DLL4 together with a PD-1 or PD-L1 blocker. Such PD-1/PD-L1 blockers may include monoclonal antibodies that disrupt the PD-1/PD-L1 signaling axis.

[00102] In addition, the present disclose also contemplates using other PD-1 and/or PD-L1 antibodies or proteins. For example, PD-1 inhibitors may include but are not limited to, Pembrolizumab (Keytruda), Nivolumab (Opdivo and Opdivo Qvantig), and/or Cemiplimab (Libtayo). Further, PD-L1 inhibitors may include but are not limited to Atezolizumab (Tecentriq and Tecentriq Hybreza), Avelumab (Bavencio), and/or Durvalumab (Imfinzi).

FORMULATIONS

[00103] The present disclosure also provides for a pharmaceutical composition comprising the combination of antibodies of the present disclosure with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. Such pharmaceutical compositions can be used in a subject having *e.g.*, cancer, as disclosed herein.

[00104] In certain embodiments, acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed. In certain embodiments, the formulation material(s) are for parenteral administration. In certain embodiments, the pharmaceutical composition can contain formulation materials for modifying, maintaining or preserving, for example, the pH, osmolality, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. In certain embodiments, the optimal pharmaceutical composition will be determined by one skilled in the art depending upon, for example, the intended route of administration, delivery format and desired dosage. *See*, for example, Remington's Pharmaceutical Sciences, supra. In certain embodiments, such compositions may influence the physical state, stability, rate of *in vivo* release and/or rate of *in vivo* clearance of the antibodies described herein.

[00105] In certain embodiments, the pharmaceutical composition can be selected for parenteral delivery. In certain embodiments, the compositions can be selected for inhalation or for delivery through the digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the ability of one skilled in the art.

[00106] In some embodiments, kits are provided for producing a single-dose administration unit. In certain embodiments, the kit can contain both a first container having a

dried protein and a second container having an aqueous formulation. In certain embodiments, kits containing single and multi-chambered pre-filled syringes (*e.g.*, liquid syringes and lyosyringes) may be included.

[00107] In certain embodiments, the effective amount of a pharmaceutical composition comprising an antibody or antibody combination as described herein to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment, according to certain embodiments, will thus vary depending, in part, upon the molecule delivered, the indication for which the antibody or antibody combination disclosed herein is being used, the route of administration, and the size (body weight, body surface or organ size) and/or condition (the age and general health) of the patient. In certain embodiments, the clinician can titer the dosage and modify the route of administration to obtain the optimal therapeutic effect.

[00108] In certain embodiments, the frequency of dosing will consider the pharmacokinetic parameters of antibodies or antibody fragments in the formulation used. In certain embodiments, a clinician will administer the composition until a dosage is reached that achieves the desired effect. In certain embodiments, the composition can therefore be administered as a single dose or as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. In certain embodiments, appropriate dosages can be ascertained through use of appropriate dose-response data.

Generation of mCTX-009, mCTX-471, Atezolizumab and isotypes (design, expression, binding profiles on chips, binding on cells)

[00109] The murine surrogate of CTX-009, referred to as mCTX-009, is a bispecific antibody developed by combining the sequences of two antibody templates. The VEGF-A targeting component was discovered by phage display technology and binds to both human and mouse VEGF to interfere with its binding to the VEGFR2 (Fuh, G., et al., *Structure-function studies of two synthetic anti-vascular endothelial growth factor Fabs and comparison with the Avastin Fab.* J Biol Chem, 2006. **281**(10): p. 6625-31; and Liang, W.C., et al., *Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies*

completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. J Biol Chem, 2006. **281**(2): p. 951-61.). In certain experiments a single targeting anti-VEGF antibody was used as a reference, here called mAvastin. The discovery and generation of the DLL4 targeting component was described in Ridgway, J., et al., *Inhibition of Dll4 signaling inhibits tumour growth by deregulating angiogenesis.* Nature, 2006. **444**(7122): p. 1083-7. Although not limited to such configuration, the murine surrogate of CTX-009, mCTX-009, may be a bispecific antibody comprising an anti-VEFG component on the N-terminus of the bispecific antibody while anti-DLL4 component may be on the C-terminus (See Fig. 1A).

***In vitro* and *ex-vivo* characterization of mCTX-009**

[00110] The Delta-like ligand 4 (DLL4)/Notch and vascular endothelial growth factor (VEGF)/VEGF-receptor signaling pathways are essential for angiogenesis, which is a meticulously coordinated process through which new blood vessels form from pre-existing vessels. Both pathways help orchestrate vascular sprouting and migration of the endothelial tip cells, and are functionally interconnected (Akil, A., et al., *Notch Signaling in Vascular Endothelial Cells, Angiogenesis, and Tumor Progression: An Update and Prospective.* Front Cell Dev Biol, 2021. **9**: p. 642352.). Angiogenesis occurs in physiological processes, such as wound healing, and it is required for tumors to grow beyond a minimal size (*See*, Dvorak, H.F., *Tumors: wounds that do not heal-redux.* Cancer Immunol Res, 2015. **3**(1): p. 1-11; and Nagy, J.A., A.M. Dvorak, and H.F. Dvorak, *Vascular hyperpermeability, angiogenesis, and stroma generation.* Cold Spring Harb Perspect Med, 2012. **2**(2): p. a006544.). Because of this, pharmacological interference with this process has been postulated to serve as an effective anticancer strategy (Folkman, J., *Tumor angiogenesis: therapeutic implications.* N Engl J Med, 1971. **285**(21): p. 1182-6.).

[00111] Indeed, neutralizing VEGF or its primary angiogenic receptor, KDR (interchangeably referred to as VEGFR-2) with biologics or small molecule kinase inhibitors respectively, can prevent the growth of many rapidly growing mouse tumors and in the clinic (Ferrara, N., *Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications.* Semin Oncol, 2002. 29(6 Suppl 16): p. 10-4; Dvorak, H.F., *Rous-Whipple Award Lecture. How tumors make bad blood vessels and stroma.* Am J Pathol, 2003. 162(6): p. 1747-57; Jain, R.K., et al., *Lessons from phase III clinical trials on anti-VEGF therapy for cancer.* Nat Clin Pract Oncol, 2006. 3(1): p. 24-40; and Hurwitz, H.,

et al., *Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer.* N Engl J Med, 2004. 350(23): p. 2335-42.). Analogously, DLL4 blockade with monoclonal antibodies leads to tumor growth inhibition in preclinical animal models, and the effect is enhanced by VEGF blockade or chemotherapy (Miles, K.M., et al., *Dll4 blockade potentiates the anti-tumor effects of VEGF inhibition in renal cell carcinoma patient-derived xenografts.* PLoS One, 2014. 9(11): p. e112371; Ridgway, J., et al., *Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis.* Nature, 2006. 444(7122): p. 1083-7; Fischer, M., et al., *Anti-DLL4 inhibits growth and reduces tumor-initiating cell frequency in colorectal tumors with oncogenic KRAS mutations.* Cancer Res, 2011. 71(5): p. 1520-5; and Hoey, T., et al., *DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency.* Cell Stem Cell, 2009. 5(2): p. 168-77.). Hence, simultaneous blockade of both signaling pathways holds the potential to enhance the efficacy of the single monotherapies.

[00112] CTX-009 (also referred to as TR009, ABL001 and HD105) is a recombinant bispecific antibody of the human IgG1 isotype which contains at least one scFv that binds to DLL4 linked to the heavy chain of a bevacizumab biosimilar that binds and neutralizes the activity of human VEGF. Preclinical and early clinical data indicate that CTX-009 exhibits robust anti-tumor activity as a monotherapy or in combination with chemotherapy across a range of solid tumors, including colorectal, gastric, cholangiocarcinoma, pancreatic, and NSCLC (*See*, Yeom, D.H., et al., *ABL001, a Bispecific Antibody Targeting VEGF and DLL4, with Chemotherapy, Synergistically Inhibits Tumor Progression in Xenograft Models.* Int J Mol Sci, 2020. 22(1); and Kim, D.H., et al., *Synergistic antitumor activity of a DLL4/VEGF bispecific therapeutic antibody in combination with irinotecan in gastric cancer.* BMB Rep, 2020. 53(10): p. 533-538.).

[00113] Presently, CTX-009 is under clinical development in patients with advanced solid tumors in the United States, South Korea and China. Moreover, a Phase 1 dose escalation and dose expansion monotherapy trial in patients with solid tumors and a Phase 1b trial of CTX-009 in combination with chemotherapy were completed in South Korea. Based on the encouraging results in patients with advanced BTC that emerged from this study, a Phase 2 trial of CTX-009 in combination with paclitaxel in patients with advanced BTC was conducted in the US (clinicaltrial.gov identifier NCT04492033). In the latter study, a 37.5% overall response rate (ORR) in patients with BTC treated in the second- and third- line settings was observed. Subgroup analysis revealed an ORR of 63.6% in patients treated in the second-line setting compared with an ORR of 14.9% in patients treated in the third-line

setting. Median progression free survival (PFS) was 9.4 months and median overall survival (OS) was 12.5 months. Consequently, two additional clinical trials were open in the United States: a Phase 2 trial of CTX-009 in patients with advanced colorectal cancer, CRC (NCT05513742), and a Phase 2/3 trial of CTX-009 in combination with paclitaxel in patients with advanced BTC (NCT05506943).

[00114] When combined with chemotherapy or immunotherapy, anti-angiogenesis drugs delay tumor progression and recurrence, and, in some instances, greatly prolong patient survival. In a study in unresectable hepatocellular carcinoma, the combination of bevacizumab (an anti VEGF-A monoclonal antibody) and Atezolizumab (an anti PD-L1 antibody) showed encouraging anti-tumor activity and safety, and this combination has since become the preferred first-line standard of care for patients with these diseases (Finn, R.S., et al., *Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma.* N Engl J Med, 2020. **382**(20): p. 1894-1905; Hsu, C., et al., *Immunotherapy in hepatocellular carcinoma: evaluation and management of adverse events associated with atezolizumab plus bevacizumab.* Ther Adv Med Oncol, 2021. **13**: p. 17588359211031141; and Raybould, A.L. and H. Sanoff, *Combination Antiangiogenic and Immunotherapy for Advanced Hepatocellular Carcinoma: Evidence to Date.* J Hepatocell Carcinoma, 2020. **7**: p. 133-142.).

[00115] As a standalone treatment, mCTX-009 demonstrated significant antitumor activity in the CT26, MC38, LLC1 and 4T1 isograft models. On the other hand, mCTX-009 in combination with CTX-471 increased its effectiveness in several isograft models, including the IO resistant LLC1 model. Additionally, antitumor activity was even seen when tumors were rendered MHC-I negative due to B2M gene deletion, which recapitulates a CPI resistance mechanism observed in post CPI patients (Zaretsky, J.M., et al., Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. N Engl J Med, 2016. 375(9): p. 819-29; and Torrejon, D.Y., et al., Antitumor Immune Responses in B2M-Deficient Cancers. Cancer Immunol Res, 2023. 11(12): p. 1642-1655.). In the $MC38^{B2mKO}$ and $CT26^{B2mKO}$ models, the combination of CTX-009 and CTX-471 maintained potent anti-tumor activity. While not wishing to be bound by any particular hypothesis, preliminary investigations into the mechanism of action suggest that this combination might potentiate a tumor rejection axis, potentially primed by the absence of MHC-I, which involves NK and CD4+ T-cells. The findings indicate that CTX-009 holds promise for enhanced clinical benefits when paired with immuno-modulating agents featuring complementary mechanisms of action, particularly CD137 agonism. This approach could be considered not only as a first-

line treatment option, but also as a viable alternative where previous immunotherapy was ineffective.

EXAMPLES

[00116] To explore the full potential of CTX-009, mouse syngeneic tumor models were tested with the combinations of CTX-009 and non-overlapping immunotherapy approaches, namely PD-1/PD-L1 blockade and CD137 agonism. In immunopharmacological investigations conducted in preclinical animal models the immune systems remained intact. To facilitate this, we developed mouse-specific surrogates for CTX-009 (mCTX-009), Atezolizumab (which natively binds to mouse PD-L1), and a mouse-optimized version of CTX-471, an anti-CD137 agonist monoclonal antibody (See, Eskiocak, U., et al., *Differentiated agonistic antibody targeting CD137 eradicates large tumors without hepatotoxicity.* JCI Insight, 2020. **5**(5).). Additionally, considering that the loss of HLA-I is a prevalent intrinsic and acquired mechanism by which tumors evade the immune system, even in patient cohorts that have previously undergone immunotherapy (post-checkpoint inhibitor scenario) (Montesion, M., et al., *Somatic HLA Class I Loss Is a Widespread Mechanism of Immune Evasion Which Refines the Use of Tumor Mutational Burden as a Biomarker of Checkpoint Inhibitor Response.* Cancer Discov, 2021. **11**(2): p. 282-292; Sari, G. and K.L. Rock, *Tumor immune evasion through loss of MHC class-I antigen presentation.* Curr Opin Immunol, 2023. **83**: p. 102329; and Dhatchinamoorthy, K., J.D. Colbert, and K.L. Rock, *Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation.* Front Immunol, 2021. **12**: p. 636568.), we sought to investigate the impact of our drugs in this specific context. To do so, we intentionally induced MHC-I deficiency in specific isogenic tumor models by deleting the *B2M* gene, a modification known to result in complete loss of MHC-I surface expression. While this genetic alteration rendered the tumors more resistant to CD8+ T-cell attacks, it concurrently heightened their vulnerability to surveillance by NK cells. Enhancing the activity of NK cells is a critical consideration in the management of post-checkpoint inhibitor patient cohorts (Cao, Y., et al., *Immune checkpoint molecules in natural killer cells as potential targets for cancer immunotherapy.* Signal Transduct Target Ther, 2020. **5**(1): p. 250.).

[00117] Our studies provide an in-depth examination of mCTX-009's *in vivo* efficacy as a monotherapy and in combination with agents targeting the PD-1/PD-L1 pathway or a CD137 agonist antibody, mCTX-471. As a standalone treatment, mCTX-009 demonstrated

effectiveness in the CT26, MC38, and LLC1 isograft models. Its combination with both Atezolizumab and mCTX-471 boosted its effectiveness not only in models already responsive to immunotherapy, but also in the challenging LLC1 model. Notably, even in cases where tumors were rendered MHC-I negative due to *B2M* gene deletion, such as $MC38^{B2mKO}$ and $CT26^{B2mKO}$, the combination of CTX-009 and mCTX-471 maintained potent anti-tumor activity, while the efficacy of Atezolizumab was considerably reduced.

[00118] Furthermore, described herein are two novel mouse models of immunotherapy resistance without the enhanced NK cell susceptibility bias conferred by complete MHC-I loss due to *B2M* gene deletion. The mouse model exhibits total MHC loss by deleting the *B2M* gene in MC38 and CT26 isografts and partial loss by deleting both H2-K1 alleles in MC38 cells (which eliminates 1/3 of the MHC-I presentation potential). To counteract potential increase susceptibility to NK killing from total MHC loss, we passaged $CT26B2^{m-/-}$ cells in vaccinated mice and selected a line with increased NK resistance ($CT26B2^{m-/-E}$ CT26 B2m knockout escapers). For the second model, the H-2k1 MHC-I locus was knocked out in MC38 cells, resulting in targeted homozygous loss. Using this approach, the expression of the other MHC-I alleles, and therefore natural resistance to NK cells, were left intact. Mouse CTX-009 surrogate bispecific antibody binds to endothelial cells and blocks both VEGF and DLL4 activity in vitro.

[00119] **A murine surrogate of a bispecific antibody that targets VEGF-A and DLL4 was developed**.

[00120] The bispecific antibody was developed by combining the sequences of two mouse/human cross-reactive templates (**Error! Reference source not found.**A). As shown in Figure 1A, the constant domains (CH and CL) of the IgG scaffold may be comprised of the murine IgG2a isotype, whereas the variable regions may be human. Furthermore, the murine bispecific antibody was developed to bind to and disrupt the interactions between DLL4 and Notch, as well as VEGF-A and KDR. To use as a comparator, a single targeting anti-VEGF antibody (mAvastin) was also developed. The equilibrium dissociation constants (KD) of the murine bispecific antibody to recombinant mouse VEGF-A and DLL4 may be around about 2.22 pM and 13.1 nM respectively. In any case, the KDs may be similar to the reported KD of human CTX-009 (See, e.g., Lee, D., et al., Simultaneous blockade of VEGF and Dll4 by HD105, a bispecific antibody, inhibits tumor progression and angiogenesis. MAbs, 2016. 8(5): p. 892-904.).

[00121] As shown in Figure 1B, mCTX-009 bound to mouse aortic endothelial cells (mAEC). In addition, Figures 1C and 1D show that mCTX-009 delayed the VEGF-dependent

reconstitution of a cellular monolayer in the mAEC scratch assay and blocked DLL4-induced NOTCH1 activity in a reporter assay. Luciferase reporter expression under the control of NOTCH1-response elements activated by plastic bound DLL4 is blocked by mCTX-009 as measured by luminescence (IC_{50}= 2.90e-8M). Further, mCTX-009 efficiently delayed the closure of scratched monolayers, suggesting the molecule was able to interfere with VEGF-induced endothelial cell migration (Figure 1C). Moreover, as shown in Figure 1E, both mCTX-009 and mAvastin blocked to a similar extent the proliferation of mAEC grown in the presence of spent CT26 cell culture medium (which contains pro-angiogenic factors such as VEGF), suggesting they were capable of functionally interfering with tumor-derived VEGF.

[00122] To evaluate the ability of mCTX-009 to functionally interfere with the activity of DLL4, a cellular *in vitro* assay in which the interaction of plastic bound human DLL4 triggers a reporter gene in cells expressing human Notch-1 was deployed (Figure 1D). The assay revealed that mCTX-009 is a cross-reactive antibody that binds to human DLL4 and inhibited in a dose dependent manner the activation of Notch-1. Under these experimental conditions, the inhibition curve produced by mCTX-009 may overlap with the curve produced by the actual CTX-009 drug (not shown). Taken together, the *in vitro* characterization data of mCTX-009 supports the notion that mCTX-009 is a functional mouse surrogate for human CTX-009.

Superior efficacy of the mCTX-009 and mCTX-471 combination in selected CPI-sensitive and CPI-refractory models.

[00123] As a standalone treatment, mCTX-009 demonstrated effectiveness in CT26, MC38, LLC1 and 4T1 isograft models. The combination of mCTX-009 with CTX-471, however, boosted its effectiveness in several isograft models, including the immunotherapy ("IO") resistant LLC1 model. Although mCTX-009 or mCTX-471 monotherapies produced a modest response in the highly refractory LLC1 model, their combination produced a markedly enhanced anti-tumor activity. Similarly, combination of mCTX-009 and mCTX-471 produced curative responses (60% and trending lower at takedown) in the immune CPI responsive CT26 model. As seen in Figure 2, mCTX-009 was dosed every 3 days (upper thick tick marks, 6.8 mpk in LLC1, 5 mpk in the CT26 model), whereas mCTX-471 was dosed weekly (2 mpk in CT26, 5 mpk in LLC1, below axis thick tick marks). Shown is the average tumor volume of 9 (LLC1) or 7 (CT26) mice per group.

B2mKO negative cell lines.

[00124] As shown in Figure 3A, loss of HLA, commonly observed in patients with resistance to CPI, can be recapitulated in cell lines, albeit in an HLA-allele non-specific manner, by elimination of the *B2M* gene (*See*, Torrejon, D.Y., et al., *Antitumor Immune Responses in B2M-Deficient Cancers*. Cancer Immunol Res, 2023. 11(12): p. 1642-1655.). Deletion of the *B2M* gene produced a MHC-I null phenotype in both CT26 and MC38 cell lines (bottom panels). Unlike the parental lines, $CT26^{B2mKO}$ and $MC38^{B2mKO}$, IFN-γ fails to further induce the expression of MHC-I. Figure 3B shows that the loss of B2M did not significantly affect proliferation *in vitro*, proliferation in mice, however, was severely affected.

NK depletion restores B2mKO cell engraftment.

[00125] As seen in Figure 4, loss of B2M did not significantly affect $CT26^{B2mKO}$ cell proliferation in vitro. Engraftment in mice, however, was affected. Mice rejected the standard cell inoculum used for engraftment of CT26 wild type ("WT") cells. Increasing the number of cells inoculated enabled $CT26^{B2mKO}$ engraftment to levels comparable to WT cells (data not shown). Treatment with NK-cell depleting antibody anti-Asialo GM1 restored engraftment to levels similar or better than seen with WT CT26. These data are consistent with literature suggesting a role for NK-cell mediated tumor cell killing in the absence of MHC-I (*See*, Torrejon, D.Y., et al., Antitumor Immune Responses in B2M-Deficient Cancers. Cancer Immunol Res, 2023. 11(12): p. 1642-1655.).

Escape of B2mKO cells as a model of CPI progression.

[00126] Mice cured from CT26 tumors by either mCTX-471 or mCTX-471 + mCTX-009 (Experienced) or age matched tumor-naïve mice were challenged with either the CT26 or $CT26^{B2mKO}$ cells (FIG. 5A-B). As shown in Figure 5A, tumor experienced mice were capable of dramatically delaying or completely suppressing the growth of both cell types, whereas tumor-naïve mice did not. In this case, $1x10^6$ $CT26^{B2mKO}$ cells were needed to produce growing tumors (n=5). $CT26^{B2mKO}$ tumors that eventually grew in experienced mice were reinoculated in naive mice (FIG. 5B). Shown in Figure 5B, a 10-fold lower inoculum was required for $CT26^{B2mKO}$ escaper ($CT26^{B2mKO\text{-}E}$) to produce tumors similar to $CT26^{B2mKO}$, suggesting the tumors acquired resistance.

Superior efficacy of the mCTX-009 + mCTX-471 combination in $MC38^{B2mKO}$ tumors.

[00127] As shown in Figure 6A, MC38 tumors were responsive to mCTX-009 + mCTX-471 treatment ($1x10^5$ cells/mouse, mCTX-471 @1.5 mpk, q7dx2). Figure 6B shows MC38 cells that were rendered MHC-I negative by *B2M* gene deletion responding to mCTX-009 (5 mpk, q3dx5) or mCTX-471 (1.5 mpk, q7dx3), with 1 (out of 8) mice completely responding in each treatment group. Efficacy was significantly increased by the combination of these antibodies which led to 62.5% cures (5 out of 8 mice). Of note, ten times more $MC38^{B2mKO}$ cells were inoculated to ensure tumor formation. In the same model, Figure 6C illustrates the combination of Atezolizumab (3 mpk, q3dx3) and mCTX-009 (5 mpk, q3dx5) controlled the tumor growth with no complete responders. Figure 6D depicts TIL analysis by flow cytometry. In MC38 tumors, mCTX-009 + mCTX-471 increased the proportion of CD8+ T cells and decreased the amount of tumor associated macrophages (TAMs). The proportions of CD8+ T-cells in control tumors were approximately twice the amount of CD4+ T cells. Combination treatment increased the CD4+T cells/CD8+T cells ratio. As shown in Figure 6E, in the control $MC38^{B2mKO}$ tumors, CD4+ TILs were slightly more numerous than CD8+ as previously shown by others (*See e.g.,* Torrejon, D.Y., et al., Antitumor Immune Responses in B2M-Deficient Cancers. Cancer Immunol Res, 2023. 11(12): p. 1642-1655.). Treatment with mCTX-009 and mCTX-471 or Atezolizumab increased the relative amount of CD8+ T cells (FIG. 6E, left panel), with a stronger effect of mCTX-009 + Atezolizumab combination. Since an increase in CD8+ T cell infiltrate did not correlate with better efficacy (*See* FIG. 6B-C), we hypothesized that the treatment altered the phenotype or the function of infiltrating T cells. Based on recent work by Lerner (Lerner, E.C., et al., CD8(+) T cells maintain killing of MHC-I-negative tumor cells through the NKG2D-NKG2DL axis. Nat Cancer, 2023. 4(9): p. 1258-1272.), we asked whether antibody combinations generated NKG2D-expressing innate killer cells. Both NKG2D+CD8+T and NKG2D+CD4-CD8- cells were detected in $MC38^{B2mKO}$ tumors, which were positive for NKG2D ligands. Moreover, mCTX-471 worked with mCTX-009 better than Atezolizumab in increasing the amount of these "innate killers". (FIG. 6E, middle two panels). Of note, the proportion of resident TAMs in $MC38^{B2mKO}$ was less than half of the total CD45+ TILs in MC38 parental cell. Both combination treatments further reduced the fraction of infiltrating TAMs, suggesting that the CTX-009 + CTX-471 combination might help eradicating $MC38^{B2mKO}$ tumors by potentiating innate cell killing and alleviating immunosuppression by infiltrating TAMs.

Rejection of CT26^{B2mKO} cells by mCTX-009 + mCTX-471 cured mice is critically dependent on both CD4 and NK cells.

[00128] As shown in Figure 7A, tumor-naïve mice were challenged with CT26^{B2mKO} cells (1x10^6 per mouse, s.c.) in NK cells or CD4+ T cell depleted animals (i.p. injections of depleting antibodies at day -1, 5 and 11 (100 μg anti-asialoGM1 and 200 μg anti-CD4 T-cells clone GK1.5 per mouse respectively, 5 mice/group, thick ticks). Figure 7B presents the same parameters as Figure 7A but in CT26-tumor-experienced mice. Since transient depletion of NK and CD4 cells rendered CT26-tumor-experienced mice more vulnerable to the MHC-I negative tumor cell challenge, it is likely that either an antibody dependent innate mechanism ("ADCC") or a mechanism involving NK cell memory might be involved in rejection of these tumors. Hence, we hypothesize that the combination of mCTX-009 and mCTX-471 might potentiate this anti-tumor axis.

Enhanced recognition threshold of IO-resistant CT26 cells by splenocytes of tumor-experienced mice.

[00129] As seen in figure 9A, splenocytes from mice previously challenged with CT26 tumors and cured via CTX-471 and CTX-009 treatment (left panel) contain precursors that react to CT26 cells, producing approximately 10 times more IFN-γ against the indicated target cell lines (x-axis) compared to splenocytes from naïve mice (right panel). Neither tumor-experienced nor naïve splenocytes recognized CT26$^{B2m-/-E}$ cells. B16F10 cells (C57Bl/6 background) served as negative controls. These findings support a model in which CT26$^{B2m-/-E}$ cells have evaded anti-tumor immunity, reducing recognition by host splenocytes. Data shown corresponds to a 10:1 E:T ratio. FIG. 9B illustrates that there is a similar percentage of CD4, CD8, B, Monocytes and NK cells in the spleens from naïve vs tumor experienced (cured) mice.

Loss of CD8 T-cells and murine dendritic cells and increase in CD4 T-cells in the CT26$^{B2m-/-E}$ model.

[00130] As shown in Figure 10, baseline tumor infiltrating lymphocytes (TILs) are characterized in the CT26 tumor series. RNA from tumors with the indicated genetic background (n = 4) was extracted, sequenced, and the relative amount of different cell types determined by CIBERSORT deconvolution (via the Timer2.0 portal). Both CT26$^{B2m-/-}$ and

CT26$^{B2m-/-E}$ had similar relative amounts of TILs and diverged from the parent by showing an increase in CD4 T cells, and a loss of both CD8 T cells and mDCs. A decrease in CD8 T cell infiltrate was also observed in the MC38$^{B2m-/-}$, which were also characterized by an increase in B cells, mDCs and macrophages (not shown).

The CTX-009/CTX-471 combination is effective in the CT26$^{B2m-/-E}$ model.

[00131] Figure 11 shows the combination of CTX-471 and CTX-009 is efficacious in CT26 models where conventional IO (anti PD-L1) exhibits reduced activity. Mice bearing the indicated tumor models were treated with the specified antibodies (mCTX-009: 5 mpk, q3dx3; mCTX-471: 0.1 mpk, q7dx2; mAtezo: 3 mpk, q3dx3) when tumors reached ~100 mm^3 tumor volumes (Y-axis) were recorded over time. The parental CT26 model was highly responsive to mCTX-471 and the combination of mCTX-471 + mCTX-009. In contrast, CT26$^{B2m-/-}$ and CT26$^{B2m-/-E}$ exhibited reduced sensitivity to monotherapies, while the combination treatment showed additive efficacy. Note: Initial tumor cell inoculum differed (CT26 and CT26$^{B2m-/-E}$: 100,000 cells/mouse; CT26$^{B2m-/-}$: 1×10^6 cells/mouse).

Changes in TIL composition following treatment of CT26$^{B2m-/-E}$ tumors.

[00132] As seen in figure 12A, RNA from whole tumors (n = 3) was extracted, sequenced, and analyzed using CIBERSORT deconvolution via the TIMER2.0 portal to determine relative content of TILs. Treatment with the CTX-009 + CTX-471 combination led to an increase in total CD4 T cells, B and NK cells, whereas, compared to the monotherapies, the combination decreased the proportion of monocytes/macrophages (Gran. = other granulocytes). Figure 12B shows IHC analysis of the extracted tumors (focus on the cortical areas of the tumors). At baseline, tumors lacked $CD8^+$ T cells but showed moderate $CD4^+$ T cell infiltration. CTX-009 and CTX-471 induced distinct infiltration patterns, with CTX-471 promoting immune cell recruitment in the tumor cortex, which strongly correlated with tumor growth inhibition. Notably, the combination treatment uniquely increased the frequency of activated immune cells, as indicated by Granzyme B staining. The discrepancy between Macrophage values (CD11b) between panels A and B might be due to different areas of the tumor considered for the analysis (total tumor for RNA vs cortical tumor area for IHC).

The CTX-009/CTX-471 combination enhances the Inflammasome, cytolysis and interferon pathways.

[00133] Bulk RNA was extracted from CT26$^{B2m-/-E}$ bearing mice (n = 3) which were treated with CTX-471, CTX-009, or a combination of CTX-471 and CTX-009. Figure 13A shows selected genes representing hypoxia (Hif1a), IFN (Isg15), EMT (Smad3), and cytotoxicity (Gzmb) plotted as fold induction over isotype-treated mice. A selection of differentially expressed genes (DEGs) highlighting those preferentially induced in the combination treatment is shown in Figure 13B. GO analysis of the DEGs, revealed significant enrichment of inflammatory and immune pathways. AIM2 inflammasome assembly, cytolysis, and pyroptotic inflammatory response showed fold enrichment >100 with highly significant p-values (e.g., 2.04E-10 for cytolysis) and FDRs (4.91E-08 to 1.44E-05), indicating strong activation of inflammatory cell death. Type II interferon signaling and its response (83.56-fold enrichment, p = 4.83E-06, FDR ≈ 5.8E-04) highlight IFN-driven effects. The CAMKK-AMPK cascade suggests metabolic regulation in immune responses, while IL-27 signaling (74.27-fold enrichment, p = 4.76E-09, FDR = 9.40E-07) points to a role in immune modulation.

The combination of CTX-471 and CTX-009 is efficacious in MC38-derived models where conventional IO (anti PD-L1) shows reduced activity.

[00134] Figure 14 depicts mice bearing the indicated tumor models treated with the specified antibodies (mCTX-009: 5 mpk, q3dx3; mCTX-471: 0.1 mpk, q7dx2; mAtezo: 3 mpk, q3dx3) when tumors reached ~80 mm³. Tumor volumes (Y-axis) were recorded over time. The parental MC38 model was highly responsive to mCTX-471 and the combination of mCTX-471 + mCTX-009. In contrast, MC38$^{B2m-/-}$ (not shown) and MC38$^{H-2k-/-}$ exhibited reduced sensitivity to monotherapies, while the combination treatment showed additive efficacy. Note: Initial tumor cell inocula differed (MC38 and MC38$^{B2m-/-E}$: 250,000 cells/mouse; MC38$^{B2m-/-}$: 1×10^6 cells/mouse).

Evidence of NK activation in CTX-471 treated patients.

[00135] Figure 15A shows an increase of circulating NK cells (primarily CD56 bright) in CD137 treated patients. Figure 15B shows an increase in the fraction of CD137 positive cells in sequential slides (pre-treatment and post-treatment) with CTX-471. Tumor upregulation of CD137 post-treatment suggests enhanced effector activity. These results suggest that CD137 agonism may drive a feedback loop, further activating cells and aligning with the mechanism of action of anti-CD137 antibodies.

[00136] To clarify the use of and to hereby provide notice to the public, the phrases "at least one of <A>, <B>, … and <N>" or "at least one of <A>, <B>, … <N>, or combinations thereof" or "<A>, <B>, … and/or <N>" are defined by the Applicant in the broadest sense, superseding any other implied definitions hereinbefore or hereinafter unless expressly asserted by the Applicant to the contrary, to mean one or more elements selected from the group comprising A, B, … and N. In other words, the phrases mean any combination of one or more of the elements A, B, … or N including any one element alone or the one element in combination with one or more of the other elements which may also include, in combination, additional elements not listed. Unless otherwise indicated or the context suggests otherwise, as used herein, "a" or "an" means "at least one" or "one or more."

[00137] While various embodiments have been described, it will be apparent to those of ordinary skill in the art that many more embodiments and implementations are possible. Accordingly, the embodiments described herein are examples, not the only possible embodiments and implementations.

CLAIMS

1. A method of treating cancer comprising administering

a bispecific antibody, wherein the bispecific antibody targets VEGF-A and DLL4, and

simultaneously administering at least one agonistic anti-CD137 antibody and/or monoclonal antibody that disrupt the PD-1/PD-L1 signaling axis.

2. A method for increasing anti-tumor efficacy comprising administering a bispecific antibody targeting VEGF-A and DLL4 together with an agonistic anti-CD137 antibody, or monoclonal antibody that disrupts the PD-1/PD-L1 signaling axis.

3. A method to increase the anti-tumor efficacy of individual treatments alone comprising simultaneously administering a combination of a bispecific antibody targeting VEGF-A and DLL4 and an agonistic anti-CD137 antibody, or monoclonal antibodies that disrupt the PD-1/PD-L1 signaling axis.

4. A method to increasing anti-tumor activity in MHC-I deficient tumors comprising administering a combination of a bispecific antibody targeting VEGF-A and DLL4 and an agonistic anti-CD137 antibody, or monoclonal antibodies that disrupt the PD-1/PD-L1 signaling axis.

5. A method for increasing the anti-tumor efficacy of the individual treatments alone comprising coupling VEGF-A/DLL4 targeting with either CD137 agonism or PD-1/PD-L1 blockade.

6. A method to enhance clinical benefits of cancer treatment using a bispecific antibody targeting VEGF-A and DLL4 comprising administering an agonistic anti-CD137 antibody or PD-1/PD-L1 blockade in combination with the bispecific antibody targeting VEGF-A and DLL4.

7. A method of treating cancer comprising administering a dual-targeting protein to a subject, wherein the dual-targeting protein comprises a protein that specifically binds to DLL4 and VEGF thereby blocking DLL4 and VEGF signaling, in combination with agonistic anti-CD137 antibody or PD-1/PD-L1 blocker.

8. A method of treating cancer comprising administering a dual-targeting protein to a subject, wherein the dual-targeting protein comprises a protein that simultaneously blocks DLL4 and VEGF signaling pathways in combination with an agonistic anti-CD137 antibody or PD-1/PD-L1 blocker.

9. A method of first line treatment of cancer comprising administering a combination of a bispecific antibody targeting VEGF-A and DLL4 and an agonistic anti-CD137 antibody, or monoclonal antibodies that disrupt the PD-1/PD-L1 signaling axis.

10. A method of increasing anti-tumor efficacy of monotherapy cancer treatment comprising administering a combination of a bispecific antibody targeting VEGF-A and DLL4 and an agonistic anti-CD137 antibody, or a monoclonal antibody that disrupts the PD-1/PD-L1 signaling axis.

11. A method of increasing potency of anti-tumor activity in a subject suffering from cancer comprising administering a combination of a bispecific antibody targeting VEGF-A and DLL4 and an agonistic anti-CD137 antibody, or a monoclonal antibody that blocks PD-1/PD-L1 signaling axis.

12. A method of oncological therapeutic treatment comprising administering a bispecific antibody that blocks DLL4/Notch and VEGF/VEGF-receptor signaling in combination with a molecule that blocks PD-1/PD-L1 pathway or an CD137 agonist.

13. A method of treating biliary tract cancer (BTC) comprising administering a bispecific antibody that blocks DLL4/Notch and VEGF/VEGF-receptor signaling in combination with an anti-PD-1/PD-L1 antibody or an CD137 agonist.

14. A method of treating colorectal cancer (CRC) comprising administering a bispecific antibody that blocks DLL4/Notch and VEGF/VEGF-receptor signaling in combination with an anti-PD-1/PD-L1 antibody or an CD137 agonist.

15. A method of enhancing the efficacy of treating biliary tract cancer (BTC), colorectal cancer (CRC), gastric cancer, cholangiocarcinoma cancer, pancreatic cancer, non-small cell lung cancer, and/or other cancers in patients that have been previously treated with one or more checkpoint inhibitors (CPI) where the cancer evaded treatment for such post-CPI patients, comprising administering a bispecific antibody that blocks DLL4/Notch and

VEGF/VEGF-receptor signaling in combination with an anti-PD-1/PD-L1 antibody or an CD137 agonist.

16. A method of treating cancer by increasing the anti-tumor efficacy of the individual cancer treatments alone comprising coupling VEGF-A/DLL4 targeting with either CD137 agonism or PD-1/PD-L1 blockade markedly increased the anti-tumor efficacy of the individual treatments alone in the CT26, MC38 and LLC1 syngeneic tumor models.

17. The method of any of claims 1-16, wherein the protein that specifically binds to VEGF is bevacizumab.

18. The method of any of claims 1-16, wherein the protein binding specifically to DLL4 is in the form of a full-length antibody, Fab', F(ab')2, Fab, Fv, IgG, or scFv.

19. The method of any of claims 1-16, wherein the protein binding specifically to VEGF is in the form of a full-length antibody, Fab', F(ab')2, Fab, Fv, IgG, or scFv.

20. The method of any of claims 1-16, wherein the cancer is biliary tract cancer (BTC), colorectal cancer (CRC), gastric cancer, cholangiocarcinoma cancer, pancreatic cancer, non-small cell lung cancer, and/or another form of cancer that has evaded treatment after a patient received one or more CPIs.

21. A method of treating a subject suffering from treatment resistant cancer, comprising

administering a bispecific antibody, wherein the bispecific antibody targets VEGF-A and DLL4, and

simultaneously administering an agonistic anti-CD137 antibody, and/or an anti-PD-1 and/or anti-PD-L1 monoclonal antibody.

22. The method of claim 21, wherein the anti-CD137 antibody comprises CTX-471, Urelumab, and/or Utomilumab.

23. The method of claim 21, wherein the anti-PD-1 and/or anti-PD-L1 monoclonal antibody comprises Pembrolizumab, Nivolumab, Cemiplimab, Atezolizumab, Avelumab, and/or Durvalumab.

24. A method of treating cancer in a subject suffering from a cancer that has escaped prior checkpoint blockade comprising

administering a bispecific antibody, wherein the bispecific antibody targets VEGF-A and DLL4, and

simultaneously administering an agonistic anti-CD137 antibody, and/or an anti-PD-1 and/or anti-PD-L1 monoclonal antibody.

25. The method of claim 24, wherein the anti-CD137 antibody comprises CTX-471, Urelumab, and/or Utomilumab.

26. The method of claim 24, wherein the anti-PD-1 and/or anti-PD-L1 monoclonal antibody comprises Pembrolizumab, Nivolumab, Cemiplimab, Atezolizumab, Avelumab, and/or Durvalumab.

27. A method of treating a solid tumor in a subject comprising:

administering a bispecific antibody, wherein the bispecific antibody specifically binds both VEGF-A and DLL4, and

concurrently administering an agonistic anti-CD137 antibody, and/or an anti-PD-1 or anti-PD-L1 monoclonal antibody.

28. The method of any one of the preceding claims, wherein the bispecific antibody that targets DLL4 and VEGF is a recombinant bispecific antibody of the human IgG1 isotype comprising at least one scFv that binds to DLL4 linked to an antibody that binds and neutralizes the activity of human VEGF-A.

29. An MHC-I negative or MHC-I deficient cell line carrying MHC defects comprising:

(a) MC38 and CT26 cells wherein the B2M gene is biallelically deleted;

(b) MC38 cells wherein both H2-K1 alleles are deleted; or

(c) $CT26^{B2m-/-}$ cells wherein such cells are passaged in tumor experienced mice to generate a line with increased NK resistance ($CT26^{B2m-/-E}$).

30. A genetically modified MHC-I negative or MHC-I deficient cell line maintained as a stable cell line in vitro and suitable for implantation in vivo into a living host subject, the MHC-I negative or MHC-I deficient cell comprising MC38 and CT26 cells, wherein the B2M gene in the MC38 and CT26 cells is biallelically deleted.

31. A genetically modified MHC-I negative or MHC-I deficient cell line maintained as a stable cell line in vitro and suitable for implantation in vivo into a living host subject, the MHC-I negative or MHC-I deficient cell line comprising MC38 cells wherein both H2-K1 alleles are deleted.

32. A genetically modified MHC-I negative or MHC-I deficient cell line maintained as a stable cell line in vitro and suitable for implantation in vivo into a living host subject, the MHC-I negative or MHC-I deficient cell line comprising $CT26^{B2m-/-}$ cells passaged in tumor experienced mice to generate a cell line with increased NK resistance ($CT26^{B2m-/-E}$).

33. The cell line of claims 29-32, wherein the cell line further comprises a lack of both class I MHC alleles; a lack of individual MHC alleles; and/or the tumor suppressor CDKN2A.

34. The cell line of claim 29 or 32, wherein the serial passaging of cells selects for escape and/or evasion anti-tumor immunity as a model of resistance to checkpoint inhibitors and/or CD137 agonists in human tumors.

35. A method of developing checkpoint inhibitors for use in the treatment of cancer comprising

producing a genetically modified MHC-I negative or MHC-I deficient cell line comprising:

(a) MC38 and CT26 cells wherein the B2M gene is biallelically deleted;

(b) MC38 cells wherein both H2-K1 alleles are deleted; or

(c) $CT26^{B2m-/-}$ cells wherein such cells are passaged in tumor experienced mice to generate a line with increased NK resistance ($CT26^{B2m-/-E}$);

implanting the MHC-I negative or MHC-I deficient cell line into a living host or test subject;

administering at least one checkpoint inhibitor to the living host or test subject; and

analyzing the efficacy of the checkpoint inhibitor in the living host or test subject.

36. A method of developing agonists for use in the treatment of cancer comprising:

producing a genetically modified MHC-I negative or MHC-I deficient cell line comprising:

(a) MC38 and CT26 cells wherein the B2M gene is biallelically deleted;

(b) MC38 cells wherein both H2-K1 alleles are deleted; or

(c) $CT26^{B2m-/-}$ cells wherein such cells are passaged in tumor experienced mice to generate a line with increased NK resistance ($CT26^{B2m-/-E}$);

implanting the MHC-I negative or MHC-I deficient cell line into a living host or test subject;

administering at least one agonist to the living host or test subject; and

analyzing the efficacy of the agonist in the living host or test subject.

37. A method of developing angiogenesis inhibitors for use in the treatment of cancer comprising:

producing a genetically modified MHC-I negative or MHC-I deficient cell line comprising:

(a) MC38 and CT26 cells wherein the B2M gene is biallelically deleted;

(b) MC38 cells wherein both H2-K1 alleles are deleted; or

(c) $CT26^{B2m-/-}$ cells wherein such cells are passaged in tumor experienced mice to generate a line with increased NK resistance ($CT26^{B2m-/-E}$);

implanting the MHC-I negative or MHC-I deficient cell line into a living host or test subject;

administering at least one angiogenesis inhibitor to the living host or test subject; and

analyzing the efficacy of the angiogenesis inhibitors in the living host or test subject.

38. The method of any one of claims 35-37, wherein efficacy is measured by a reduction in cancer cells, a reduction in tumor size, and/or a reduction in symptoms.