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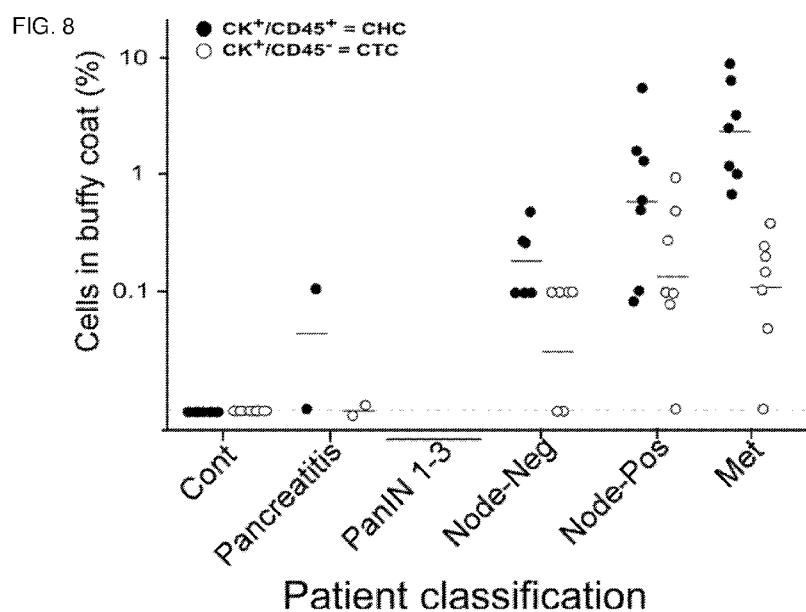
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(54) Title: DETECTING AND/OR SUBTYPING CIRCULATING HYBRID CELLS THAT CORRELATE WITH STAGE AND SURVIVAL



(57) Abstract: Provided are methods of detecting and differentiating/grading Circulating Hybrid Cells (CHCs) in samples, such as blood samples, as well as uses of CHCs in diagnosing and tracking disease, and in determining and tracking treatments. Methods of treatment are also described, as are kits and devices that can be used in carrying out methods provided herein.



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DETECTING AND/OR SUBTYPING CIRCULATING HYBRID CELLS THAT CORRELATE WITH STAGE AND SURVIVAL

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 62/730,519 filed on September 12, 2018, and to U.S. Provisional Application No. 62/843,094 filed on May 3, 2019, both of which are incorporated herein by reference in their entirety as if fully set forth herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under CA172334 awarded by the National Institutes of Health. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Historic dogma describing tumor evolution is based on outgrowth and expansion of clonal tumor populations. However, it is now appreciated that both genetic and non-genetic mechanisms drive tumor evolution, fostering phenotypic variability of neoplastic cells and their clones. These changes underlie aggressive tumor growth, metastatic spread, therapeutic response and resistance (Heppner, *Cancer Res.* 44(6):2259-2265, 1984; Marusyk & Polyak, *Biochim Biophys Acta.* 1805(1):105-117, 2010). While understanding of the molecular and cellular mechanisms contributing to intratumoral heterogeneity has significantly expanded, there are no effective therapies quelling heterogeneity to improve patient stratification or response to anti-cancer therapies, highlighting the need for advances in this area.

[0004] Heterotypic cell fusion is a fundamental developmental mechanism serving to enhance cellular diversity; the most notable and best studied example is fusion of sperm and egg. In adult murine intestines, fusion between hematopoietic and epithelial cells is readily detected in response to injury (Rizvi *et al.*, *Proc Natl Acad Sci U S A.* 103(16):6321-6325, 2006; Davies *et al.*, *PLoS One.* 4(8):e6530, 2009); similar findings have been reported with various other cells including hepatocytes, cardiomyocytes, and skeletal muscles (Alvarez-Dolado *et al.*, *Nature.* 425(6961):968-73, 2003; Camargo *et al.*, *Nat Med.* 9(12):1520-7, 2003; Terada *et al.*, *Nature.* 416(6880):542-5, 2002; Wang *et al.*, *Nature.* 422(6934):897-901, 2003). In cancer, however, despite a century-old hypothesis (Aichel, *Vorträge und Aufsätze über Entwicklungsmechanik Der Organismen*, ed. Roux, W. (Wilhelm Engelmann, Leipzig), pp. 92–111, 1911) that cell fusion contributes to tumor initiation (Carter, *J Natl Cancer Inst.* 100(18):1279-81, 2008; Pawelek, *Lancet Oncol.* 6(12):988-93, 2005; Dittmar & Zanker, *Int J Mol Sci.* 16(12):30362-81, 2015) and acquisition of metastatic behaviors (Pawelek, *Lancet Oncol.* 6(12):988-93, 2005; Powell *et al.*, *Cancer Res.* 71(4):1497-505, 2011; Lazova *et al.*, *PLoS One.*

8(6):e66731, 2013), few experimental studies have mechanistically addressed the functional underpinnings or consequences of cell fusion in the etiology of malignant progression.

[0005] Reports using various approaches identify human tumor cells with immune and malignant characteristics (Lazova *et al.*, *PLoS One*. 8(6):e66731, 2013; Adams *et al.*, *Proc Natl Acad Sci U S A*. 111(9):3514-9, 2014; Cogle *et al.*, *Stem Cells*. 25(8):1881-7, 2007; Grimm *et al.*, *Oral Surg Oral Med Oral Pathol Oral Radiol*. 121(3):301-6, 2016; LaBerge *et al.*, *PLoS One*. 12(2):e0168581, 2017; Shabo *et al.*, *Int J Cancer*. 125(8):1826-31, 2009; Yilmaz *et al.*, *Bone Marrow Transplant*. 35(10):1021-1024, 2005). Etiologic mechanism for these cells is attributed to cell fusion, developmental mimicry, trans-differentiation, or other unidentified mechanisms. These studies do not address the biologic significance of the hybrid tumor cells, or present evidence from experimental models to support the mechanism. Since underlying mechanisms for these cells cannot easily be determined in human subjects, murine models and *in vitro* studies provide a more appropriate and tractable platform for investigation.

[0006] Lustberg *et al.* (*Breast Cancer Res*. 16:R23, 2015) previously describes CD45+/ cytokeratin+ cells in mammary cancer patients. However, they do not show any biology, or speculate on the nature of these cells, except to say they are found abundantly and that they express the macrophage marker CD68. Further, they also state that these cells have previously been reported but have always been thought to be an artifact (Stott *et al.*, *PNAS* 107(43):18392-18397, 2010; van de Stolpe *et al.*, *Cancer Res*. 71(18):5955, 5960, 2011).

[0007] There remains a need for understanding of fused cells and their role in cancer biology.

SUMMARY OF ASPECTS OF THE DISCLOSURE

[0008] In prior studies, *in vivo* fusion between intestinal epithelial cells and macrophages (MΦs) was reported to yield hybrid offspring retaining epithelial characteristics defined by their gene expression profile (Powell *et al.*, *Cancer Res*. 71(4):1497-505, 2011). Based on this, and recognizing that MΦs are inherently migratory, physiologic relevance of cell fusion to tumor heterogeneity was studied by enhanced somatic diversity of neoplastic hybrids, through increased migratory or invasive properties, and instilling a selective metastatic advantage. Described herein is a systematic analysis of MΦ-neoplastic cell fusion (referred to as MΦ-cancer cell fusion, or fusion hybrid) using *ex vivo* and *in vivo* murine cancer models to provide evidence that hybrids acquire functional MΦ-associated phenotypes that enhance tumor progression. Analyses of human tumor biopsies and peripheral blood reveal a novel circulating hybrid cell population (defined as cells harboring hematopoietic and epithelial/tumor properties), whose numbers correlates with disease stage and predicts overall outcome, thereby representing a biomarker for patient stratification. See also PCT/US2016/057389 and U.S. Application Publication No. 2017/0106101.

[0009] Thus, described herein is the discovery that macrophages can fuse with cancer (and other) cells and create a new cell that has retained the genes and properties of both of the original “parent” cells, the tumor cell and the macrophage (Gast *et al.*, *Sci. Adv.* 4:3aa67828, 1-15, 2018). Because the macrophage (MΦ) is an immune cell that normally functions to travel throughout the body, attracted by cytokines, traffic in and out of vessels, invade distant organs, it is believed that fusion between a macrophage and tumor cell results in cancer cells that have enhanced metastatic capacity.

[0010] Bone marrow-derived cells fuse with epithelial cells (Rizvi *et al.*, *Proc Natl Acad Sci U S A.* 103(16):6321-5, 2006); the fusion partners have been defined as macrophages and cells that are actively proliferating (Powell *et al.*, *Cancer Res.* 71(4):1497-505, 2011; Davies *et al.*, *PLoS One.* 4(8):e6530, 2009). The inventors have demonstrated that these macrophage-tumor cell fusion hybrids harbor properties of metastatic cancer cells and when compared to cancer cells that have not fused, out-perform them in a number of metastatic cancer cell assays (see below for details; Gast *et al.*, *Sci. Adv.* 4:3aa67828, 1-15, 2018). Importantly, these fused cells have been found in the blood of patients with various stages of cancer. They are more abundant in the blood than cancer cells that have not fused. It has been confirmed that these patient circulating hybrid cells (CHCs) are the product of macrophage-tumor cell (or blood cell-tumor cell) fusion in a mouse models of glioblastoma, melanoma, mammary cancer, and colorectal cancer. These novel CHCs are identified by expression of cytokeratin (and optionally one or more of EpCAM, MUC4, MASPIN, GFAP, Nestin, ECAD, MelanA, NKI/Btep) and CD45 (and additionally one or more of CD68, CD11c, CD163, CD14, CD16, CD11b, CSF1R).

[0011] Epithelial-immune cell fusion hybrids (more generally, Circulating Hybrid Cells; CHCs) have also been found in peripheral blood of patients with inflammatory conditions such as inflammatory bowel disease (IBD) and pancreatitis. These hybrid cells have different protein expression profiles than cancer-derived hybrids. It is therefore believed that CHCs can be used as a monitor of disease pathologic state across the disease axis based upon discrete protein expression profiling. This enables assays for early detection of disease, such as cancer, and pre-disease, such as pre-cancer risk; and may be especially useful in high-risk cohorts such as patients with diabetes or pancreatitis with a risk of pancreatic cancer, and IBD patients with greater risk of colorectal cancer. Panels of antibodies enable subtyping of different classes of CHCs that will underlie assays for early detection of cancer, thus differentiating inflammatory-derived CHCs from cancer-derived CHCs.

[0012] High lethality rates associated with metastatic cancer highlight an urgent medical need for improved understanding of biologic mechanisms driving metastatic spread, and identification of biomarkers predicting late-stage progression. Numerous neoplastic cell intrinsic and extrinsic mechanisms are known to fuel tumor progression; however, mechanisms driving heterogeneity of neoplastic cells in solid tumors remain obscure. Increased mutational rates of neoplastic cells in stressed environments are clearly implicated, but cannot explain all aspects of tumor heterogeneity. Presented herein is further evidence that fusion of neoplastic cells with leukocytes (e.g. macrophages)

contributes to tumor heterogeneity, resulting in cells exhibiting increased metastatic behavior. Fusion hybrids are readily detectable in cell culture and tumor-bearing mice. Further, hybrids (cells harboring hematopoietic and epithelial properties) in peripheral blood of human cancer patients correlate with disease stage and predict overall survival. This unique population of neoplastic cells provides a novel biomarker for tumor staging, as well as a potential therapeutic target for intervention.

[0013] Thus, the current disclosure provides a method of detecting Circulating Hybrid Cells (CHCs) in a patient (such as a human patient), the method including: obtaining a blood sample from the (human) patient; and detecting whether CHCs are in the blood sample by contacting the sample with an anti-source cell antibody; and contacting the sample with an anti-immune cell antibody; wherein specific binding of both antibodies in the same cell indicates the presence of CHCs (or identifies the detected cell as a CHC).

[0014] Another embodiment is a method of diagnosing a solid tumor in a (human) patient, the method including: obtaining a blood sample from the (human) patient; and detecting whether CHCs are in the blood sample by: contacting the sample with an antibody specific for a protein found on cells from a tissue from which the solid tumor is derived; contacting the sample with an antibody specific for an immune cell; and diagnosing the patient as having a solid tumor wherein specific binding of both antibodies in the same cell indicates the presence of one or more CHCs. Examples of this method embodiment also include contacting the sample with at least two different antibodies specific for proteins found on cells from a tissue from which the solid tumor is derived, with at least two different antibodies specific for an immune cell, or with both, and diagnosing the patient as having a solid tumor wherein specific binding of antibodies specific for proteins found on cells from a tissue from which the solid tumor is derived and antibodies to immune cells in the same cell (which is a CHC) indicates the presence of one or more CHCs.

[0015] Another embodiment is a method of diagnosing metastatic cancer (or the potential to become metastatic) in a patient, such as a human patient, the method including: obtaining a blood sample from the (human) patient; and detecting whether CHCs are in the blood sample by contacting the sample with an anti-(source of cancer) antibody; contacting the sample with an anti-immune cell antibody; and diagnosing the patient as having a metastatic cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more CHCs.

[0016] Also provided are methods of differentiating disease status of a (human) patient, which methods include: obtaining a blood sample from the human patient; and typing CHCs are in the blood sample by contacting the sample with at least two panels of antibodies, each panel including at least two antibodies, wherein: a first panel of antibodies including at least one anti-immune cell antibody and at least one antibody specific for a first source cell type; and a second panel of antibodies including at least one anti-immune cell antibody and at least one antibody specific for a second source cell type, wherein the first source cell type and the second source cell type represent two stages of a disease

progression; identifying the disease status of the patient based on detection of circulating cells that exhibit specific binding to both an anti-immune cell antibody and an antibody specific for either the first source cell type or the second source cell type. In embodiments of these methods of differentiating disease states, the first source cell type is a cancer cell and the second source cell type is a non-cancerous cell of the same origin as the cancer cell. In specific examples of such method embodiments, the first cell type is an epithelial-derived cancer cell and the at least one antibody specific for the first cell type is specific for one of MUC4 or MASPIN; and the second cell type is an epithelial cell and the at least one antibody specific for the first cell type is specific for one of ECAD, EpCAM, or CK.

[0017] Another provided embodiment is a method of treating cancer, or metastatic cancer, or cancer at higher risk for metastasis, in a (human) patient, the method including: obtaining a blood sample from the (human) patient; detecting whether CHCs are in the blood sample by contacting the sample with an anti-(source of the metastatic cancer) antibody; contacting the sample with an anti-immune cell antibody; diagnosing the patient as having metastatic cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more Circulating Hybrid Cells; and administering to the patient in need thereof a pharmaceutically effective amount of an anti-cancer agent. In such treatment methods, the anti-cancer agent in some embodiments is a CSF1R inhibitor, such as a CSF1R inhibitor selected from pexidartinib, PLX7486, LY3022855, MC-CS4, chiauranib, SNDX6352, JNJ-40346527, DCC-3014, linifanib, IMC-CS4, AMG820, BLZ945, TK-1258, dovitinib, vatalinib, sunitinib, ARRY-3882, 5-(3-methoxy-4-((4-methoxybenzyl)oxy)benzyl)pyrimidine-2,4-diamine, CEP-32496, or 3-((quinolin-4-ylmethyl)amino)-N-(4-(trifluoromethoxy)phenyl)thiophene-2-carboxamide; or a pharmaceutically acceptable salt thereof; or selected from pexidartinib, chiauranib, linifanib, dovitinib, vatalinib, or sunitinib; or a pharmaceutically acceptable salt thereof; or a CSF1R inhibitors is an anti-CSF1R antibody (such as cabiralizumab or emactuzumab).

[0018] In examples of any of the provided methods, the anti-source cell antibody can be an epithelial cell antibody that specifically binds to an epitope on a biomarker selected from the group of EpCAM, E-cadherin, cytokeratin, MUC4, MASPIN, Glypican-1 (GPC1), GFAP, Nestin, gp100, and MAGEA1.

[0019] In examples of any of the provided methods, detecting whether CHCs are in the blood sample includes contacting the sample with two or more, three or more, or four or more anti- antibodies, each of which specifically binds to an epitope on a biomarker selected from the group of EpCAM, E-cadherin, cytokeratin, MUC4, MASPIN, Glypican-1 (GPC1), GFAP, Nestin, gp100, MAGEA1, MelanA and NKI/Btep. Of these, GFAP and Nestin are glioblastoma markers; MelanA and NKI/Btep are uveal melanoma markers; and the remaining are epithelial markers.

[0020] In examples of any of the provided methods, the anti-immune cell antibody specifically binds to an epitope on a biomarker selected from CD45, CD14, CD16, CD11b, CD11c, CD64, CD163, CD68, CD66b, CD71, CD80, CD86, CSF1R, or CCR5.

[0021] In examples of any of the provided methods, detecting whether CHCs are in the blood sample includes contacting the sample with two or more, three or more, or four or more anti-immune cell antibodies, each of which specifically binds to an epitope on a biomarker selected from the group of CD45, CD14, CD16, CD11b, CD11c, CD64, CD163, CD68, CD66b, CD71, CD80, CD86, CSF1R, and CCR5.

[0022] In examples of any of the provided methods, the anti-immune cell antibody specifically binds to an epitope on a biomarker selected from CD14, CD16, CD45, CD64, CD68, CD71, or CCR5.

[0023] In examples of any of the provided methods, detecting whether CHCs are in the blood sample includes contacting the sample with two or more, three or more, or four or more anti-immune cell antibodies, each of which specifically binds to an epitope on a biomarker selected from CD14, CD16, CD45, CD64, CD68, CD71, or CCR5.

[0024] In examples of any of the provided methods, the anti-(source) antibody is an anti-epithelial antibody that specifically binds to an epitope on a biomarker from an epithelial-derived cell. Optionally, the biomarker is selected from (or includes one or more of) EpCAM, E-cadherin, cytokeratin, MUC4, MASPIN, or Glypican-1 (GPC1).

[0025] Additional embodiments provided methods of diagnosing or staging a condition or disease in a subject, such as a human subject. In examples of such embodiments, the method includes: obtaining a blood sample from the subject; characterizing Circulating Hybrid Cells (CHCs) in the blood sample by: contacting the sample with an antibody specific for an antigen found on an immune cell (such as CD45), contacting the sample with one or more antibodies specific for an antigen from a cell that originates from a tissue type involved with the condition or disease (a source cell) (for instance, an antibody specific for an epithelial antigen, such as ECAD, EpCAM, and/or CK); contacting the sample with one or more antibodies specific a cancerous cell (for instance, antibodies specific for MUC4 and/or MASPIN); identifying the blood sample as containing: *inflammation-indicative CHCs* when the immune cell antigen-specific antibody(s) (such as an anti-CD4 antibody) and the source cell antigen-specific antibody(s) (for instance, epithelial-cell specific antibody(s)), but not cancer-cell specific antibodies specifically bind the same cell in the blood sample; *cancer-indicative CHCs* when at least one immune cell antigen-specific antibody(s) (such as an anti-CD4 antibody), the source cell antigen-specific antibody(s) (for instance, epithelial-cell specific antibody(s)), and the cancer-cell specific antibodies all specifically bind the same cell in the blood sample; and diagnosing the subject as: having or at risk for cancer when the number of cancer-indicative CHCs outnumber (for instance, by two-fold) the number of inflammation indicative CHCs; having or at risk of chronic inflammation when the number of inflammation-indicative CHCs is great than (for instance, by two fold) the number of cancer-indicative CHCs; or having neither cancer nor chronic inflammation when the number of inflammation-indicative CHCs and the number of cancer-indicative CHCs is equal and fewer than a threshold level. Without intending to be limited in any way, the threshold level for instance may be fewer than 1000

CHCs/20,000 nuclei in the sample, or fewer than 100 CHCs/20,000 nuclei in the sample, or fewer than 25 CHCs/20,000 nucleic in the sample.

[0026] Yet another embodiment is a method of treating cancer in a human patient, the method including: obtaining a sample from the human patient; detecting whether Circulating Hybrid Cells (CHCs) are in the sample by contacting the sample with an anti-(source of the cancer) antibody; contacting the sample with an anti-immune cell antibody; diagnosing the patient as having cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more Circulating Hybrid Cells; and administering to the patient in need thereof a pharmaceutically effective amount of an anti-cancer agent.

[0027] Also provided is a method of detecting Circulating Hybrid Cells (CHCs) in a human patient, the method including: obtaining a sample from the human patient; and detecting whether CHCs are in the sample by contacting the sample with an anti-source cell antibody that recognizes a marker other than cytokeratin (CK); and contacting the sample with an anti-immune cell antibody; wherein specific binding of both antibodies in the same cell indicates the presence of CHCs.

[0028] Another method described herein is a method of diagnosing a solid tumor in a human patient, the method including: obtaining a sample from the human patient; and detecting whether Circulating Hybrid Cells (CHCs) are in the sample by: contacting the sample with an antibody specific for a protein found on cells from a tissue from which the solid tumor is derived; contacting the sample with an antibody specific for an immune cell; and diagnosing the patient as having a solid tumor wherein specific binding of both antibodies in the same cell indicates the presence of one or more CHCs.

[0029] Another embodiment is a method of diagnosing metastatic cancer in a human patient, the method including: obtaining a sample from the human patient; and detecting whether Circulating Hybrid Cells (CHCs) are in the sample by contacting the sample with an anti-(source of cancer) antibody; contacting the sample with an anti-immune cell antibody; and diagnosing the patient as having a metastatic cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more CHCs.

[0030] Yet another embodiment is a method of differentiating disease status of a human patient, the method including: obtaining a sample from the human patient; and typing Circulating Hybrid Cells (CHCs) are in the sample by contacting the sample with at least two panels of antibodies, each panel including at least two antibodies, wherein: a first panel of antibodies including at least one anti-immune cell antibody and at least one antibody specific for a first source cell type; and a second panel of antibodies including at least one anti-immune cell antibody and at least one antibody specific for a second source cell type, wherein the first source cell type and the second source cell type represent two stages of a disease progression; identifying the disease status of the patient based on detection of circulating cells that exhibit specific binding to both an anti-immune cell antibody and an antibody specific for either the first source cell type or the second source cell type.

[0031] Also provided is a method of treating metastatic cancer in a human patient, the method including: obtaining a sample from the human patient; detecting whether Circulating Hybrid Cells (CHCs) are in the sample by contacting the sample with an anti-(source of the metastatic cancer) antibody; contacting the sample with an anti-immune cell antibody; diagnosing the patient as having metastatic cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more Circulating Hybrid Cells; and administering to the patient in need thereof a pharmaceutically effective amount of an anti-cancer agent.

[0032] In any of the described method embodiments, there are examples in which at least one of the antibodies is conjugated to a fluorescent label, and the detecting includes fluorescence activated cell sorting (FACS) analysis

[0033] In any of the described method embodiments, the sample can include blood, plasma, serum, lymph, another blood fraction, a tumor aspirate, a tumor biopsy, peritoneal fluid, a secretions, urine, or another biological sample that contains or is believed to contain immune cells.

[0034] Another embodiment is a chromatographic assay device including: a panel of two or more capture antibodies or antigen-binding fragment thereof, each of which specifically binds a different marker protein in Table 2 or Table 3.

[0035] Also described are kits that include: a panel of two or more antibodies or antigen-binding fragment thereof, each of which specifically binds a different marker protein in Table 2 or Table 3.

[0036] Yet another embodiment is an antibody cocktail that allows for separation of CHCs via a flow based or DEP based assay. Examples of this antibody cocktail embodiment include: two or more antibodies or antigen-binding fragment thereof, each of which specifically binds a different marker protein in Table 2 or Table 3.

[0037] Also provided are methods of use and uses of the assay device embodiments, the kit embodiments, and the antibody cocktail embodiments to detect one or more CHCs in a sample from a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0038] FIGs. 1A-1E. A histological section demonstrating cell fusion in human tumors. Solid tumors from women ($n = 7$) with previous sex-mismatched bone marrow transplantation (BM4T) permits analysis of cell fusion. (FIG. 1A) PDAC tumor section with cytokeratin, the Y chromosome (Y chr), and Hoechst detection revealed areas of cytokeratin-positive cells with Y chr-positive nuclei (white arrowheads). Boxed representative areas in FIG. 1A are enlarged in FIGs. 1B to 1E. Scale bars, 25 μ m.

[0039] FIGs. 2A-2G. Human CTCs. FIG. 2A depicts data from a sex-mismatched BMT patient who acquired a solid tumor (PDAC). Peripheral blood was analyzed for the presence of cell fusion. Two panels displaying cell fusion hybrids (arrowheads) that co-stain for EPCAM and CD45 and have a Y

chromosome (white dot) in their nuclei are shown. Arrows denote leukocytes. FIG. 2B depicts results for CHCs and CTCs analyzed from $n = 4$ patients with PDAC. CHCs (CK+/CD45+) also express MΦ proteins (cocktail: CD68, CD163, CD66b, and CSF1R), while CTCs (CK+/CD45–ve) do not. CHCs also express the tumor-specific protein MUC4. FIG. 2C depicts data for CHCs and CTCs analyzed by flow cytometry for CD14, CD16, CD11c, and CD163 expression or the cancer-specific protein MUC4 ($n = 4$ patients). FIG. 2D depicts results for human pancreatic cancer patient peripheral blood analyzed for cytokeratin+ (CK) and CD45+ expression using *in situ* analyses and digital scanning. FIG. 2E depicts data for CK+/CD45+ and CK+/CD45– cells quantified in patient blood across cancer stages [analysis of variance (ANOVA), $*P < 0.023$]. FIG. 2F depicts a Kaplan-Meier curve showing that dichotomized biomarkers based on median value of CHC was associated with statistically significant increased risk of death ($P = 0.0029$). FIG. 2G depicts a Kaplan-Meier curve showing that dichotomized biomarkers based on median value of CTC was not associated with statistically significant increased risk of death ($P = 0.95$).

[0040] FIGs. 3A-3F. Cell fusion in PanIN and tumors from other organ sites. Solid tumors from women with previous sex-mismatched bone marrow transplantation permits analysis of cell fusion. (FIG. 3A) Hematoxylin and Eosin stain of pancreatic ductal adenocarcinoma (PDAC) section, (FIG. 3B) with cytokeratin, the Y-chromosome (Ychr) and Hoechst detection. Boxed region enlarged in (FIG. 3C) contains pancreatic intraepithelial neoplasia (PanIN). (FIGs. 3D-3F) Renal cell carcinoma, head and neck squamous carcinoma (HNSCC), and lung tumors analyzed for cytokeratin-positive cells with Y-chromosome-positive nuclei, white arrowhead. Representative areas boxed in white are enlarged. Bar = 10 μ m.

[0041] FIGs. 4A-4B. Control blood samples for immunohistochemical and FISH analyses. FIG. 4A depicts male and female tissue stained with Y-chromosome FISH probe (white arrow). Quantification of Y-chromosome-positive cells in male, female, and the PDAC tumor from FIG. 1A was performed using confocal microscopy to survey through nucleus. A total of 1532 nuclei in female tissue and 1057 nuclei in male tissue were analyzed. FIG. 4B depicts male and female peripheral blood analyzed for expression of cytokeratin (CK), CD45 and the FISH probe to Y-chromosome (white dot). Only male cells are positive for the Y-chromosome. A total of 253 nuclei were analyzed in female cells and 638 nuclei in male cells.

[0042] FIG. 5 (part 1 and part 2) depicts a flow cytometry gating scheme for analyses of human CHCs. Isolated human peripheral blood cells were stained and subjected to FACS. Gating scheme was established based upon single color controls and/or Fluorescence Minus One (FMO) controls.

[0043] FIG. 6 depicts an example of a “cell in a well” microfluidic device for cell capture.

[0044] FIG. 7 depicts an example of a “through-hole well” microfluidic device for cell capture.

[0045] FIG. 8 depicts analysis of CHCs in patients with benign pancreatic tumors or pancreatitis.

[0046] FIG. 9A-9C depict CHCs identified in multiple different cancer organ sites, by flow cytometry of stained peripheral blood mononuclear cells (PBMCs) collected from patients with cancer, or by imaging of antibody-stained PBMCs processed onto glass slides. Quantification of imaged PBMCs is performed from digitally scanned images and with software that can identify and enumerate CHCs. CHCs are defined as cytokeratin+ (CK+)/CD45+ for pancreatic ductal adenocarcinoma (PDAC) (FIG. 9A), esophageal, lung, breast (FIG. 9B), colorectal cancer. CHCs also harbor cancer specific protein expression such as MUC4 and MASPIN1 for PDAC. CHCs are defined as gp100+/CD45+ in uveal melanoma (FIG. 9A), and GFAP+, Nestin+/CD45+ in glioblastoma (FIG. 9C). CHCs are also detected in head and neck squamous carcinoma, colorectal cancer, pediatric high grade glioma, cutaneous melanoma, and prostate cancer, but are not pictured here. In each case, CHCs outnumber CTCs (e.g. CK+/CD45-) cells. CTCs are enumerated in the right-hand bar (graphs in FIG. 9A) or the left-hand bar (FIG. 9B). CHCs are detectable in early stage PDAC and uveal melanoma cancer (FIG. 9A), but CTCs are undetectable.

[0047] FIG. 10 shows that CHCs (defined herein by CK+/CD45+) are detected at higher numbers in cancer patients relative to healthy controls. Note that healthy controls have a low baseline of ~0.005%. This chart compares CHCs from early and late stage breast cancer patients relative to healthy controls. Early stage breast cancer patients have detectable CHCs above background.

[0048] FIG. 11 shows that basic (CK+/CD45+) CHC enumeration in lung cancer patients supports increasing numbers of detectable CHCs with increasing disease burden. What is interesting about this data is that a patient diagnosed with early stage lung cancer (*i.e.* stage 1a, designated by asterisk) had levels of CHCs that were consistent with late stage disease. The patient passed away 1 month after diagnosis, and it was determined he had metastatic tumors, and thus should have been diagnosed with late stage disease (stage IV). All cancer patients harbored CHCs above the baseline of CHCs detected in healthy normal controls.

[0049] FIG. 12A-12C. CHCs can be subtyped into different “phenotypes” that correlate with different disease pathologies. Two types of subtyping panels have been developed, one that recognizes cancer-derived CHCs (exemplified cancer-Abs for pancreatic cancer: MUC4+, MASPIN+) and one that recognizes epithelial-derived hybrids generated from inflammation of the epithelium (exemplified epithelial-Abs: ECAD+, EpCAM+, CK+). When applied to patient peripheral blood cells, the cancer Abs and Epithelial Abs can stratify/distinguish patients with chronic inflammation (pancreatitis; a high risk pathology for cancer) from PDAC. Healthy normal control (in this case, subjects that do not have cancer) subject peripheral blood does not harbor cancer-derived CHCs, but does have very low baseline epithelial-derived hybrids. In the top panels of FIG. 12A, arrow indicates a CHC among stained PBMCs that expresses CD45, cancer antibody cocktail, and epithelial antibody cocktail. In the bottom panels of FIG. 12A, arrow indicates a CHC among stained PBMCs that co-expresses CD45 and epithelial antibody cocktail, but not cancer antibodies. (FIG. 12B) Graphical analysis of two CHC

subtypes (cancer-derived, ▲; epithelial-derived, ● in people with pancreatitis, PDAC, or normal healthy controls. People with disease pathology can clearly be distinguished from each other, and importantly from healthy people. (FIG. 12C) Cancer-derived CHCs (and epithelial-derived CHCs, not pictured) can be distinguished by flow cytometry or FACS sorting as well as from a solid platform (shown in FIG. 12A).

[0050] FIG. 13 Cell signaling pathway activation can be assessed in CHCs. Here activation of the ERK signaling pathway is identified in CHCs derived in cancer and pancreatitis patients. PBMCs were processed and adhered to glass slides, as described in Gast *et al.* (*Sci. Adv.* 4:3aa67828, 1-15, 2018). PBMCs were stained with CK and CD45 antibodies, as well as phospho-ERK (pERK) antibodies. The left panels shows cell populations that are positive for CK+/CD45+ (encircled) and are therefore considered to be CHCs. Light grey events are from pancreatitis patients, dark grey events are from PDAC patients, and non-circled events are control cells). The right panel depicts cells that are both expressing pERK and CK+ (box). CHCs from both PDAC and Pancreatitis patients have activated ERK signaling.

[0051] FIGs. 14A-14B depict the heterogeneity among CHCs that can be identified by analyses of multiple proteins and immunohistochemical analyses. PBMCs from breast cancer patients are processed and stained with antibodies to identify CHCs (here CK+/CD45+) are also stained with antibodies to ECAD, estrogen receptor (ER), androgen receptor (AR), CD68, Ki67, CD44 to establish their hormone receptor status, proliferative status and stem state. (FIG. 14A) PBMCs are stained with CD45, CK, ER, CD44. Two CHCs are found in this field of view and are boxed. Enlarged images are shown to the right. (FIG. 14B) CHCs are indicated by arrow, two different CHCs are depicted.

[0052] FIG. 15 CHCs analyzed over the course of a patient's treatment change in phenotype. The figure depicts three different timepoints of analyzed CHCs normalized to 50,000 cells. The white and black grids depict CHCs with different protein expression (each column is one CHC, white indicated protein expression and black is absence of protein expression), and the change in these phenotypes over time. This indicates that CHCs can be used to analyze treatment responsiveness. In order, the proteins detected are: CK, ECAD, CD45, CD68, AR, ER, Ki67, CD44.

[0053] FIG. 16 Longitudinal assess of CHCs across regional and systemic treatment of metastatic colorectal cancer. CHCs (in solid black, CK+/CD45+) track with treatment resistance and their presence precedes detection of disease progression by radiographic means (black arrow), and detection of protein biomarkers, CEA (lower line). Black dashed line depicts CTCs (CK+/CD45-). Day of treatment is listed on the x-axis.

[0054] FIG. 17 depicts analysis of CHCs in patients with head and neck squamous cell carcinoma. Pre-surgery levels of CHCs strongly correlate with patients that convert to pN1+ (right hand column). Further, patients that are designated pN0 have a 30% risk of disease recurrence within 2 years after surgery, meaning that they most likely had undetectable positive nodes. All of the pN0 patients in the

study are under 2 years post-surgery. The circle indicated with the arrow denotes a patient that has developed recurrent disease. The dashed line designates the value (from a ROC curve analysis) that predicts aggressive disease.

REFERENCE TO SEQUENCE LISTING

[0055] The nucleic acid sequences described herein and provided in the accompanying Sequence Listing are shown using standard letter abbreviations for nucleotide bases, as defined in 37 C.F.R. §1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included in embodiments where it would be appropriate. A computer readable text file, entitled "25T3943.txt (Sequence Listing.txt)" created on or about September 8, 2019, with a file size of 8 KB, contains the sequence listing for this application and is hereby incorporated by reference in its entirety. In the accompanying Sequence Listing:

[0056] SEQ ID NO: 1 is the nucleotide sequence of the forward RFP primer:

5'-CAGTTCAGTACGGCTCCAAG-3'

[0057] SEQ ID NO: 2 is the nucleotide sequence of the reverse RFP primer:

5'- CCTCGGGGTACATCCGCTC-3'

[0058] SEQ ID NO: 3 is the nucleotide sequence of the forward Actin primer:

5'-GAAGTACCCCATTTGAACATGGC-3'

[0059] SEQ ID NO: 4 is the nucleotide sequence of the reverse Actin primer:

5'-GACACCGTCCCCAGAATCC-3'

DETAILED DESCRIPTION

[0060] Provided herein are methods of identifying, characterizing and/or quantifying the number/concentration of Circulating Hybrid Cells (CHCs) and discrete disease-specific CHC subtypes in blood. The presence of CHCs provides functional and clinical utility to patients in several ways: 1) CHCs possess greater tumor initiating capacity than Circulating Tumor Cells (CTCs); 2) CHC levels provide a prognostic indication of overall survival of cancer patients, such as pancreatic cancer patients, regardless of cancer stage; 3) CHCs can be monitored along with patient treatment and provide a non-invasive indication of tumor growth and tumor response to treatment; 4) subtyping of CHCs can differentiate different disease pathologies across the cancer continuum, including high risk pathologies versus cancer; 5) CHCs can be used to monitor extent of inflammatory conditions (such as inflammatory bowel disease or pancreatitis) in a non-invasive fashion, and to determine if treatment is effective; and 6) CHCs of discrete subtypes can be used to differentiate "pseudo-progression" from true tumor progression that is measured by imaging modalities. As such, the levels of CHCs in a patient blood sample can be an early predictor of aggressive disease that will allow medical professionals to stratify patients and direct them into the most efficacious treatment methods.

[0061] As CHCs provide a non-invasive biomarker of treatment response in patients (for instance, in cancer patients in some embodiments), they also provide opportunities to sequence the mutational profile of a patient's tumor and serve as an early prediction of therapeutic response or disease recurrence, allowing for a more tailored treatment decisions.

[0062] CHCs or macrophage-tumor fusions isolated from murine mammary cancer displayed greater tumor initiating function than unfused tumor cells. Detection of the presence of CHCs in peripheral blood therefore may indicate greater tumor initiating capacity and metastatic disease.

[0063] Though exemplified herein in certain embodiments using fusions between epithelial cells and immune cells, it is recognized that CHCs are of broader importance and applicability than that. With the discoveries presented herein, there are enabled methods of detecting CHCs that are derived from other cell and tissue types. Thus, it is contemplated that CHCs may be derived from any tissue or cell type that is undergoing a pathologic state, such as tissue regeneration, tissue (e.g., chronic) inflammation, cancerous transformation, and so forth. In each instance, the resultant associated CHCs are a fusion between a cell of that pathologic tissue and an immunological cell (e.g., a myeloid cell or a lymphoid cell). In view of this, CHCs can be identified by screening for circulating cells that express one or more antigens typically associated with expression in/on an immunological cell concurrent with one or more antigens typically associated with cells/tissues that are associated with the target pathologic state, condition, or disease. Identification of a CHC thus may be diagnostic of the pathologic tissue – for instance, a tumor type or other disease state can be identified by identifying the cell/tissue type that has fused with an immune cell to produce a CHC. Thus, there are contemplated herein diagnostic screening panels that detect CHCs as a circulating cell that expresses one or more (for instance, two, three, four, five, or more) antigens usually associated with an immune cell (for instance, associated with a macrophage, neutrophil, fibroblast, dendritic cell, basophil, mast cell, eosinophil, B cell, NK cell, T cell, or dendritic cell) along with one or more (for instance, two, three, four, five, or more) antigens from a non-immune cell, which panels provide for instance a number of different non-immune cell targets. Such non-immune targets (that is, non-immune cell types that may have fused to form a CHC, and which can be detected using a screening panel) include for instance epithelial cells, nerve cells (such as neurons, glia, astrocytes, oligodendrocytes, microglial cells, ependymal cells, Schwann cells, satellite cells), bone cells (such as osteoclasts osteoblasts, osteocytes), muscle cells (such as skeletal, cardiac and smooth muscle myocytes), skin cells (such as keratinocytes, melanocytes, Langerhans/dendritic cells, Merkel cells), pancreatic cells, intestinal cells, liver cells, cardiac cells, kidney cells, lung cells, adipose cells, thymus cells, breast cells, reproductive/gonadal system cells, cartilage (e.g., chondrocytes), vascular (e.g., endothelial cells) and so forth. Those of ordinary skill in the art will be able to recognize specific antigens that could be used to identify fusions cells (CHCs) that contain any type of a non-immune cell fused to an immune-derived cell; in addition, specific examples are provided herein.

[0064] Provided herein are methods of detecting Circulating Hybrid Cells in a patient, such as a human patient. Examples of this method involve obtaining a blood sample from the human patient; and detecting whether Circulating Hybrid Cells are in the blood sample by (i) contacting the sample with an anti-source-cell (non-immune cell) antibody; and (ii) contacting the sample with an anti-immune cell antibody; wherein specific binding of both antibodies on/to/in the same cell indicates the presence of a Circulating Hybrid Cell, and wherein the term “source cell” refers to a cell originating (sourced from) a tissue undergoing a pathologic condition or state.

[0065] Also provided is a method of diagnosing a solid tumor in a human patient, the method involving obtaining a blood sample from the human patient; and detecting whether Circulating Hybrid Cells are in the blood sample by (i) contacting the sample with an anti-cancer cell antibody (where the specific type of cancer cell antibody will be dependent on the source of the cancer); (ii) contacting the sample with an anti-immune cell antibody; and diagnosing the patient as having a solid tumor wherein specific binding of both antibodies on/to/in the same cell indicates the presence of one or more Circulating Hybrid Cells.

[0066] Also provided is a method of diagnosing metastatic cancer in a human patient, the method involving obtaining a blood sample from the human patient; and detecting whether Circulating Hybrid Cells are in the blood sample by (i) contacting the sample with an anti-(source of tumor) antibody; (ii) contacting the sample with an anti-immune cell antibody; and diagnosing the patient as having a metastatic tumor wherein specific binding of the both antibodies in the same cell indicates the presence of one or more Circulating Hybrid Cells.

[0067] Also provided is a method of diagnosing a solid tumor in a human patient, the method involving obtaining a blood sample from the human patient; detecting whether Circulating Hybrid Cells are in the blood sample by (i) contacting the sample with an anti-(source of tumor) antibody; (ii) contacting the sample with an anti-immune cell antibody; diagnosing the patient as having a solid tumor wherein specific binding of both antibodies in the same cell indicates the presence of one or more Circulating Hybrid Cells; and administering to the patient in need thereof a pharmaceutically effective amount of a cancer treatment. By way of example, such a treatment involve administering a CSF1R inhibitor.

[0068] Also provided is a method of diagnosing metastatic cancer in a human patient, the method involving obtaining a blood sample from the human patient; detecting whether Circulating Hybrid Cells are in the blood sample by (i) contacting the sample with an anti-(source of tumor) antibody; (ii) contacting the sample with an anti-immune cell antibody; diagnosing the patient as having metastatic cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more Circulating Hybrid Cells; and administering to the patient in need thereof a pharmaceutically effective amount of a therapeutic agent (e.g., an anti-cancer agent). For instance, in some embodiments, the therapeutic agent is a CSF1R inhibitor.

[0069] In specific examples of the provided methods, the cancer (or metastatic cancer) is of an epithelial origin. In such embodiments, the anti-(source of tumor) antibody is an anti-epithelial antibody. Within the methods of detecting epithelial-derived CHCs, there is a further embodiment in which the anti-epithelial antibody specifically binds to an epitope on a biomarker selected from EpCAM, E-cadherin, cytokeratin, MUC4, MASPIN, and Glypican-1. In some embodiments, the method include contacting the blood sample with two or more anti-epithelial antibodies selected from the same group.

[0070] In other embodiments, the circulating hybrid cell is of pancreatic epithelial origin and the anti-epithelial antibody binds to an epitope on a biomarker selected from MUC4, MASPIN, and Glypican-1 (GPC1).

[0071] In other embodiments, the CHC is of melanoma origin (that is, the target cell/tissue participating in production of fusions with immune cells is melanoma) and the anti-cancer antibody binds to an epitope on a melanoma biomarker, such as gp100/ NK1B220, MageA1, and MelanA.

[0072] In yet other embodiments, the CHC is of glioblastoma origin (that is, the target cell/tissue participating in production of fusions with immune cells is glioblastoma) and the anti-cancer antibody binds to an epitope on a glioblastoma biomarker, such as GFAP or Nestin.

[0073] In another embodiment, the CHC is of head and neck squamous cell carcinoma (HNSCC) origin (that is, the target cell/tissue participating in production of fusions with immune cells is HNSCC) and the anti-cancer antibody binds to an epitope on a HNSCC biomarker.

[0074] Within the methods of detecting CHCs, there is a further embodiment in which the anti-myeloid antibody specifically binds to an epitope on a biomarker selected from CD45, CD14, CD16, CD11b, CD11c, CD64, CD163, CD68, CD66b, CD71, CD80, CD86, CSF1R, and CCR5. In some embodiments the myeloid epitope (or immune cell epitope) to be detected is selected from an epitope on a biomarker selected from CD14, CD16, CD45, CD64, CD68, CD71, and CCR5. In some embodiments, the method includes contacting the blood sample with two or more anti-myeloid antibodies selected from the same group.

[0075] In some embodiments that involve detecting the presence of cytokeratin with an anti-cytokeratin antibody (for instance, an anti-pan-cytokeratin antibody), the presence of cytokeratin uniformly expressed across the cell in question indicates the presence of a CHC (or identifies that cell as a CHC). Cytokeratin found as a punctate presence only in specific locations or vacuoles within a cell may be used to identify CAMLs. Other methods for distinguishing or identifying CAMLs are provided herein and/or known to those of skill in the art.

[0076] CSF1R inhibitors that may be used in the methods herein include pexidartinib (PLX3397, PLX108-01), PLX7486, LY3022855, MC-CS4, chauranib, SNDX6352, JNJ-40346527, DCC-3014, linifanib (ABT-869), IMC-CS4, AMG820, BLZ945, TK-1258, dovitinib, vatalinib, sunitinib (Sutent®), ARRY-3882, 5-(3-methoxy-4-((4-methoxybenzyl)oxy)benzyl)pyrimidine-2,4-diamine (GW2580), CEP-32496, and 3-((quinolin-4-ylmethyl)amino)-N-(4-(trifluoromethoxy)phenyl) thiophene-2-carboxamide

(OSI-930), as well as cabiralizumab (FPA008), emactuzumab, and other anti-CSF1R antibodies. It is understood that each of the CSF1R inhibitors may be administered in the methods herein in doses and regimens for which they are known to treat the relevant cancer(s) by those skilled in the art.

[0077] Discussion of Terms

[0078] Administration means to provide or give a subject an agent, such as a composition including an active compound (such as a therapeutic compound) by any effective route. Exemplary routes of administration include, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), oral, sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes.

[0079] Antibodies are polypeptides (and polypeptide complexes) including at least a light chain or heavy chain immunoglobulin variable region that specifically recognizes and binds an epitope of an antigen or a fragment thereof (a target of the antibody). The heavy and light chain have a variable region, termed the variable heavy (V_H) region and the variable light (V_L) region. Together, the V_H region and the V_L region are responsible for binding the antigen recognized by the antibody. The V_H and V_L regions can be further segmented into complementarity determining regions (CDRs) and framework regions. The CDRs (also termed hypervariable regions) are the regions within the V_H and V_L responsible for antibody binding.

[0080] The term "antibody" encompasses intact immunoglobulins, as well the variants and portions thereof, such as Fab fragments, Fab' fragments, F(ab)'₂ fragments, single chain Fv proteins ("scFv"), and disulfide stabilized Fv proteins ("dsFv"). A scFv protein is a fusion protein in which a light chain variable region of an immunoglobulin and a heavy chain variable region of an immunoglobulin are bound by a linker. In dsFvs the chains have been mutated to introduce a disulfide bond to stabilize the association of the chains. The term also includes genetically engineered forms such as chimeric antibodies, hetero-conjugate antibodies (such as, bispecific antibodies). See also, Pierce Catalog and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, Ill.); Kuby, Immunology, 3rd Ed., W.H. Freeman & Co., New York, 1997. The term also includes monoclonal antibodies (all antibody molecules have the same V_H and V_L sequences and therefore the same binding specificity) and polyclonal antisera (the antibodies vary in V_H and V_L sequence but all bind a particular antigen, such as CD45.)

[0081] Binding means an association between two substances or molecules, such as the association of an antibody with a polypeptide. Stable binding (or detectable binding) means that a macromolecule (such as an antibody) can bind to another macromolecule (such as a polypeptide) in a manner that can be detected. Binding can be detected by any procedure known to one skilled in the art, such as by physical or functional properties. Binding can also be detected by visualization of a label (such as a fluorescent label) conjugated to one of the molecules.

[0082] Specific binding means that a macromolecule (such as an antibody) binds to members of a class of macromolecules to the exclusion of macromolecules not in that class (binding to non-specific antibody binding macromolecules such as protein A, Fc receptors, etc. is excepted). A class of macromolecules can include macromolecules related by sequence or structure. For example, a pan-cytokeratin specific antibody can bind to some or all cytokeratins to the exclusion of other intermediate filament proteins. An antibody that binds specifically to a particular cytokeratin (such as cytokeratin 8) binds to that cytokeratin to the exclusion of other cytokeratins.

[0083] Molecular, biological or physical attributes that characterize a physiological, cellular, or disease state (or lack of that disease state) and that can be objectively measured to detect or define disease progression or predict or quantify therapeutic responses are referred to as biomarkers (or markers). A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. It may be any molecular structure produced by a cell or organism. A biomarker may be expressed inside any cell or tissue; accessible on the surface of a tissue or cell; structurally inherent to a cell or tissue such as a structural component; secreted by a cell or tissue; produced by the breakdown of a cell or tissue through processes such as necrosis, apoptosis or the like; or any combination of these. A biomarker may be any protein, carbohydrate, fat, nucleic acid, catalytic site, or any combination of these such as an enzyme, glycoprotein, cell membrane, virus, cell, organ, organelle, or any uni- or multi-molecular structure or any other such structure now known or yet to be disclosed whether alone or in combination. A biomarker can also be a discrete cellular entity such as a circulating tumor cell expressing particular cell surface markers including one or more of the markers described herein.

[0084] Biological samples as the phrase is used herein can include any sample from a subject, in which it is possible to detect and/or quantify CHCs. Such samples may include blood, plasma, serum, other blood fractions, lymph, tumor aspirates or biopsies, peritoneal fluids, secretions (such as pancreatic duct secretions, bile duct secretions), urine, and any other biological sample that contains or may be believed to contain immune cells. In particular embodiments, a biological sample includes a test sample derived from a subject that is tested in an assay to measure and/or detect the presence of CHCs, for instance using one or a set of the biomarkers described herein. In particular embodiments, a biological sample includes a test sample derived from a subject that is tested in an assay to diagnose an inflammatory disorder in the subject, or a cancer in the subject. Particular embodiments of "derived from" refer to a biological (or test) sample being obtained from a subject or other source and including any modification to the sample, addition to the sample, or removal from the sample, as long as the presence and/or quantity of CHCs can be determined from the sample using the systems and methods of the present disclosure.

[0085] Cancer is a disease or condition in which abnormal cells divide without control and are able to invade other tissues. Cancer cells spread to other body parts through the blood and lymphatic

systems. Cancer is a term for many diseases. There are more than 100 different types of cancer in humans. Most cancers are named after the organ in which they originate. For instance, a cancer that begins in the colon can be termed a colon cancer. However, the characteristics of a cancer, especially with regard to the sensitivity of the cancer to therapeutic compounds, are not limited to the organ in which the cancer originates. A cancer cell is any cell derived from any cancer, whether *in vitro* or *in vivo*. A malignant tumor is characterized by abnormal or uncontrolled cell growth. Other features often associated with cancer include metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels and suppression or aggravation of inflammatory or immunological response, invasion of surrounding or distant tissues or organs, such as lymph nodes, etc.

[0086] The term “Cancer associated macrophage-like cell (CAML)” refers to a macrophage or monocyte that has phagocytosed a cancer cell, but is found in the peripheral blood. See, for instance, Adams *et al.*, *PNAS USA* 111:3514-3519, 2014. Thus, CAMLs often contain digested tumor cells within them; they are multinucleated.

[0087] A Circulating Hybrid Cell (CHC) is a fusion hybrid between an immune cell (e.g., a myeloid cell or a lymphoid cell) and a cell that is derived from a tissue involved in a pathologic state (such as tissue regeneration, tissue (acute or chronic) inflammation, cancer, and so forth), with the CHC being found in the peripheral blood (that is, in circulation). CHCs may be derived from any tissue or cell type that is undergoing a pathologic state. The resultant pathologic-state-associated CHCs are a fusion between a cell of that pathologic tissue and an immunological cell. By way of example, a CHC that arises from the fusion of an immune cell and a cell of epithelial origin can be referred to as an epithelial-derived CHC, or eCHC. Similarly, a CHC that arises from the fusion of an immune cell and a neoplastic (cancer) cell (no matter what the underlying origin of the neoplastic cell) can be referred to as a neoplastic-derived CHC, or nCHC. Other specific but non-limiting examples of CHCs include those derived from melanoma, or glioblastoma, wherein the resultant CHC is a melanocyte-immune cell or neuronal cell-immune cell fusion, respectively. CHCs may also be considered as tumor cells that have leukocyte genes or leukocyte protein expression. Generally, CHCs are notably smaller than CAMLs. For instance, CHCs associated with pancreatic ductal adenocarcinoma (PDAC) may fall in a size range of from 10-20 μm , whereas the corresponding PDAC-associated CAMLs may fall in range from 30-100 μm . In general, a CHC has greater migratory capacity than do CAMLs, exhibits responsiveness to macrophage ligands, shows greater growth and seeding in metastatic sites, in some instances evades the immune system, and can extravasate.

[0088] As is described herein, it has surprisingly been discovered that CHCs can be identified in subjects that do not have cancer. In some instances, these subjects are undergoing a precancerous disease or disease state, and the identification of CHCs can be used as an early diagnostic for possible progression to cancer. In some instances, the subject has an inflammatory condition, such as a chronic

inflammatory condition. The chronic inflammatory condition may or may not be one that is known to give rise to a higher incidence of cancer. Specific inflammatory disorders include pancreatitis and inflammatory bowel disease.

[0089] CHCs can be identified by screening for circulating cells that express one or more antigens typically associated with expression in/on an immunological cell concurrent with one or more antigens typically associated with cells/tissues that are associated with the originating (source) pathologic state, condition, or disease. Identification of a CHC thus may be diagnostic of the pathologic tissue – for instance, a tumor type or other disease state can be identified by identifying the cell/tissue type that has fused with an immune cell to produce a CHC. Thus, there are contemplated herein diagnostic screening panels that detect CHCs as a circulating cell that expresses one or more (for instance, two, three, four, five, or more) antigens usually associated with an immune cell (for instance, associated with a macrophage, neutrophil, fibroblast, dendritic cell, basophil, mast cell, eosinophil, B cell, NK cell, T cell, or dendritic cell) along with one or more (for instance, two, three, four, five, or more) antigens from a non-immune cell, which panels provide for instance a number of different non-immune cell targets. Such non-immune targets (that is, non-immune cell types that may have fused to form a CHC, and which can be detected using a screening panel) include for instance epithelial cells, skin cells, nerve cells, bone cells, muscle cells, skin cells, pancreatic cells, intestinal cells, liver cells, cardiac cells, kidney cells, lung cells, adipose cells, thymus cells, breast cells, reproductive/gonadal system cells, and so forth. Those of ordinary skill in the art will be able to recognize specific antigens that could be used to identify fusions cells (CHCs) that contain any type of a non-immune cell fused to an immune-derived cell; in addition, specific examples are provided herein.

[0090] CHCs can be identified based upon a cocktail of antibodies (or an antibody panel) that are specific to different states across the disease continuum. For example, pancreatitis, pre-cancer and cancer would contain detectable CHCs that express epithelial cell identity (EpCAM, ECAD, Cytokeratin) and CD45. Cancer- or precancer-derived CHCs express cancer specific proteins (such as, in some embodiments, MUC4, MASPIN, and/or Glypican-1), whereas non-cancerous-derived CHCs (such as pancreatitis-derived CHCs) do not. Moreover, pre-cancer (*i.e.* PanIN, MUC) and pancreatitis can be differentiated by detecting antigens that are expressed only or preferentially in one or the other cell type. For instance, transformation of the epithelium is associated with activation of discrete cell signaling pathways including Tgf β , and therefore these cells would express detectable phosphorylated proteins that indicate pathway activation.

[0091] A “Circulating Tumor Cell (CTC)” is a tumor cell that dissociates from a primary tumor and circulates in the blood, particularly in the peripheral blood, and does not express myeloid markers. CTCs are cells that are present in the circulation of patients with different solid malignancies. In some examples, they are derived from clones of the primary or metastatic tumor and are malignant. CTCs can be considered an independent diagnostic for cancer progression of carcinomas (Beitsch & Clifford,

Am. J. Surg. 180, 446-449, 2000 (breast); Feezor et al, *Ann. Oncol. Surg.* 9, 944-53, 2002 (colorectal); Ghossein et al, *Diagn. Mol. Pathol.* 8, 165-175 (1999) (melanoma, prostate, thyroid); Glaves, *Br. J. Cancer* 48, 665-673, 1983 (lung); Matsunami et al, *Ann. Surg. Oncol.* 10, 171-175, 2003 (gastric)).

[0092] Contacting means placing within an environment where direct physical association occurs, including contacting of a solid with a solid, a liquid with a liquid, a liquid with a solid, or either a liquid or a solid with a cell or tissue, whether *in vitro* or *in vivo*. Contacting can occur *in vitro* with isolated cells or tissue or *in vivo* by administering to a subject.

[0093] As used herein, a “control” (sample or subject) or “normal control” refers to a standard, for instance a subject that is not affected by the disease(s) that are the subject of the comparison for which the control is used. For instance, in embodiments of the current disclosure a control may be a healthy subject (or a sample from such a subject) that is not affected by cancer (for instance, a subject who does not have breast cancer, or does not have melanoma, or does not have a head and neck squamous cell carcinoma, and so forth), or a subject (or sample from such a subject) who does not have an inflammatory response, and so forth. In particular embodiments, a control sample includes plasma, serum, blood, or blood fraction(s) derived from one or more healthy subjects.

[0094] Cytokeratins are structural proteins that belong to the intermediate filament (IF) family of proteins with a number of uses in epithelial cells. At least 23 types of cytokeratin are known with different cytokeratins having been shown to be markers of particular types of cancer and/or cancer activities including cytokeratin 5, cytokeratin 7, cytokeratin 8, cytokeratin 10, cytokeratin 13, cytokeratin 17, and cytokeratin 18 (Moll et al; *Cell* 31: 11-24, 1982; Varadhachary et al, *Cancer* 100, 1776-1785, 2004; Gusterson et al, *Breast Cancer Res* 7, 143-148, 2005; Kanaji et al, *Lung Cancer* 55, 295-302, 2007; Moll et al., *Virchows Arch B Cell Pathol Incl Mol Pathol*, 58, 129-145, 1989; Rugg et al., *J Invest Dermatol*, 127: 574-580, 2007; Betz et al., *Am J Human Genet* 78, 510-519, 2006; Ramaekers et al., *Am J Pathol* 136, 641-655, 1990; Yabushita et al, *Liver* 21, 50-55, 2001; Chatzipantelis et al., *Hepatol Res* 36, 182-187, 2006; Galameau et al, *Exp Cell Res* 313, 179-194, 2007; Ku & Omary, *J Cell Biol* 174, 115-125, 2006; Lau & Chiu, *Cancer Res* 67, 2107-2113, 2007; Linder et al, *Cancer Lett* 214, 1-9, 2004; van Dorst et al, *J Clin Pathol* 51, 679-684, 1988; Maddox et al, *J Clin Pathol* 52, 41-46, 1999; Toyoshima et al, *J Cancer Res Clin Oncol*, 134: 515-521, 2008; Deshpande et al, *Am J Surg Pathol* 28, 1145-1153, 2004; Park et al, *J Korean Med Sci* 22, 621-628, 2007; Barroeta et al, *Endocr Pathol* 17, 225-234, 2006; Ignatiadis et al, *J Clin Oncol* 25, 5194-5202, 2007; Lindberg & Rheinwald, *Am J Pathol* 134, 89-98, 1989; antibodies-online.com/resources/18/624/cytokeratins-in-the-detection-- of-tumors/ last accessed 10 Oct. 2016). Cytokeratins can be detected through the use of specific antibodies such as an antibody to a specific human or mouse cytokeratin or through the use of a pan-cytokeratin antibody that detects more than one, more than three, more than 5, more than 10, more than 12, more than 15, or more than 20 cytokeratin molecules using a single antibody.

[0095] Fluorescent proteins are proteins usually characterized by a barrel structure that allows the protein to absorb light and emit it at a particular wavelength. Fluorescent proteins include green fluorescent protein (GFP), modified GFPs and GFP derivatives, and other fluorescent proteins, such as EGFP, EBFP, YFP, RFP, BFP, CFP, ECFP, mCherry, as well as circularly permuted fluorescent proteins such as cpVenus.

[0096] The term “hybrid” as used herein refers to the product of heterogenous cell fusion, for instance between a tumor or epithelial cell and an immune cell (such as a macrophage; MΦ).

[0097] The phrase immune cell encompasses any and all cells that are part of the immune response, including both myeloid cells and lymphoid cells. Specifically contemplated immune cells (which may be a fusion partner in forming a CHC) include macrophage, neutrophil, fibroblast, dendritic, basophil, mast, eosinophil, B, NK, T, and dendritic cells.

[0098] A label can be any substance capable of aiding a machine, detector, sensor, device, column, or enhanced or unenhanced human eye from differentiating a labeled composition from an unlabeled composition. Labels may be used for any of a number of purposes and one skilled in the art will understand how to match the proper label with the proper purpose.

[0099] Examples of uses of labels include purification of biomolecules, identification of biomolecules, detection of the presence of biomolecules, detection of protein folding, and localization of biomolecules within a cell, tissue, or organism. Examples of labels include: radioactive isotopes or chelates thereof; dyes (fluorescent or non-fluorescent), stains, enzymes, nonradioactive metals, magnets, protein tags, fluorescent proteins, any antibody epitope, any specific example of any of these; any combination between any of these, or any label now known or yet to be disclosed. A label may be covalently attached to a biomolecule or bound through hydrogen bonding, Van Der Waals or other forces. A label may be covalently or otherwise bound to the N-terminus, the C-terminus or any amino acid of a polypeptide or the 5' end, the 3' end or any nucleic acid residue in the case of a polynucleotide.

[0100] One particular example of a label is a small molecule fluorescent dye. Such a label can be conjugated to an antibody such as an antibody that binds a macrophage or tumor cell marker. One of skill in the art would be able to identify and select any appropriate fluorescent dye or combination of fluorescent dyes for use in the disclosed methods.

[0101] Another particular example of a label is a protein tag. A protein tag includes a sequence of one or more amino acids that may be used as a label as discussed above, particularly for use in protein purification. In some examples, the protein tag is covalently bound to the polypeptide. It may be covalently bound to the N-terminal amino acid of a polypeptide, the C-terminal amino acid of a polypeptide or any other amino acid of the polypeptide. Often, the protein tag is encoded by a polynucleotide sequence that is immediately 5' of a nucleic acid sequence coding for the polypeptide such that the protein tag is in the same reading frame as the nucleic acid sequence encoding the polypeptide. Protein tags may be used for all of the same purposes as labels listed above and are well

known in the art. Examples of protein tags include chitin binding protein (CBP), maltose binding protein (MBP), glutathione-S-transferase (GST), poly-histidine (His), thioredoxin (TRX), FLAG™, V5, c-Myc, HA-tag, and so forth. A His-tag facilitates purification and binding to on metal matrices, including nickel matrices, including nickel matrices bound to solid substrates such as agarose plates or beads, glass plates or beads, or polystyrene or other plastic plates or beads. Other protein tags include BCCP, calmodulin, Nus, Thioredoxin, Streptavidin, SBP, and Ty, or any other combination of one or more amino acids that can work as a label described above.

[0102] Another particular example of a label is biotin. Biotin is a natural compound that tightly binds proteins such as avidin or streptavidin. A compound labeled with biotin is said to be `biotinylated`. Biotinylated compounds can be detected with avidin or streptavidin when that avidin or streptavidin is conjugated another label such as a fluorescent, enzymatic, radioactive or other label.

[0103] A macrophage (interchangeably abbreviated herein as MΦ) is a phagocytic, mononuclear, myeloid cell of the immune system. Macrophages can be found in and purified from the peripheral blood, spleen, and lymph nodes. Alternatively, human and mouse macrophage cell lines are available. Macrophages can be identified by cell surface markers including CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, CD163, and others known in the art. Macrophages in stationary form are found in tissues or as a mobile white blood cell, especially at sites of infection. Macrophages have properties similar to metastatic cancer cells.

[0104] Macrophage-tumor cell fusion hybrids are fusions between a tumor cell and a macrophage that occurs when a macrophage and a tumor come in contact with one another. Macrophage-tumor cell fusion hybrids can be isolated from human or animal subjects as described in detail herein. Alternatively, macrophage-tumor cell fusion hybrids can be constructed *in vitro* by contacting primary macrophages or a macrophage cell line with a primary tumor or tumor cell line. Cellular fusions between a macrophage and a tumor cell artificially produced *in vitro* using a reagent that promotes membrane fusion (such as fusogenic or syncytium forming viruses or polypeptides derived from those viruses) are not considered to be macrophage-tumor cell fusion hybrids as described herein.

[0105] Macrophage-tumor cell fusion hybrids (which are a type of CHC) can be identified by the expression of one or more macrophage markers such as those described above and one or more tumor cell markers such as such as one or more of the cytokeratins, CD49F, CD24, CD325, CD44, CD11b, CSF1R, or CD36, as well as others known in the art or yet to be disclosed.

[0106] Metastases refers to the process through which a tumor in a primary site releases single tumor cells that seed other distant organ sites. This phase of tumorigenesis is most fatal (90% mortality). Metastatic disease or metastasis refers to cancer cells that have left the original tumor site and migrate to other parts of the body for example via the bloodstream or lymph system. The "pathology" of cancer includes all phenomena that compromise the well-being of the subject. This includes, abnormal or

uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, neoplasia, premalignancy, malignancy, invasion of surrounding or distant tissues or organs, such as lymph nodes, etc.

[0107] Reference to “myeloid cells” or “myeloid origin” is understood to include macrophage or neutrophil cells or origins.

[0108] A polypeptide is any chain of amino acids, regardless of length or posttranslational modification (such as glycosylation, methylation, ubiquitination, phosphorylation, or the like). The term polypeptide is used interchangeably with peptide or protein, and is used to refer to a polymer of amino acid residues. The term residue refers to an amino acid or amino acid mimetic incorporated in a polypeptide by an amide bond or amide bond mimetic.

[0109] Purification of a cell can be achieved by any method known in the art including by use of methods that involve the use of labeled antibodies that bind cell surface antigens such as fluorescence activated cell sorting, sorting through the use of magnetic beads, or on purification columns. Purification does not require absolute purity (that is the purified cells are exactly 100% cells of the desired type). Instead, a purified population of cells can include at least 60%, 70%, 80%, 90%, 95%, 98%, 99% 99.9%, or 99.99% cells of the desired type.

[0110] The term subject is intended to mean a living multicellular vertebrate organism, a category that includes, for example, mammals and birds. A mammal includes both human and non-human mammals, such as mice. In some examples, a subject is a patient, such as a patient diagnosed with cancer or with an inflammatory disease or condition. In other examples, a subject is a patient yet to be diagnosed.

[0111] The term “therapeutically effective amount” or “pharmaceutically effective amount” refers to an amount that is sufficient to effect treatment, as defined below, when administered to a subject (e.g., a mammal, such as a human) in need of such treatment. The therapeutically or pharmaceutically effective amount will vary depending upon the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. For example, a “therapeutically effective amount” or a “pharmaceutically effective amount” of a compound (such as a CSF1R inhibitor), or a pharmaceutically acceptable salt or co-crystal thereof, is an amount sufficient to modulate expression or activity of the target inhibited by that agent (such as, for instance, CSF1R), and thereby treat a subject (e.g., a human) exhibiting or suffering an indication, or to ameliorate or alleviate the existing symptoms of the indication. For example, a therapeutically or pharmaceutically effective amount may be an amount sufficient to decrease a symptom of a disease or condition responsive to inhibition of CSF1R activity. In other embodiments, the protein being inhibited is another protein that is associated with a cancer.

[0112] The term tumor encompasses all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. Tumor markers include polynucleotides and polypeptides expressed by tumors to a greater extent than they are expressed by non-tumor cells, including cell surface or cytoplasmic or nuclear tumor antigens.

[0113] Examples of types of tumors from which cells can be derived include acute lymphoblastic leukemia; acute myeloid leukemia; adrenocortical carcinoma; AIDS-related cancers; AIDS-related lymphoma; anal cancer; appendix cancer; astrocytoma cerebellar or cerebral; basal cell carcinoma; extrahepatic bile duct cancer; bladder cancer; bone cancer, osteosarcoma/malignant fibrous histiocytoma; brainstem glioma; brain tumor; brain tumor, cerebellar astrocytoma; brain tumor, cerebral astrocytoma/malignant glioma; brain tumor, ependymoma; brain tumor, medulloblastoma; brain tumor, supratentorial primitive neuroectodermal tumors; brain tumor, visual pathway and hypothalamic glioma; breast cancer; bronchial adenomas/carcinoids; Burkitt lymphoma; carcinoid tumor; carcinoid tumor, gastrointestinal; carcinoma of unknown primary; central nervous system lymphoma, primary; cerebellar astrocytoma; cerebral astrocytoma/malignant glioma; cervical cancer; childhood cancers; chronic lymphocytic leukemia; chronic myelogenous leukemia; chronic myeloproliferative disorders; colon cancer; cutaneous T-cell lymphoma; Desmoplastic small round cell tumor; endometrial cancer; ependymoma; esophageal cancer; Ewing's sarcoma in the Ewing family of tumors; extracranial germ cell tumor; extragonadal germ cell tumor; extrahepatic bile duct cancer; eye cancer, intraocular melanoma; eye cancer, retinoblastoma; gallbladder cancer; gastric (stomach) cancer; gastrointestinal carcinoid tumor; gastrointestinal stromal tumor (GIST); germ cell tumor: extracranial, extragonadal, or ovarian; gestational trophoblastic tumor; glioma of the brain stem; glioma cerebral astrocytoma; glioma visual pathway and hypothalamic; gastric carcinoid; hairy cell leukemia; head and neck cancer; heart cancer; hepatocellular (liver) cancer; Hodgkin lymphoma; hypopharyngeal cancer; hypothalamic and visual pathway glioma; intraocular melanoma; islet cell carcinoma (endocrine pancreas); Kaposi sarcoma; kidney cancer (renal cell cancer); laryngeal cancer; leukemias; acute lymphoblastic leukemia (also called acute lymphocytic leukemia); acute myeloid leukemia (also called acute myelogenous leukemia); leukemia, chronic lymphocytic (also called chronic lymphocytic leukemia); chronic myelogenous leukemia (also called chronic myeloid leukemia); hairy cell leukemia; lip and oral cavity cancer; liver cancer (primary); non-small cell lung cancer; small cell lung cancer; lymphomas; AIDS-related lymphoma; Burkitt lymphoma; cutaneous t-cell lymphoma; Hodgkin lymphoma; lymphomas, non-Hodgkin lymphoma (an old classification of all lymphomas except Hodgkin's); primary central nervous system lymphoma; Marcus whittle, deadly disease; malignant fibrous histiocytoma of bone/osteosarcoma; medulloblastoma; melanoma; intraocular (eye) melanoma; Merkel cell carcinoma; mesothelioma; metastatic squamous neck cancer with occult primary; mouth cancer; multiple endocrine neoplasia syndrome; multiple myeloma/plasma cell neoplasm; mycosis fungoides; myelodysplastic syndromes; myelodysplastic / myeloproliferative

diseases; myelogenous leukemia, chronic; myeloid leukemia acute; myeloid leukemia acute; myeloma, multiple (cancer of the bone-marrow); chronic myeloproliferative disorders; nasal cavity and paranasal sinus cancer; nasopharyngeal carcinoma; neuroblastoma; non-Hodgkin lymphoma; non-small cell lung cancer; oral cancer; oropharyngeal cancer; osteosarcoma/malignant fibrous histiocytoma of bone; ovarian cancer; ovarian epithelial cancer (surface epithelial-stromal tumor); ovarian germ cell tumor; ovarian low malignant potential tumor; pancreatic cancer; islet cell pancreatic cancer; paranasal sinus and nasal cavity cancer; parathyroid cancer; penile cancer; pharyngeal cancer; pheochromocytoma; pineal astrocytoma; pineal germinoma; pineoblastoma and supratentorial primitive neuroectodermal tumors; pituitary adenoma; plasma cell neoplasia/multiple myeloma; pleuropulmonary blastoma; primary central nervous system lymphoma; prostate cancer; rectal cancer; renal cell carcinoma (kidney cancer); renal pelvis and ureter, transitional cell cancer; retinoblastoma; rhabdomyosarcoma; salivary gland cancer; sarcoma, Ewing family of tumors; Kaposi sarcoma; soft tissue sarcoma; uterine sarcoma; Sezary syndrome; skin cancer (nonmelanoma); skin cancer (melanoma); skin carcinoma, Merkel cell; small cell lung cancer; small intestine cancer; soft tissue sarcoma; squamous cell carcinoma; squamous neck cancer with occult primary, metastatic; stomach cancer; supratentorial primitive neuroectodermal tumor; cutaneous T-Cell lymphoma; testicular cancer; throat cancer; thymoma; thymoma and thymic carcinoma; thyroid cancer; thyroid cancer; transitional cell cancer of the renal pelvis and ureter; gestational trophoblastic tumor; ureter and renal pelvis, transitional cell cancer; urethral cancer; uterine cancer, endometrial; uterine sarcoma; vaginal cancer; visual pathway and hypothalamic glioma; vulvar cancer; Waldenstrom macroglobulinemia, and Wilms tumor.

[0114] Methods of Use

[0115] Provided herein are methods of identifying, characterizing and/or quantifying the number/concentration of Circulating Hybrid Cells (CHCs) and discrete disease-specific CHC subtypes in a biological sample from a subject (such as blood, plasma, serum, other blood fractions, lymph, tumor aspirates or biopsies, peritoneal fluids, secretions (such as pancreatic duct secretions, bile duct secretions), urine, and any other biological sample that contains or may be believed to contain immune cells). The presence of CHCs provides functional and clinical utility to patients in several ways, including: 1) CHCs possess greater tumor initiating capacity than Circulating Tumor Cells (CTCs); 2) CHC levels provide a prognostic indication of overall survival of cancer patients, such as pancreatic cancer patients, regardless of cancer stage; 3) CHCs can be monitored along with patient treatment and provide a non-invasive indication of tumor growth and tumor response to treatment; 4) subtyping of CHCs can differentiate different disease pathologies across the cancer continuum, including high risk pathologies versus cancer; 5) CHCs can be used to monitor extent of inflammatory conditions (such as inflammatory bowel disease or pancreatitis) in a non-invasive fashion, and to determine if

treatment is effective; 6) CHCs of discrete subtypes can be used to differentiate “pseudo-progression” from true tumor progression that is measured by imaging modalities; and 7) phenotyping (metabolic, mutational, and/or epigenetic) of CHCs can reveal vulnerabilities of the tumor in regard to therapeutic strategies. As such, the levels of CHCs in a patient blood sample can be used as an indicator/predictor of disease aggressiveness that permits medical professionals to stratify patients and direct them into the most efficacious treatment methods.

[0116] As CHCs provide a non-invasive biomarker of treatment response in patients (for instance, in cancer patients), they also provide opportunities to sequence the mutational profile of a patient’s tumor (via the tumor cell that fused to form the CHC) and serve as an early prediction of therapeutic response or disease recurrence, allowing for a tailored (personalized) treatment decisions.

[0117] With the discoveries presented herein, there are enabled methods of detecting CHCs that that are derived from myriad cell and tissue types. CHCs may arise from any tissue or cell type that is undergoing a pathologic state, such as tissue regeneration, tissue inflammation, cancer initiation, chronic inflammation, cancerous transformation, and so forth. In each instance, the resultant CHCs are a fusion between a cell of (originating in) that pathologic tissue and an immunological cell (*e.g.*, a myeloid cell or a lymphoid cell).

[0118] Also enabled are methods of detecting and quantifying both CHCs and CTCs from a sample from a subject, and comparing the number (or relative number) of these two cell types.

[0119] CHCs can be identified by screening for (for instance, using antibody binding to) circulating cells that express one or more antigens typically associated with expression in/on an immunological cell co-incident with one or more antigens typically associated with cells/tissues that are associated with the target pathologic state, condition, or disease. Identification of a CHC may be diagnostic of the pathologic tissue – for instance, a tumor type or other disease state can be identified by identifying the cell/tissue type that has fused with an immune cell to produce a CHC. Based on this, embodiments provide diagnostic screening panels (that is, collections of antibodies or antigen-binding fragments thereof, or nucleic acid probes (directed to DNA or RNA)) that detect CHCs as circulating cells that express (on the same cell) one or more (for instance, two, three, four, five, or more) antigens usually associated with an immune cell (for instance, associated with a macrophage, neutrophil, fibroblast, dendritic cell, basophil, mast cell, eosinophil, B cell, NK cell, T cell, or dendritic cell) along with one or more (for instance, two, three, four, five, or more) antigens from a non-immune cell. In examples of such diagnostic screening panels, the panels include antigens from a number of different non-immune cell types, such that the panel (or a set of panels) can be used to distinguish different types of CHCs. Such non-immune targets (that is, non-immune cell types that may have fused to form a CHC, and which can be detected using a screening panel) include for instance epithelial cells, skin cells, nerve cells, bone cells, muscle cells, skin cells, pancreatic cells, intestinal cells, liver cells, cardiac cells, kidney cells, lung cells, adipose cells, thymus cells, breast cells, reproductive/gonadal system cells,

and so forth. Those of ordinary skill in the art will be able to recognize specific antigens that could be used to identify fusions cells (CHCs) that contain any particular type of a non-immune cell fused to an immune-derived cell; in addition, specific examples are provided herein (including in Tables 2 and 3).

[0120] There are also contemplated herein embodiments in which antibody screening panels are used to stratify samples (and thus, subjects from which the samples are obtained) based on changes in CHC profiles, for instance over time. Such stratification can be progression, for instance disease state progression: from healthy (that is, not having a specific disease) to progression toward the disease, to progression through stages of disease severity, to progression from one disease to another. Other examples of stratification/differentiation that can be made based on differential CHC profiles (that is, detection of different CHCs in a subject, or different amounts or proportions of CHCs) include:

1. Differentiating high risk disease state (such as diabetes, pancreatitis, inflammatory bowel disease, which have epithelial-based CHCs, eCHCs) from corresponding cancer (which has neoplastic CHCs, nCHCs); such differentiation provides opportunities for early cancer detection assays, including detection of subjects with a chronic inflammatory condition or other high-risk pathology, or disease progresses to or transitions into cancer.
2. Diagnoses and prognoses, for instance by:
 - a) Collecting and downstream sequencing of specific CHCs to obtain genomic profiles (for instance, in uveal melanoma, though myriad other specific diseases could benefit from this)
 - b) Subtyping CHCs in patients to identify the types of metastatic lesions the patient is at risk for; this can be based on antibody profile data from single CHCs that exhibit different protein expression (that is, individual CHS within a heterogenous cell population); distinct detection panes that are expected include panels specific subclasses of tumors, or signaling states
3. Distinguishing pseudo progression from actual progression. Pseudo-progression is typically a response seen on imaging that looks like the tumor is expanding after treatment, but the expansion is actually inflammation (*i.e.*, recruited immune cells). Detection of CHCs of discrete subtypes can be used to differentiate such “pseudo-progression” from true tumor progression.
4. Treatment response, for instance by enabling a practitioner to differentiate inflammation- or surgery-derived CHCs from tumor CHCs.
5. Subtyping of CHCs can differentiate different disease pathologies. For example high risk pathologies that precede cancer can be detected, such as pancreatitis or diabetes. Detection of cancer-specific CHCs in patients with epithelial-derived CHCs would allow for monitoring of high risk patients and detection of conversion to cancer at an early stage, for instance.
6. Subtyping of CHCs can provide information for therapeutic response to treatment. An increase in CHCs with stem cell-like feature(s), or high proliferative index, might indicate that the treatment paradigm is not effective.

7. Subtyping of CHCs would identify subsets of CHCs with high metastatic potential. Even though the primary tumor may be responding to therapeutic treatment, dissemination of cells with discrete features may be a harbinger of metastatic disease.
8. Subtyping of CHCs would identify potential new targets for treatment. For example, the data provided in FIGs. 14 & 15 indicate subtype switching. The corresponding biopsy did not harbor a high percentage of ER positive cells in the initial biopsy, but the disseminated CHCs had a high percentage of ER+ suggesting that metastatic tumors that might arise from these cells could be susceptible to tamoxifen. It is predicted that response to targeted therapies can be monitored by examining pathway activation in CHCs (see the pERK data), and detection of activation of bypass signaling pathways will be detectable, indicating treatment resistance. Longitudinal assay of CHCs will provide real-time analyses of therapeutic treatment.

[0121] Also contemplated are embodiments where distinguishing different types (categories) of CHCs allows stratification of disease state, between: pancreatitis, diabetes, Intraductal papillary mucinous neoplasm (IPMN), and Pancreatic ductal adenocarcinoma (PDAC); or between Irritable bowel disease (IBD), Crohn's, and colorectal cancer (CRC); or between chronic obstructive pulmonary disease (COPD), smoker's lung, and lung cancer. Additional stratification is also enabled, including distinguishing subjects with more or less severe disease, for instance more or less severe cancer or cancer progression.

[0122] CHC distinction panels are also contemplated that will permit distinguishing early stage breast cancer and colorectal cancer, including to identify patients with high likelihood of recurrence after treatment versus those that make be cured.

[0123] Rapid detection of CHCs can also be used in assays to detect people that cannot or do not come for disease (e.g., cancer) screening. For instance, people who are below the "traditional" screening age for a disease, such as the age for screening for certain types of cancer (breast cancer, CRC, etc.) or for non-invasive screening in relatively low-risk populations (such as screening for lung cancer in non-smokers). By way of particular example, the rise in lethal CRC in people under 40 indicates that they are people who could beneficially be screened earlier to allow early detection and possible prevention of disease or disease progression.

[0124] The discoveries described herein also enable and functionalize detection of cancer heterogeneity, for instance as exhibited in genetic, epigenetic, mitochondrial, protein, and/or antigenic heterogeneity of CHCs. Heterogeneity is a major issue for cancer treatment. It could be helpful for diagnosis (and even prognosis) to be able to use CHCs to detect heterogenous (or homogenous) cancers. For instance, if a cancer is more homogeneous it might be earlier stage and may be more readily treated with a single agent. If heterogeneous, different profiles may indicate unique combinations of therapeutics (combination therapy) for a more effective eradication of the cancer.

[0125] CHC are more abundant in circulation than circulating tumor cells (CTCs). In metastatic cancer patients, CHCs outnumber CTCs by at least an order of magnitude. Use of CHCs provides the opportunity to FACS-isolate this discrete population and perform -omic analyses. Circulating cells provide opportunities for DNA, RNA and protein analyses, whereas cell free DNA (cf-DNA) has limitations in that it can only provide DNA, is diminishing low in circulation, and analyses of cellular heterogeneity is not feasible. Such -omic analysis of CHCs enables characterization of the underlying cancer from which the fusion cells arose; for instance, such characterization enables identification of markers (such as cancer markers) that can be correlated with viable treatment(s) for the underlying cancer as well as metastases thereof.

[0126] Biomarkers in the peripheral blood (such as the herein described CHCs) provide an opportunity to monitor the disease state of solid tumor over time in a non-invasive fashion. Detection of CHCs also provides non-invasive biomarker assays for temporal analyses of cancer treatment, response and recurrent disease. Subtyping panels of antibodies are developed for early detection of cancer in high risk patient cohorts. Subtyping panels of antibodies can be used to detect cancer in people with stage I cancer.

[0127] In addition to identifying appropriate treatment(s), phenotyping of CHCs with discrete antibody panels (including those provided herein) also can be used to identify treatment resistance.

[0128] Without intending to be limited in any way, the following specific types of CHCs are believed to be useful in methods described herein:

[0129] *Cancer-derived hybrids* (neoplastic cell-derived CHC, nCHC), are fusions between any neoplastic cell and an immune cell (such as specifically a macrophage, a neutrophil, or a fibroblast), or a neoplastic cell fused with any blood cell. In the context of nCHCs, the term “neoplastic” encompasses any epithelial-based cancer (e.g., breast cancer, lung, esophageal, head and neck, colorectal cancer, pancreatic cancer, prostate cancer) as well as brain cancers (in adult and pediatric subjects), melanoma (both cutaneous and uveal), and sarcoma.

[0130] *Epithelial-derived hybrids* (epithelial derived CHC, eCHC) are fusions between any epithelial cell (whether or not it is neoplastic) and an immune cell such as specifically a macrophage, a neutrophil, or a fibroblast), or an epithelial cell fused with any blood cell. Within this category there are more tissue-specific hybrids, such as tissue specific hybrids derived from tissues in the brain and nervous system: ***Neuronal-derived hybrids***, that are a fusion between any neuronal cell and an immune cell.

[0131] By way of example, Table 1 provides a non-limiting list of types of cells (from a tissue or cell type involved in the pathology) that can be found fused in CHCs in the listed representative diseases/conditions/pathologies. Any of these “source” cells can be found fused with any type of immune cell (such as macrophage or neutrophils) to form a CHC, as described herein.

[0132] Table 1: Representative “Source” Cells with Representative Pathologies

Disease/Condition/Pathology	Cell Types (“Source” cell types)
Diabetes	Epithelial cell
Pancreatitis	Epithelial cell
Pancreatic cancer	Neoplastic epithelial cell
Inflammatory bowel disease (e.g., Crohn’s Disease, Ulcerative colitis)	Epithelial cell
Breast cancer	Neoplastic epithelial cell
Lung cancer	Neoplastic epithelial cell
Colorectal cancer (CRC)	Neoplastic epithelial cell
Prostate cancer	Neoplastic prostate cell
Melanoma	Melanoma cell
Glioma	Neuronal cell
Radiation exposure	Epithelial cell
Obesity	Epithelial cell
Aging	Epithelial cell
Alcohol use/abuse	Epithelial cell

[0133] Table 2 represents panels of markers (and antibodies that recognize each), which define or subtype CHCs, in order for them to be used as biomarkers of disease. For epithelial cancers, general “CHC identity” markers define CHC as either epithelial-derived or cancer-derived and are identified by one or more of: CD45, CD68, EpCAM, ECAD, CK, MUC4, and/or MASPIN. Beyond disease state definition, CHCs are cancer cells disseminated from the tumor and therefore reflect the key features of malignant cells. These feature can be identified in CHCs to provide a prognostic outlook for the tumor, specifically if the CHC has stem cell characteristics as defined by co-expression of proteins associated with stem cell states (such as CD44, Ki67, pHH3, CD166, Sox9, and/or PDX1) and have a high propensity for migration (such as Vimentin, MMP11, and/or ECAD). In addition, tumor cells are receptive to cell signaling pathway cues and activation of discrete pathways is defined by the increase in phosphor-protein expression. CHCs retain the tumor’s activated cell signaling state (see FIG. 13), and therefore cell signaling state can be defined by the markers in Table 2 (“cell signaling pathway activation” markers). This is of importance because tumors are often treated with inhibitors of discrete cell signaling pathways. In these cases CHCs and their cell signaling activation state can be used to determine if a particular targeted therapy is effective, or if the tumor has acquired additional targetable vulnerabilities.

[0134] Thus, by way of example, Table 2 provides non-limiting lists of markers useful in determining the cell subtype and signaling pathway status of a cell, including a cell found fused in a CHC. Any of

these markers, or combinations of these markers, can be used to characterize CHCs, as described herein (see, for instance, FIG. 13 and accompanying text). The representative antibody clones listed with for each protein marker are available commercially; the source company and catalog number are provided in the Table. One of ordinary skill in the art will recognize that myriad other antibodies are available, or can be made using well known techniques, that can be used to detect the presence of these markers.

[0135] Table 2: Representative Cell-Subtype and Signalling Marker List

	Marker Protein	Representative Ab Clone Supplier: Catalog #		Marker Protein	Representative Ab Clone Supplier: Catalog #
CHC identity	CD45	EP322Y abcam: ab214437	Cell signaling pathway activation	KRAS	Polyclonal AbCam: ab180772
	CD68	KP1 Biolegend: 916104		pKRAS	Polyclonal CST: 2231S
	EpCAM	E144 abcam: ab32392		RAF	EPR16208 AbCam: ab200653
	ECAD	EP700Y abcam: ab201499		pRAF	Polyclonal CST: 9421
	panCK	AE3 InVitrogen: MA 1-92041		MEK 1/2	L38C12 CST: 4694
	MUC4	8G7 Millipore: MABT395		pMEK 1/2	EPR3338 AbCam: ab96379
	MASPIN	Polyclonal Sigma Aldrich: HPA019025		MAPK	EPR6528(N) AbCam: ab205926
Stem/ Development	CD44	EPR3094 abcam: ab218582		pMAPK	A16016A BioLegend: 690202
	Ki67	D3B5 CST: 12075		ERK 1/2	Polyclonal AbCam: ab17942
	pHH3	D2C8 CST: 3465		pERK1/2	197G2 CST: 13148
	CD166	Polyclonal R&D Sys.: AF1172		PI3K	EPR18702 AbCam: ab191606
	Sox9	Polyclonal Millipore: ABE571		pPI3K	EP383Y AbCam: ab40776
	PDX1	Polyclonal abcam: ab47383		pS6RP	D57.2.2E CST: 3985
Migratory	Vimentin	D21H3 CST: 9854		AKT	40D4 CST: 2920S
	MMP11	Polyclonal Sigma Aldrich: HPA068864		pAKT	D9E CST: 4075
	ECAD	EP700Y abcam: ab201499		pSTAT3	D3A7 CST: 9145S
				TGFβ	TB21 AbCam: ab27969
				SMAD4	D3R4N CST: 46535
				EGFR	D38B1 CST: 5108S
				pEGFR	D7A5 CST: 3777S

[0136] Cancers in distinct organ sites are defined by their discrete protein expression in many instances. CHCs harbor organ specific identity (see Table 3, for instance). This is significant for cases where cancer is detected but the originating organ site is unknown, and/or where the tumor type or tumor subtype/genotype is not fully characterized. Appropriate treatment can be selected based on this knowledge. CHCs could be used to identify the tissue-specificity of the cancer. By way of example, Table 3 provides non-limiting lists of markers useful in determining the organ from which a cell originates, or the cell type, and in some instances the type of cancer or disease with which the marker is associated. Any of these markers, or combinations of these markers, can be used to characterize CHCs, as described herein. One of ordinary skill in the art will recognize that myriad antibodies are available commercially, or can be made using well known techniques, that can be used to detect the presence of these markers. In addition, markers in Table 3 can be used in panels for identification of, for instance, cell type and/or cell source when CHCs are analyzed.

[0137] Table 3: Organ-Specific and Cancer-Linked Marker List

	Protein	Category		Protein	Category
Lung	UCHL	Cancer	CRC**	PTPRO	Cancer
	SFTPb	Cancer		TANGO6	Cancer
	MUC4	Cancer		TMEM211	Cancer
	WDR72	Cancer		APOBEC1	Cancer
	RAGE	Cancer		CST5	Cancer
	EGFR	Cancer		PRSS22	Cancer
PDAC*	ITGA3	Cancer		DPEP1	Cancer
	COL17A1	Cancer		PCCA	Cancer
	MUC4	Cancer		CHST4	Cancer
	MMP11	Cancer		NOX1	Cancer
	MUC16	Cancer		CD166	Cancer
	AHNAK2	Cancer		DCAMKL1	Cancer
	AGAP1	Cancer		TROP2	Cancer, colitis
	DAS-1	IPMN	Epithelial	ZO1	Epithelial
	LEMD1	Cancer		EpCAM	Epithelial
	MSLN	Cancer		ECAD	Epithelial
	KLCK10	Cancer		pan-Cytokeratin	Epithelial
	TFF1	Cancer	Non-epithelial	GFAP	Glioblastoma
	PDX1	Cancer		Nestin	Glioblastoma
	MASPIN	Cancer		gp100	Melanoma
	GPC1	Cancer		MageA1	Melanoma
Breast	HER2	Cancer		MelanA	Melanoma
	ER	Cancer		TYR	Melanoma
	AR	Cancer			

*PDAC: Pancreatic ductal adenocarcinoma; **CRC: colorectal cancer.

[0138] By way of example, macrophages (or macrophage character in a fusion cell, such as a CHC) can be identified by the presence of one or more cell surface markers, such as CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, CD163, and others known in the art.

[0139] Treatments, Pharmaceutical Compositions and Administration Formulations

[0140] With the discovery of CHCs described herein, it is now possible to tailor or modify treatment of diseases and conditions (such as diseases and conditions involving inflammatory response, and particularly cancers) based on the identification and/or characterization of CHCs in a subject. In a very general sense, identification and/or characterization of CHCs (or populations of heterogenous CHCs) from a subject permits identification of the type of disease or cancer that resulted in generation of the CHC (by characterizing the fusion cell(s)), enables selection of treatment(s) for that subject. For instance, identification of which type of cancer gave rise to cells that have fused to form CHCs permits election of anti-cancer treatment(s) based on conventional cancer treatment(s) for that cancer. By way of example, estrogen receptor therapy may be selected as appropriate if a subject is found to have a CHC population that is ER+ (see, for instance, FIG. 14); EGFR targeted therapy may be selected as appropriate if a CHC population expresses EGFR; and so forth. Likewise, it is believed that metastatic tumor(s) arising from (seeded from) circulating CHCs will be susceptible to treatments based on characteristics the originating CHC(s). Thus, antigen expression in a CHC (or population of CHCs) can be characterized similarly to characterization of tumor biopsies, to customize anti-cancer treatment for a subject.

[0141] In some instances, in some instances the CHC population in a subject may provide information that is not available through analysis of the originating tumor alone. By way of example, data presented in FIG. 14 indicated that the patient could respond to estrogen receptor therapy because the CHCs are ER+ (the majority of CHCs expressed ER). However the patient's initial primary tumor biopsy indicated the sample was ER-negative, and thus she was treated with a therapy that is used for triple negative patients. She did not respond to that therapy. However, the CHC analysis would have suggested at least some of the tumor load would be susceptible to therapy(s) used in ER-positive situations.

[0142] It is noted that CHC populations can be quite heterogenous. By way of example, CSF1R positivity has been detected in about 20% of the CHC population from a single subject. Based on this, the subject could be treated with anti-CSF1R antibodies, but it is important to know that this would target only a subset of cells. Thus, analysis of a CHC population (and its heterogeneity) from a sample from a subject can be used to tailor treatment to the best fit the array of cell populations detected.

[0143] In addition, the art recognizes various tumor type specific treatments. If a tumor in a subject is of unknown origin, characterization of CHCs from that subject could help define the cancer tumor type

and thereby influence selection of anti-cancer treatments even for the primary tumor. For instance, melanoma is most effectively treated with checkpoint inhibitors, whereas colorectal cancer is effectively treated with FOLFOX chemotherapy, and so forth. Only colorectal cancers that have mismatched repair defects respond well to checkpoint inhibition. Characterization of CHCs can be used to define the status of the source tumor and/or resultant metastases (i.e. for CRC, to see if they are mismatch repair deficient). Such analysis may rely not on detection of expressed proteins using antibodies, but by looking at DNA in the CHCs. Even so, an antibody panel may still be employed in isolation of the CHCs so that their DNA could be analyzed.

[0144] Provided herein are methods and compositions for treatment, for instance treatment of cancer, and/or treatment of inflammatory diseases or conditions. Appropriate active/therapeutic compounds for such treatments are discussed herein, and additional appropriate active compounds are known to those of ordinary skill in the art.

[0145] When formulated in a pharmaceutical composition, a therapeutic compound can be admixed with a pharmaceutically acceptable carrier or excipient. As used herein, the phrase “pharmaceutically acceptable” refers to molecular entities and compositions that are generally believed to be physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human or veterinary subject.

[0146] The term “pharmaceutically acceptable derivative” as used herein means any pharmaceutically acceptable salt, solvate or prodrug, e.g. ester, of the desired active agent, which upon administration to the recipient is capable of providing (directly or indirectly) the desired active agent, or an active metabolite or residue thereof. Such derivatives are recognizable to those skilled in the art, without undue experimentation. Nevertheless, reference is made to the teaching of Burger’s Medicinal Chemistry and Drug Discovery, 5th Edition, Vol 1: Principles and Practice. Pharmaceutically acceptable derivatives include salts, solvates, esters, carbamates, and phosphate esters.

[0147] While it is possible to use a composition for therapy as is, it may be preferable to administer it in a pharmaceutical formulation, e.g., in admixture with a suitable pharmaceutical excipient, diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice. Accordingly, in one aspect, pharmaceutical composition or formulation includes at least one active composition, or a pharmaceutically acceptable derivative thereof, in association with a pharmaceutically acceptable excipient, diluent and/or carrier. The excipient, diluent and/or carrier is “acceptable” in the sense of being compatible with the other ingredient(s) of the formulation and not significantly deleterious to the recipient thereof.

[0148] Any composition formulation disclosed herein can advantageously include any other pharmaceutically acceptable carriers which include those that do not produce significantly adverse, allergic, or other untoward reactions that outweigh the benefit of administration, whether for research, prophylactic and/or therapeutic treatments. Exemplary pharmaceutically acceptable excipients,

diluents, and carriers for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington: The Science and Practice of Pharmacy. Lippincott Williams & Wilkins (A.R. Gennaro edit. 2005), and in n Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990. Moreover, formulations can be prepared to meet sterility, pyrogenicity, general safety and purity standards as required by United States FDA Office of Biological Standards and/or other relevant foreign regulatory agencies. The pharmaceutical excipient(s), diluent(s), and carrier(s) can be selected with regard to the intended route of administration and standard pharmaceutical practice.

[0149] Such pharmaceutical formulations may be presented for use in a conventional manner with the aid of one or more suitable excipients, diluents, and carriers. Pharmaceutically acceptable excipients assist or make possible the formation of a dosage form for a bioactive material and include diluents, binding agents, lubricants, glidants, disintegrants, coloring agents, and other ingredients. Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, ascorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used. An excipient is pharmaceutically acceptable if, in addition to performing its desired function, it is non-toxic, well tolerated upon ingestion, and does not interfere with absorption of bioactive materials.

[0150] Exemplary generally used pharmaceutically acceptable carriers include any and all bulking agents or fillers, solvents or co-solvents, dispersion media, coatings, surfactants, antioxidants (e.g., ascorbic acid, methionine, vitamin E), preservatives, isotonic agents, absorption delaying agents, salts, stabilizers, buffering agents, chelating agents (e.g., EDTA), gels, binders, disintegration agents, and/or lubricants.

[0151] Exemplary buffering agents include citrate buffers, succinate buffers, tartrate buffers, fumarate buffers, gluconate buffers, oxalate buffers, lactate buffers, acetate buffers, phosphate buffers, histidine buffers and/or trimethylamine salts.

[0152] Exemplary preservatives include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides, hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

[0153] Exemplary isotonic agents include polyhydric sugar alcohols including trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol, or mannitol.

[0154] Exemplary stabilizers include organic sugars, polyhydric sugar alcohols, polyethylene glycol; sulfur-containing reducing agents, amino acids, low molecular weight polypeptides, proteins, immunoglobulins, hydrophilic polymers, or polysaccharides.

[0155] The pharmaceutical compositions can be formulated for administration in any convenient way for use in human or veterinary medicine. Exemplarily modes of administration are discussed herein.

[0156] A therapeutically effective amount means the amount of a compound that, when administered to an animal subject or treating a state, disorder or condition, is sufficient to effect such state, disorder, or condition. The therapeutically effective amount will vary depending on the compound, the disease and its severity and the age, weight, physical condition and responsiveness of the mammal to be treated. In certain cases, the phrase therapeutically effective amount is used to mean an amount or dose sufficient to modulate, *e.g.*, increase or decrease a desired activity *e.g.*, by 10 percent, by 50 percent, or by 90 percent. Generally, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the subject following a therapeutic regimen involving one or more therapeutic agents. The concentration or amount of the active ingredient depends on the desired dosage and administration regimen, as discussed below. Suitable dosages may range from 0.01 mg/kg to 100 mg/kg of body weight per day, week, or month.

[0157] The actual dose amount administered to a particular subject can be determined by a physician, veterinarian, or researcher taking into account parameters such as physical, physiological and psychological factors including target, body weight, stage of cancer, the type of cancer, previous or concurrent therapeutic interventions, idiopathy of the subject, and route of administration.

[0158] Exemplary doses can include 0.05 mg/kg to 5.0 mg/kg of the active compounds (drugs) disclosed herein. The total daily dose can be 0.05 mg/kg to 30.0 mg/kg of an agent administered to a subject one to three times a day, including administration of total daily doses of 0.05-3.0, 0.1-3.0, 0.5-3.0, 1.0-3.0, 1.5-3.0, 2.0-3.0, 2.5-3.0, and 0.5-3.0 mg/kg/day of administration forms of a drug using 60-minute oral, intravenous or other dosing. In one particular example, doses can be administered QD or BID to a subject with, *e.g.*, total daily doses of 1.5 mg/kg, 3.0 mg/kg, or 4.0 mg/kg of a composition with up to 92-98% wt/v of the compounds disclosed herein.

[0159] Additional useful doses can often range from 0.1 to 5 µg/kg or from 0.5 to 1 µg /kg. In other examples, a dose can include 1 µg/kg, 10 µg/kg, 20 µg /kg, 40 µg/kg, 80 µg/kg, 200 µg/kg, 0.1 to 5 mg/kg or from 0.5 to 1 mg/kg. In other examples, a dose can include 1 mg/kg, 10 mg/kg, 20 mg/kg, 40 mg/kg, 80 mg/kg, 200 mg/kg, 400 mg/kg, 450 mg/kg, or more.

[0160] Therapeutically effective amounts can be achieved by administering single or multiple doses during the course of a treatment regimen (*e.g.*, hourly, every 2 hours, every 3 hours, every 4 hours, every 6 hours, every 9 hours, every 12 hours, every 18 hours, daily, every other day, every 3 days, every 4 days, every 5 days, every 6 days, weekly, every 2 weeks, every 3 weeks, or monthly).

[0161] A therapeutically effective amount of the desired active agent can be formulated in a pharmaceutical composition to be introduced parenterally, transmucosally (*e.g.*, orally, nasally, or rectally), or transdermally. In some embodiments, administration is parenteral, for instance, via intravenous injection, or intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration.

[0162] In another embodiment, the active ingredient can be delivered in a vesicle, in particular a liposome (see Langer, *Science*, 1990;249:1527-1533; Treat et al, in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss: New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327).

[0163] The effective amounts of compounds containing active agents include doses that partially or completely achieve the desired therapeutic, prophylactic, and/or biological effect. The actual amount effective for a particular application depends on the condition being treated and the route of administration. The effective amount for use in humans can be determined from animal models. For example, a dose for humans can be formulated to achieve circulating and/or gastrointestinal concentrations that have been found to be effective in animals.

[0164] For administration, therapeutically effective amounts (also referred to herein as doses) can be initially estimated based on results from *in vitro* assays and/or animal model studies. Such information can be used to more accurately determine useful doses in subjects of interest. Particularly useful pre-clinical tests include measure of cell growth, cell death, and/or cell viability. In particular, measurement of (T) cell exhaustion may be beneficial.

[0165] The pharmaceutical compositions may also include other biologically active compounds.

[0166] Compositions can also be administered with anesthetics including ethanol, bupivacaine, chloroprocaine, levobupivacaine, lidocaine, mepivacaine, procaine, ropivacaine, tetracaine, desflurane, isoflurane, ketamine, propofol, sevoflurane, codeine, fentanyl, hydromorphone, marcaine, meperidine, methadone, morphine, oxycodone, remifentanyl, sufentanyl, butorphanol, nalbuphine, tramadol, benzocaine, dibucaine, ethyl chloride, xylocaine, and/or phenazopyridine.

[0167] In particular embodiments, the compositions disclosed herein can be used in conjunction with other cancer treatments, such as chemotherapeutic agents, radiation therapy, and/or immunotherapy. The compositions described herein can be administered (except as discussed regarding checkpoint inhibition therapy, which is administered subsequent to cessation of the preconditioning) simultaneously with or sequentially with another treatment within a selected time window, such as within 10 minutes, 1 hour, 3 hour, 10 hour, 15 hour, 24 hour, or 48 hour time windows or when the complementary treatment is within a clinically-relevant therapeutic window.

[0168] The compositions described herein can be administered by, a variety of routes.

[0169] For injection, compositions can be made as aqueous solutions, such as in buffers such as Hanks' solution, Ringer's solution, or physiological saline. The solutions can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the composition can be in lyophilized and/or powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

[0170] Compositions can also be formulated for oral administration. For ingestion, compositions can take the form of tablets, pills, lozenges, sprays, liquids, and capsules formulated in conventional

manners. Ingestible compositions can be prepared using conventional methods and materials known in the pharmaceutical art. For example, U.S. Pat. Nos. 5,215,754 and 4,374,082 relate to methods for preparing swallowable compositions. U.S. Pat. No. 6,495,177 relates to methods to prepare chewable supplements with improved mouthfeel. U.S. Pat. No. 5,965,162, relates to compositions and methods for preparing comestible units which disintegrate quickly in the mouth.

[0171] Ingestible compositions may have a shape containing no sharp edges and a smooth, uniform and substantially bubble free outer coating. Coatings of ingestible compositions can be derived from a polymeric film. Such film coatings reduce the adhesion of the compositions to the inner surface of the mouth and can aid in masking potential unpleasant tastes. Coatings can also protect the compositions from atmospheric degradation. Exemplary polymeric films include vinyl polymers, cellulose, acrylates and methacrylates, natural gums and resins such as zein, gelatin, shellac and acacia. Other common excipients used in ingestible compositions include sucrose, fructose, lactose, glucose, lycasin, xylitol, lactitol, erythritol, mannitol, isomaltose, dextrose, polydextrose, dextrin, compressible cellulose, compressible honey, compressible molasses, fondant or gums, vegetable oils, animal oils, alkyl polysiloxanes, corn starch, potato starch, pre-gelatinized starches, stearic acid, calcium stearate, magnesium stearate, zinc stearate, benzoic acid, and colorants.

[0172] For administration by inhalation (e.g., nasal or pulmonary), the compositions can be formulated as aerosol sprays for pressurized packs or a nebulizer, with the use of suitable propellants, e.g. dichlorodifluoromethane, trichlorofluoromethane, or dichlorotetrafluoroethane.

[0173] Screening, Detection, Panels, and Arrays

[0174] With the provision herein of the link between CHCs and disease conditions, there are enabled methods of using detection molecules (such as antibodies) specific for markers that detect and/or identify/characterize CHCs in a sample from a subject. Such methods include diagnosis, screening, selecting or sorting subjects for treatment (including tailoring treatments to subjects) and so forth, as described herein. These methods can be carried out using individual markers, or combinations of markers, including panels and arrays of multiple markers. Such panels and arrays may include sets of detection molecules that bind specifically to markers that are indicative of the mere presence of a CHC (of any type), characterization of the cells that fused to result in a CHC, characterization of the signalling pathway(s) active in a CHC, the tissue source of the cell(s) fused to form a CHC, and/or the type of diseased tissue (e.g., the type of cancer) from which arose one of the cells that fused

to form the CHC. Table 2, for instance, provides examples of markers that identify a CHC, that provide information on the stem cell property(s) of the CHC (which provides insight regarding possible metastatic potential, for instance), and that provide information on the cell signalling pathway(s) that are activated in a CHC or other cell being analyzed. Table 3 provides, for instance, example markers that can be used to identify the tissue source (or diseased tissue source) of a cell or CHC. Panels and arrays that include detection molecules corresponding to fewer than all of the listed markers for any category (for instance, markers specific for lung tissue) are contemplated. Similarly, panels and arrays that include at least one detection molecule specific for a marker for each type of tissue, or for at least two types of tissue, are envisioned; such panels and arrays can be used to distinguish the tissue (or tumor) source of CHC(s) in a sample, including possibly characterizing heterogeneous populations of CHCs from a subject.

[0175] The presence or quantity of biomarker(s) or panels of markers, as indicated herein for particular markers, can be assessed by comparing a value to a relevant reference level. For example, the quantity of one or more markers can be indicated as a value. The value can be one or more numerical values resulting from the assaying of a sample, and can be derived, e.g., by measuring level(s) of the marker(s) in the sample by an assay performed in a laboratory, or from a dataset obtained from a provider such as a laboratory, or from a dataset stored on a server. The markers disclosed herein in many embodiments are protein marker(s), though nucleic acid markers (for instance, a gene encoding a protein marker) are also contemplated.

[0176] In the broadest sense, the value may be qualitative or quantitative. As such, where detection is qualitative, the systems and methods provide a reading or evaluation, e.g., assessment, of whether or not the marker is present in the sample being assayed. In yet other embodiments, the systems and methods provide a quantitative detection of whether the marker is present in the sample being assayed, i.e., an evaluation or assessment of the actual amount or relative abundance of the marker in the sample being assayed. In such embodiments, the quantitative detection may be absolute or, if the method is a method of detecting two or more different markers in a sample, relative. As such, the term “quantifying” when used in the context of quantifying a marker in a sample can refer to absolute or to relative quantification; it is recognized that the quantity of a marker can be used as correlative to the number of cells in/on which that marker is expressed. Thus, quantification may simply

refer to counting the number of cells that are labeled with an agent that binds specifically to a marker expressed on/in or associated with that cell type.

[0177] Absolute quantification can be accomplished by inclusion of known concentration(s) of one or more control markers and referencing, e.g., normalizing, the detected level of the marker with the known control markers (e.g., through generation of a standard curve). Alternatively, relative quantification can be accomplished by comparison of detected levels or amounts between two or more different markers to provide a relative quantification of each of the two or more markers, e.g., relative to each other. The actual measurement of values of the markers can be determined at the protein or nucleic acid level using any method known in the art. In some embodiments, a marker is detected by contacting a sample with reagents (e.g., antibodies or nucleic acid primers), generating complexes of reagent and marker(s), and detecting the complexes.

[0178] The reagent can include a probe. A probe is a molecule that binds a target, either directly or indirectly. The target can be a marker, a fragment of the marker, or any molecule that is to be detected. In embodiments, the probe includes a nucleic acid or a protein. As an example, a protein probe can be an antibody. An antibody can be a whole antibody or a fragment of an antibody. A probe can be labeled with a detectable label. Examples of detectable labels include fluorsceners, chemiluminescers, dyes, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, enzyme subunits, metal ions, and radioactive isotopes.

[0179] "Protein" detection includes detection of full-length proteins, mature proteins, pre-proteins, polypeptides, isoforms, mutations, post-translationally modified proteins and variants thereof, and can be detected in any suitable manner.

[0180] Those skilled in the art will be familiar with numerous specific immunoassay formats and variations thereof which can be useful for carrying out the methods disclosed herein. See, e.g., E. Maggio, Enzyme-Immunoassay (1980), CRC Press, Inc., Boca Raton, Fla; and U.S. Pat. Nos. 4,727,022; 4,659,678; 4,376,110; 4,275,149; 4,233,402; and 4,230,797.

[0181] Antibodies can be conjugated to a solid support suitable for a diagnostic assay (e.g., beads such as protein A or protein G agarose, microspheres, plates, slides or wells formed from materials such as latex or polystyrene) in accordance with known techniques, such as passive binding. Antibodies can be conjugated to detectable labels or groups such as radiolabels (e.g., ³⁵S, ¹²⁵I, ¹³¹I), enzyme labels (e.g., horseradish peroxidase, alkaline phosphatase), and fluorescent labels (e.g., fluorescein, Alexa, green fluorescent protein, rhodamine) in accordance with known techniques.

[0182] Examples of suitable immunoassays include immunoblotting, immunoprecipitation, immunofluorescence, chemiluminescence, electro-chemiluminescence (ECL), and/or enzyme-linked immunoassays (ELISA).

[0183] Antibodies may also be useful for detecting post-translational modifications of markers. Examples of post-translational modifications include tyrosine phosphorylation, threonine phosphorylation, serine phosphorylation, citrullination, and glycosylation (e.g., O-GlcNAc). Such antibodies specifically detect the phosphorylated amino acids in marker proteins of interest. These antibodies are well-known to those skilled in the art, and commercially available. Post-translational modifications can also be determined using metastable ions in reflector matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). See Wirth *et al.*, *Proteomics* 2002, 2(10):1445-1451.

[0184] Up- or down-regulation of genes (which are a type of marker) also can be detected using, for example, cDNA arrays, cDNA fragment fingerprinting, cDNA sequencing, clone hybridization, differential display, differential screening, FRET detection, liquid microarrays, PCR, RT-PCR, quantitative real-time RT-PCR analysis with TaqMan assays, molecular beacons, microelectric arrays, oligonucleotide arrays, polynucleotide arrays, serial analysis of gene expression (SAGE), and/or subtractive hybridization.

[0185] As an example, Northern hybridization analysis using probes that specifically recognize one or more marker sequences can be used to determine gene expression. Alternatively, expression can be measured using RT-PCR; e.g., polynucleotide primers specific for the differentially expressed marker mRNA sequences reverse-transcribe the mRNA into DNA, which is then amplified in PCR and can be visualized and quantified. Marker RNA can also be quantified using, for example, other target amplification methods, such as transcription mediated amplification (TMA), strand displacement amplification (SDA), and nucleic acid sequence based amplification (NASBA), or signal amplification methods (e.g., bDNA), and the like. Ribonuclease protection assays can also be used, using probes that specifically recognize one or more marker mRNA sequences, to determine gene expression.

[0186] Further hybridization technologies that may be used are described in, for example, U.S. Pat. Nos. 5,143,854; 5,288,644; 5,324,633; 5,432,049; 5,470,710; 5,492,806; 5,503,980; 5,510,270; 5,525,464; 5,547,839; 5,580,732; 5,661,028; and 5,800,992 as well as WO 95/21265; WO 96/31622; WO 97/10365; WO 97/27317; EP 373 203; and EP 785 280.

[0187] Proteins and nucleic acids can be linked to chips, such as microarray chips. See, for example, U.S. Pat. Nos. 5,143,854; 6,087,112; 5,215,882; 5,707,807; 5,807,522; 5,958,342;

5,994,076; 6,004,755; 6,048,695; 6,060,240; 6,090,556; and 6,040,138. Microarray refers to a solid carrier or support that has a plurality of molecules bound to its surface at defined locations. The solid carrier or support can be made of any material. As an example, the material can be hard, such as metal, glass, plastic, silicon, ceramics, and textured and porous materials; or soft materials, such as gels, rubbers, polymers, and other non-rigid materials. The material can also be nylon membranes, epoxy-glass and borofluorate-glass. The solid carrier or support can be flat, but need not be and can include any type of shape such as spherical shapes (e.g., beads or microspheres). The solid carrier or support can have a flat surface as in slides and micro-titer plates having one or more wells.

[0188] Binding to proteins or nucleic acids on microarrays can be detected by scanning the microarray with a variety of laser or CCD-based scanners, and extracting features with software packages, for example, Imagen (Biodiscovery, Hawthorne, CA), Feature Extraction Software (Agilent), Scanalyze (Eisen, M. 1999. SCANALYZE User Manual; Stanford Univ., Stanford, CA Ver 2.32.), or GenePix (Axon Instruments).

[0189] Embodiments disclosed herein can be used with high throughput screening (HTS). Typically, HTS refers to a format that performs at least 100 assays, at least 500 assays, at least 1000 assays, at least 5000 assays, at least 10,000 assays, or more per day. When enumerating assays, either the number of samples or the number of protein (or nucleic acid) biomarkers assayed can be considered.

[0190] Generally HTS methods involve a logical or physical array of either the subject samples, or the protein or nucleic acid markers, or both. Appropriate array formats include both liquid and solid phase arrays. For example, assays employing liquid phase arrays, e.g., for hybridization of nucleic acids, binding of antibodies or other receptors to ligand, etc., can be performed in multiwell or microtiter plates. Microtiter plates with 96, 384, or 1536 wells are widely available, and even higher numbers of wells, e.g., 3456 and 9600 can be used. In general, the choice of microtiter plates is determined by the methods and equipment, e.g., robotic handling and loading systems, used for sample preparation and analysis.

[0191] HTS assays and screening systems are commercially available from, for example, Zymark Corp. (Hopkinton, MA); Air Technical Industries (Mentor, OH); Beckman Instruments, Inc. (Fullerton, CA); Precision Systems, Inc. (Natick, MA), and so forth. These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide HTS as well as a high degree of flexibility

and customization. The manufacturers of such systems provide detailed protocols for the various methods of HTS.

[0192] As stated previously, obtained marker values can be compared to a reference level. Reference levels can be obtained from one or more relevant datasets. A "dataset" as used herein is a set of numerical values resulting from evaluation of a sample (or population of samples) under a desired condition. The values of the dataset can be obtained, for example, by experimentally obtaining measures from a sample and constructing a dataset from these measurements. As is understood by one of ordinary skill in the art, the reference level can be based on *e.g.*, any mathematical or statistical formula useful and known in the art for arriving at a meaningful aggregate reference level from a collection of individual datapoints; *e.g.*, mean, median, median of the mean, and so forth. Alternatively, a reference level or dataset to create a reference level can be obtained from a service provider such as a laboratory, or from a database or a server on which the dataset has been stored.

[0193] A reference level from a dataset can be derived from previous measures derived from a population. A "population" is any grouping of subjects or samples of like specified characteristics. The grouping could be according to, for example, clinical parameters, clinical assessments, therapeutic regimens, disease status, severity of condition, etc.

[0194] Subjects include humans, veterinary animals (dogs, cats, reptiles, birds, hamsters, etc.) livestock (horses, cattle, goats, pigs, chickens, etc.), research animals (monkeys, rats, mice, fish, etc.) and other animals, such as zoo animals (*e.g.*, bears, giraffe, elephant, lemurs, etc.).

[0195] In particular embodiments, conclusions are drawn based on whether a sample value is statistically significantly different or not statistically significantly different from a reference level. A measure is not statistically significantly different if the difference is within a level that would be expected to occur based on chance alone. In contrast, a statistically significant difference or increase is one that is greater than what would be expected to occur by chance alone. Statistical significance or lack thereof can be determined by any of various methods well-known in the art. An example of a commonly used measure of statistical significance is the p-value. The p-value represents the probability of obtaining a given result equivalent to a particular datapoint, where the datapoint is the result of random chance alone. A result is often considered statistically significant (not the result of random chance) at a p-value less than or equal to 0.05.

[0196] In one embodiment, values obtained about the markers and/or other dataset components can be subjected to an analytic process with chosen parameters. The parameters of the analytic process may be those disclosed herein or those derived using the guidelines described herein. The analytic process used to generate a result may be any type of process capable of providing a result useful for classifying a sample, for example, comparison of the obtained value with a reference level, a linear algorithm, a quadratic algorithm, a decision tree algorithm, or a voting algorithm. The analytic process may set a threshold for determining the probability that a sample belongs to a given class. The probability preferably is at least at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or higher.

[0197] In embodiments, the relevant reference level for a particular marker is obtained based on the particular marker in control subjects. Control subjects are those that are healthy and do not have the pathology being assayed, for instance not having the target inflammatory condition or the target cancer. As an example, the relevant reference level can be the quantity of the particular biomarker in control subject(s).

[0198] Kits

[0199] Compounds and/or compositions required to carry out a method described herein, for instance one or more antibodies (or other detection molecules) specific for a biomarker, or a panel of biomarkers as discussed herein, can be provided as kits. Kits can include one or more containers including (containing) one or more or more detection or other compounds as described herein, optionally along with one or more agents for use in sample analysis and/or one or more agents for use in therapy. For instance, some kits will include an amount of at least one anti-cancer or at least one anti-inflammatory composition.

[0200] Any component in a kit may be provided in premeasured dosage(s), though this is not required; and it is expected that example kits will include more than one dose.

[0201] Embodiments of kits can contain, in separate containers, marker binding agents either bound to a matrix, or packaged separately with reagents for binding to a matrix. In particular embodiments, the matrix is, for example, a porous strip. In some embodiments, measurement or detection regions of the porous strip can include a plurality of sites containing marker binding agents. In some embodiments, the porous strip can also contain sites for negative and/or positive controls. Alternatively, control sites can be located on a separate strip from the porous strip. Optionally, the different detection sites can contain different amounts of

marker binding agents, e.g., a higher amount in the first detection site and lesser amounts in subsequent sites. Upon the addition of test sample, the number of sites displaying a detectable signal provides a quantitative indication of the amount of marker present in the sample. The detection sites can be configured in any suitably detectable shape and can be, e.g., in the shape of a bar or dot spanning the width (or a portion thereof) of a porous strip.

[0202] In some embodiments the matrix can be a solid substrate, such as a "chip." See, e.g., U.S. Pat. No. 5,744,305. In some embodiments the matrix can be a solution array; e.g., xMAP (Luminex, Austin, TX), Cyvera (Illumina, San Diego, CA), RayBio Antibody Arrays (RayBiotech, Inc., Norcross, GA), CellCard (Vitra Bioscience, Mountain View, CA) and Quantum Dots' Mosaic (Invitrogen, Carlsbad, CA).

[0203] Additional embodiments can include control formulations (positive and/or negative), and/or one or more detectable labels, such as fluorescein, green fluorescent protein, rhodamine, cyanine dyes, Alexa dyes, luciferase, and radiolabels, among others. Instructions for carrying out the assay, including, optionally, instructions for generating a score, can be included in the kit; e.g., written, tape, VCR, or CD-ROM.

[0204] In particular embodiments, the kits include materials and reagents necessary to conduct an immunoassay (e.g., ELISA). In particular embodiments, the kits include materials and reagents necessary to conduct hybridization assays (e.g., PCR). In particular embodiments, materials and reagents expressly exclude equipment (e.g., plate readers). In particular embodiments, kits can exclude materials and reagents commonly found in laboratory settings (pipettes; test tubes; distilled H₂O).

[0205] Kits can also include a notice in the form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use, or sale for human administration. The notice may state that the provided active ingredients can be administered to a subject. The kits can include further instructions for using the kit, for example, instructions regarding administration; proper disposal of related waste; and the like. The instructions can be in the form of printed instructions provided within the kit or the instructions can be printed on a portion of the kit itself. Instructions may be in the form of a sheet, pamphlet, brochure, CD-ROM, or computer-readable device, or can provide directions to instructions at a remote location, such as a website. In particular embodiments, kits can also include some or all of the necessary medical supplies needed to use the kit effectively, such as applicators, ampules, sponges, sterile adhesive strips, Chloraprep, gloves, and the like. Variations in

contents of any of the kits described herein can be made. The instructions of the kit will direct use of the active ingredient(s) included in that kit to effectuate a clinical and/or therapeutic use described herein.

[0206] Chromatographic Assay.

[0207] The methods described herein, for identifying or detecting the presence of CHCs in a sample from a subject, can be carried out using a chromatographic assay such as a dipstick or other flow-based assay system. A chromatographic assay includes any device which is capable of having a sample applied thereto and which allows the sample to diffuse or be transported along one or more of its dimensions. This includes any of the known dipstick formats suitable for testing and analyzing biological samples which would be familiar to a person skilled in the art. A representative example dipstick device is gRAD (generic rapid assay device) OneDetection Kit from BioPorto Diagnostics A/S, Hellerup, Denmark where the antibodies are provided by the user.

[0208] A chromatographic assay can involve a quick and convenient one-step procedure which may be carried out anywhere and at any time. There is no need for special training in order to carry out the test using a chromatographic assay, as the results are obtained in a short space of time and are readily interpreted by means of a visible result on a chromatography support which allows the applied sample to chromatograph along one or more dimensions of the chromatographic device. Thus, diagnosis and assessment may conveniently be carried out during a visit to a general practitioner's clinic or even during a home visit to a patient, and could be undertaken by a nurse or doctor without the need to use expensive laboratory equipment or send away samples for analysis by an outside laboratory.

[0209] In particular embodiments, a chromatographic assay can be a solid chromatography support test device. The chromatography support (e.g., dipstick) can include a sample application zone, a test zone, and a control zone. Zones can be arranged on the chromatography support in the same plane in a manner such that the material (e.g. sample fluid and/or reagents) can flow from the first to the subsequent zones, preferably sequentially from zone to zone. In particular embodiments, the chromatography support is in the form of a strip or in any form that permits separate zones for performing the various functions, as described herein. The configuration of the chromatography support can be such that the direction of flow is generally parallel to the length of the chromatography support. Particular

embodiments of the chromatography support of the disclosure can be akin to the immunochromatography test strips which are known in the art.

[0210] The sample application zone allows sample to enter the chromatography support (e.g., dipstick), and may be of any format and made from any material which allows this. In particular embodiments, a sample application zone includes a sample pad. The sample pad can, for example, retard sample penetration and/or help to distribute the sample over the test and control zones. In particular embodiments, the sample pad is able to remove particulates from the sample, adjust the pH or viscosity of the sample solution, facilitate the release of the capture antibody-antigen-detection antibody complex, or separate plasma or serum from whole blood. Thus, the sample pad effectively prepares the sample for analysis in the rest of the chromatography support.

[0211] Sample pads may commonly be made of a variety of materials such as glass fiber filter, cellulose (paper), woven fibers (meshes) or non-woven filters. Glass fiber filters are available in a wide range of product varieties, are very wettable, have moderately low protein binding characteristics, may have a moderate to high bed volume but have a low tensile strength, especially when wet. Cellulose (paper) are also available in a wide range of product varieties, are very wettable, have very low protein binding characteristics, may have a moderate to high bed volume but have a very low tensile strength, especially when wet. Woven fibers (meshes) are available in a more limited range of product varieties, are very wettable, have very low protein binding characteristics and very low bed volume, but have the advantage of a high tensile strength, even when wet. Non-woven filters also have a high tensile strength, even when wet, are available in a wide range of product varieties, but are not intrinsically wettable and have moderate protein binding characteristics. The sample application zone, as mentioned above, also may conveniently contain volume-determining means. This may take the form, for example, of a sample pad having a predetermined size and/or void volume. Such a pad may optionally be provided with a temporary liquid barrier, which allows the sample pad to be saturated before the liquid dissolves the barrier and the sample is able to flow further into the dipstick. Examples of suitable barriers include dried carbohydrates, proteins, nucleic acids, and organic or inorganic salts.

[0212] A test zone on a chromatography support can provide a means whereby an analyte, e.g., a target biomarker protein described herein, may subsequently be detected. The test zone can include a capture reagent. The capture reagent serves to capture a complex including a detector reagent in the test zone to enable detection of the detector reagent and

measurement of signals from the detector reagent. In particular embodiments, the test zone can include an immobilized reagent to capture complexes of the capture antibody-target protein-detection antibody. The detector reagent thus includes the detection antibody, and detection can include visualization of the detection moiety on the detection antibody. In particular embodiments, the test zone can be located in close proximity to the sample application zone and can be in contact either directly or indirectly with said sample application zone such that the sample may flow into said test zone. The test zone can be arranged to be in capillary flow communication with the sample application zone. Methods and means of achieving such functional requirements are a common feature of chromatography technology and are known in the art.

[0213] A reagent to capture complexes of the capture antibody-analyte-detection antibody may be immobilized by binding or coupling to a solid support. The solid support can be a part of the basic chromatography support structure itself or it may be a component which is provided in or on the chromatography support. Different forms of solid support or matrix include particles, sheets, gels, filters, membranes, fibers, capillaries, microtiter strips, etc. For particular use in the form of a dipstick, the solid support may generally take the form of a sheet, strip, membrane or particles. In particular embodiments, the solid support is a nitrocellulose membrane. Techniques for binding or coupling of the reagent to the solid support are also well known and widely described in the literature (see for example Immobilized Affinity Ligand Techniques, Ed Hermanson, Mallia and Smith, Academic Press Inc.).

[0214] In particular embodiments, the reagent to capture analyte (e.g., marker protein)-antibodies complexes may conveniently be covalently coupled directly to the pads of the test zone, using any convenient or desired coupling chemistry, e.g., a linker, such as cyanogen bromide. Alternatively, the reagent to capture analyte (e.g., marker protein)-antibodies complexes may be coupled to particles, e.g., latex particles. A pad can, for example, be dipped into a solution containing a reagent-latex conjugate, and then dried. The particles are preferably larger than the pore size of the pad in order to ensure that they are not released from the pad. Other coupling or immobilization methods for molecules are also well known in the art. The capture reagent can be immobilized in a recognizable pattern in the test zone, e.g., a strip oriented transverse to the direction of flow of the sample, to form a detectable "positive line" as the sample migrates past the test zone. In particular embodiments, the reagent is biotin-binding protein that binds a biotin-conjugated antibody complexed with the

target marker protein. Without limitation, target marker proteins are described herein, including in Tables 2 and 3.

[0215] The detection moiety or label on a detection antibody will be of a nature which allows it to be readily visualized for detection and/or quantitation purposes. Some labels will require the addition of other reagents for visualization, and others may require the use of a particular instrument for this purpose. For example, where the detection moiety or label is an enzyme, a substrate reagent for that enzyme can be added. Fluorescent molecules may be detected by means of standard excitation/radiation techniques which are well-known in the art. Where reflectometers are used to detect electromagnetic radiation or where scanners are used to detect radioisotopes, instrumentation can be used to achieve visualization. On the other hand, colored substances may be detected and visualized directly by the person carrying out the assay. In particular embodiments, the detection moieties are gold nanoparticles conjugated to the detection antibody and can be visualized by eye. In particular embodiments, the detection moieties are gold nanoparticles conjugated to the detection antibody and can be quantified by generation of a calibration curve with control protein levels.

[0216] A chromatography support of the present disclosure can include a control zone including an immobilized reagent bound to the membrane in a recognizable pattern, which captures the detector reagent and gives a detectable signal, such as the formation of a colored line, if the test has been used properly. The control zone will give an identifiable signal whether or not there is an identifiable positive line, *i.e.* the control zone develops a detectable signal if the test has been used properly, regardless of whether any analyte was present in the sample being analyzed. In particular embodiments, a control zone on a chromatography support includes any immobilized reagent that binds to a detection antibody used in the chromatographic assay. In particular embodiments, a control zone on a chromatography support includes an immobilized antibody that binds to a detection antibody used in the chromatographic assay. In particular embodiments, a control zone on a chromatography support includes an anti-mouse antibody that binds to uncomplexed gold nanoparticle-conjugated anti-target (marker) protein antibody.

[0217] In particular embodiments, the analyte is quantified by detecting the label conjugated to the detection antibody, and thus the quantity of analyte arriving at the test zone and remaining immobilized in the test zone is proportional to the amount of analyte in the sample. For example, where the detection antibody is conjugated to a visible detectable label, such

as gold nanoparticles, the intensity of signal which develops on the positive line is generally proportional to the concentration of analyte in the sample.

[0218] A further optional feature of a chromatographic assay is an absorbent pad which is conveniently placed at the end of the chromatography support, preferably beyond the test and control zones at the opposite end to the sample application zone. The absorbent pad is designed to absorb the sample after it has passed through the test and control zones, and the capacity of the absorbent pad can determine the volume of sample tested.

[0219] A chromatography support may include a plastic backing to which one or all of the sample pad, test zone, control zone, and absorbent pad are attached directly or indirectly, for example by means of an adhesive.

[0220] Quantification of a marker protein in a sample can be relative or absolute. An index, ratio, percentage or any other indication of the level or amount, or presence or absence, of marker protein in a test sample can be measured, for example, relative to a control.

[0221] In particular embodiments, color intensity of a test line on a chromatography support (e.g., dipstick) can be captured by, for example, a digital camera or a flatbed scanner. Target protein level can be quantified with image analysis software in a computer, for instance using a dedicated reader or a generalized detection device.

[0222] Signal amplification systems may be used in connection with the detector reagent/detection means of the present disclosure, according to principles well known in the art. In particular embodiments, the amplification system may include an amplification reagent including a binding ligand that binds the detectable moiety of the detector reagent (e.g., the detectable label on a detection antibody). As there can be multiple detectable labels (e.g., gold nanoparticles) on a detection antibody, a plurality of amplification reagents can bind each detector reagent, and hence signal amplification may be achieved.

[0223] Other signal amplification systems may be used. For example, detection antibodies conjugated with enzyme reporters can allow signal amplification due to particular substrates used, such as chemiluminescent substrates. Such systems may be useful in the context of low concentration analytes.

[0224] The chromatography support may contain other reagents which are capable of preventing non-specific binding of the capture antibody, the detection antibody, or the analyte. Blocking agents can be pre-loaded into the chromatography support, such that they are released during the assay and flow into the sample application zone, test zone, and/or control zone. Examples of blocking agents include albumin, casein and gamma globulin. Other

standard blocking agents known in the art may also be used. In particular embodiments, bovine serum albumin (BSA) is used. Other suitable blocking agents include polyvinyl alcohol, SDS, and other materials known in the art.

[0225] Suitable methods, materials, and examples used in the practice and/or testing of embodiments of the disclosed invention are described herein. Such methods and materials are illustrative only and are not intended to be limiting. Other methods, materials, and examples similar or equivalent to those described herein can be used.

[0226] The Exemplary Embodiments and Examples below are included to demonstrate particular embodiments of the disclosure. Those of ordinary skill in the art should recognize in light of the present disclosure that many changes can be made to the specific embodiments disclosed herein and still obtain a like or similar result without departing from the spirit and scope of the disclosure.

[0227] Exemplary Embodiments.

1. A method of treating cancer in a human patient, the method including: obtaining a sample from the human patient; detecting whether Circulating Hybrid Cells (CHCs) are in the sample by contacting the sample with an anti-(source of the cancer) antibody; contacting the sample with an anti-immune cell antibody; diagnosing the patient as having cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more Circulating Hybrid Cells; and administering to the patient in need thereof a pharmaceutically effective amount of an anti-cancer agent.
2. The method of embodiment 1, wherein the cancer includes: breast cancer, prostate cancer, head and neck squamous cell carcinoma, lung cancer, pancreatic ductal adenocarcinoma, colorectal cancer, glioblastoma, or melanoma.
3. A method of detecting Circulating Hybrid Cells (CHCs) in a human patient, the method including: obtaining a sample from the human patient; and detecting whether CHCs are in the sample by contacting the sample with an anti-source cell antibody that recognizes a marker other than cytokeratin (CK); and contacting the sample with an anti-immune cell antibody; wherein specific binding of both antibodies in the same cell indicates the presence of CHCs.
4. A method of diagnosing a solid tumor in a human patient, the method including: obtaining a sample from the human patient; and detecting whether Circulating Hybrid Cells (CHCs) are in the sample by: contacting the sample with an antibody specific for a protein found on cells from a tissue from which the solid tumor is derived; contacting the sample with an antibody specific for an immune cell; and diagnosing the patient as having a solid tumor wherein specific binding of both antibodies in the same cell indicates the presence of one or more CHCs.

5. The method of embodiment 4, including: contacting the sample with at least two different antibodies specific for proteins found on cells from a tissue from which the solid tumor is derived; contacting the sample with at least two different antibodies specific for an immune cell; and diagnosing the patient as having a solid tumor wherein specific binding of antibodies specific for proteins found on cells from a tissue from which the solid tumor is derived and antibodies to immune cells in the same cell indicates the presence of one or more CHCs.
6. The method of embodiment 4 or embodiment 5, wherein the solid tumor is a glioblastoma, a melanoma, a head and neck squamous cell carcinoma, a pancreatic ductal adenocarcinoma, a colorectal cancer, a prostate cancer tumor, or a breast cancer tumor
7. A method of diagnosing metastatic cancer in a human patient, the method including: obtaining a sample from the human patient; and detecting whether Circulating Hybrid Cells (CHCs) are in the sample by contacting the sample with an anti-(source of cancer) antibody; contacting the sample with an anti-immune cell antibody; and diagnosing the patient as having a metastatic cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more CHCs.
8. A method of differentiating disease status of a human patient, the method including: obtaining a sample from the human patient; and typing Circulating Hybrid Cells (CHCs) are in the sample by contacting the sample with at least two panels of antibodies, each panel including at least two antibodies, wherein: a first panel of antibodies including at least one anti-immune cell antibody and at least one antibody specific for a first source cell type; and a second panel of antibodies including at least one anti-immune cell antibody and at least one antibody specific for a second source cell type, wherein the first source cell type and the second source cell type represent two stages of a disease progression; identifying the disease status of the patient based on detection of circulating cells that exhibit specific binding to both an anti-immune cell antibody and an antibody specific for either the first source cell type or the second source cell type.
9. The method of embodiment 8, wherein the first source cell type is a cancer cell and the second source cell type is a non-cancerous cell of the same origin as the cancer cell.
10. The method of embodiment 8, wherein: the first cell type is an epithelial-derived cancer cell and the at least one antibody specific for the first cell type is specific for one of MUC4 or MASPIN; and the second cell type is an epithelial cell and the at least one antibody specific for the first cell type is specific for one of ECAD, EpCAM, or CK.
11. A method of treating metastatic cancer in a human patient, the method including: obtaining a sample from the human patient; detecting whether Circulating Hybrid Cells (CHCs) are in the sample by contacting the sample with an anti-(source of the metastatic cancer) antibody; contacting the sample with an anti-immune cell antibody; diagnosing the patient as having metastatic cancer wherein specific binding of both antibodies in the same cell indicates the

presence of one or more Circulating Hybrid Cells; and administering to the patient in need thereof a pharmaceutically effective amount of an anti-cancer agent.

12. The method of any one of embodiments 1-11, wherein at least one of the antibodies is conjugated to a fluorescent label, and the detecting includes fluorescence activated cell sorting (FACS) analysis
13. The method of any one of embodiments 1-12, wherein the sample includes blood, plasma, serum, lymph, another blood fraction, a tumor aspirate, a tumor biopsy, peritoneal fluid, a secretions, urine, or another biological sample that contains or is believed to contain immune cells.
14. The method of any of embodiments 1-11, wherein the anti-source cell antibody is an epithelial cell antibody that specifically binds to an epitope on a biomarker selected from the group of EpCAM, E-cadherin, cytokeratin, MUC4, MASPIN, and Glypican-1 (GPC1).
15. The method of any of embodiments 1-10, wherein the anti-source cell antibody is a glioblastoma cell antibody that specifically binds to an epitope on a biomarker selected from the group of GFAP and Nestin.
16. The method of any of embodiments 1-10, wherein the anti-source cell antibody is a melanoma cell antibody that specifically binds to an epitope on a biomarker selected from the group of gp100, MelanA, TYR, and MAGEA1.
17. The method of any of embodiments 1-10, wherein detecting whether CHCs are in the sample includes contacting the sample with two or more, three or more, or four or more anti-epithelial antibodies, each of which specifically binds to an epitope on a biomarker selected from the group of EpCAM, E-cadherin, cytokeratin, MUC4, MASPIN, Glypican-1 (GPC1), GFAP, Nestin, gp100, and MAGEA1.
18. The method of any of embodiments 1-17, wherein the anti-immune cell antibody specifically binds to an epitope on a biomarker selected from CD45, CD14, CD16, CD11b, CD11c, CD64, CD163, CD68, CD66b, CD71, CD80, CD86, CSF1R, or CCR5.
19. The method of any of embodiments 1-17, wherein detecting whether CHCs are in the sample includes contacting the sample with two or more, three or more, or four or more anti-immune cell antibodies, each of which specifically binds to an epitope on a biomarker selected from the group of CD45, CD14, CD16, CD11b, CD11c, CD64, CD163, CD68, CD66b, CD71, CD80, CD86, CSF1R, and CCR5.
20. The method of any of embodiments 1-17, wherein the anti-immune cell antibody specifically binds to an epitope on a biomarker selected from CD14, CD16, CD45, CD64, CD68, CD71, or CCR5.
21. The method of any of embodiments 1-17, wherein detecting whether CHCs are in the sample includes contacting the sample with two or more, three or more, or four or more anti-immune cell antibodies, each of which specifically binds to an epitope on a biomarker selected from CD14, CD16, CD45, CD64, CD68, CD71, or CCR5.

22. The method of embodiment 11, wherein the anti-cancer agent is selected at least in part based on gene or protein expression in the CHC.
23. The method of embodiment 11, wherein the anti-cancer agent is a CSF1R inhibitor selected from pexidartinib, PLX7486, LY3022855, MC-CS4, chiauranib, SNDX6352, JNJ-40346527, DCC-3014, linifanib, IMC-CS4, AMG820, BLZ945, TK-1258, dovitinib, vatalinib, sunitinib, ARRY-3882, 5-(3-methoxy-4-((4-methoxybenzyl)oxy)benzyl)pyrimidine-2,4-diamine, CEP-32496, 3-((quinolin-4-ylmethyl)amino)-N-(4-(trifluoromethoxy)phenyl)thiophene-2-carboxamide; or a pharmaceutically acceptable salt thereof.
24. The method of embodiment 11, wherein the anti-cancer agent is a CSF1R inhibitor is selected from pexidartinib, chiauranib, linifanib, dovitinib, vatalinib, or sunitinib; or a pharmaceutically acceptable salt thereof.
25. The method of embodiment 11, wherein the anti-cancer agent is a CSF1R inhibitors is an anti-CSF1R antibody.
26. The method of embodiment 25, wherein the anti-CSF1R antibody is cabiralizumab or emactuzumab.
27. The method of any of embodiments 1-17, wherein the anti-(source) antibody is an anti-epithelial antibody that specifically binds to an epitope on a biomarker selected from MUC4, MASPIN, or Glypican-1 (GPC1).
28. A method of diagnosing a subject, including: obtaining a sample from the subject; characterizing Circulating Hybrid Cells (CHCs) in the sample by: contacting the sample with an antibody specific for CD45, contacting the sample with epithelial antibodies specific for ECAD, EpCAM, and CK; contacting the sample with cancer antibodies specific MUC4 and MASPIN; identifying the sample as containing: inflammation-indicative CHCs when the CD45 antibody and epithelial antibodies but not cancer antibodies specifically bind the same cell in the sample; cancer-indicative CHCs when the CD4 antibody, the epithelia antibodies, and the cancer antibodies specifically bind the same cell in the sample; and diagnosing the subject as: having or at risk for cancer when the number of cancer-indicative CHCs is at least twice the number of inflammation indicative CHCs; having or at risk of chronic inflammation when the number of inflammation-indicative CHCs is at least twice the number of cancer-indicative CHCs; or having neither cancer nor chronic inflammation when the number of inflammation-indicative CHCs and the number of cancer-indicative CHCs is about equal and fewer than 25 CHCs/20,000 nuclei in the sample.
29. The method of embodiment 28, wherein at least one of the antibodies is conjugated to a fluorescent label, and the characterizing includes fluorescence activated cell sorting (FACS) analysis.
30. The method of embodiment 28, wherein the sample includes blood, plasma, serum, another blood fraction, lymph, a tumor aspirate, a tumor biopsy, peritoneal fluid, a secretions, urine, or another biological sample that contains or is believed to contain immune cells

31. A chromatographic assay device including: a panel of two or more capture antibodies or antigen-binding fragment thereof, each of which specifically binds a different marker protein in Table 2 or Table 3.
32. The chromatographic assay device of embodiment 31, wherein the panel includes two or more capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of: CD45, CD68, EpCAM, ECAD, CK, MUC4, and MASPIN (*CHC-identity panel*); CD44, Ki67, pHH3, CD166, Sox9, and PDX1 (*stem/development panel*); Vimetin, MMP11, and ECAD (*migratory panel*); KRAS, pKRAS, RAF, pRAF, MEK 1/2, pMEK 1/2, MAPK, pMAPK, ERK 1/2, pERK1/2, PI3K, pPI3K, pS6RP, AKT, pAKT, pSTAT3, TGF β , SMAD4, EGFR, and pEGFR (*cell signalling pathway panel*); UCHL, SFTPB, MUC4, WDR72, RAGE, and EGFR (*lung panel*); ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNAK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) panel*); HER2, ER, and AR (*breast cancer panel*); PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) panel*); ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial panel*); GFAP and Nestin (*glioblastoma panel*); gp100, MageA1, MelanA, and TYR (*melanoma panel*); and/or CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage panel*).
33. The chromatographic assay device of embodiment 31, wherein the panel includes a set of capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of: at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*); and at least one cell-type or cancer-type specific marker protein in Table 3 or at least one macrophage-specific marker.
34. The chromatographic assay device of embodiment 33, wherein the at least one cell-type or cancer-type specific marker protein in Table 3 includes: at least one of UCHL, SFTPB, MUC4, WDR72, RAGE, or EGFR (*lung markers*); at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNAK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*); at least one of HER2, ER, and AR (*breast cancer markers*); at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*); at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*); at least one of GFAP and Nestin (*glioblastoma markers*); at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and/or at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).

35. The chromatographic assay device of embodiment 34, wherein the panel includes at least nine antibodies or antigen-binding fragments thereof, which individually specifically bind to: at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*); at least one of UCHL, SFTPb, MUC4, WDR72, RAGE, or EGFR (*lung markers*); at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*); at least one of HER2, ER, and AR (*breast cancer markers*); at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*); at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*); at least one of GFAP and Nestin (*glioblastoma markers*); at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).
36. A kit including: a panel of two or more antibodies or antigen-binding fragment thereof, each of which specifically binds a different marker protein in Table 2 or Table 3.
37. The kit of embodiment 36, wherein the panel includes two or more capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of: CD45, CD68, EpCAM, ECAD, CK, MUC4, and MASPIN (*CHC-identity panel*); CD44, Ki67, pHH3, CD166, Sox9, and PDX1 (*stem/development panel*); Vimetin, MMP11, and ECAD (*migratory panel*); KRAS, pKRAS, RAF, pRAF, MEK 1/2, pMEK 1/2, MAPK, pMAPK, ERK 1/2, pERK1/2, PI3K, pPI3K, pS6RP, AKT, pAKT, pSTAT3, TGF β , SMAD4, EGFR, and pEGFR (*cell signalling pathway panel*); UCHL, SFTPb, MUC4, WDR72, RAGE, and EGFR (*lung panel*); ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) panel*); HER2, ER, and AR (*breast cancer panel*); PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) panel*); ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial panel*); GFAP and Nestin (*glioblastoma panel*); gp100, MageA1, MelanA, and TYR (*melanoma panel*); and/or CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage panel*).
38. The kit of embodiment 36, wherein the panel includes a set of capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of: at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*); and at least one cell-type or cancer-type specific marker protein in Table 3 or at least one macrophage-specific marker.
39. The kit of embodiment 38, wherein the at least one cell-type or cancer-type specific marker protein in Table 3 includes: at least one of UCHL, SFTPb, MUC4, WDR72, RAGE, or EGFR (*lung*

- markers); at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*); at least one of HER2, ER, and AR (*breast cancer markers*); at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*); at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*); at least one of GFAP and Nestin (*glioblastoma markers*); at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and/or at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).
40. The kit of embodiment 39, wherein the panel includes at least nine antibodies or antigen-binding fragments thereof, which individually specifically bind to: at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*); at least one of UCHL, SFTPB, MUC4, WDR72, RAGE, or EGFR (*lung markers*); at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*); at least one of HER2, ER, and AR (*breast cancer markers*); at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*); at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*); at least one of GFAP and Nestin (*glioblastoma markers*); at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).
41. An antibody cocktail that allows for separation of CHCs via a flow based or DEP based assay.
42. The antibody cocktail of embodiment 41, which includes: two or more antibodies or antigen-binding fragment thereof, each of which specifically binds a different marker protein in Table 2 or Table 3.
43. The antibody cocktail of embodiment 42, wherein the cocktail includes two or more capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of: CD45, CD68, EpCAM, ECAD, CK, MUC4, and MASPIN (*CHC-identity panel*); CD44, Ki67, pHH3, CD166, Sox9, and PDX1 (*stem/development panel*); Vimetin, MMP11, and ECAD (*migratory panel*); KRAS, pKRAS, RAF, pRAF, MEK 1/2, pMEK 1/2, MAPK, pMAPK, ERK 1/2, pERK1/2, PI3K, pPI3K, pS6RP, AKT, pAKT, pSTAT3, TGF β , SMAD4, EGFR, and pEGFR (*cell signalling pathway panel*); UCHL, SFTPB, MUC4, WDR72, RAGE, and EGFR (*lung panel*); ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) panel*); HER2, ER, and AR (*breast cancer panel*); PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) panel*); ZO1,

EpCAM, ECAD, and Cytokeratin (*epithelial panel*); GFAP and Nestin (*glioblastoma panel*); gp100, MageA1, MelanA, and TYR (*melanoma panel*); and/or CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage panel*).

44. The antibody cocktail of embodiment 42, wherein the cocktail includes a set of capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of: at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*); and at least one cell-type or cancer-type specific marker protein in Table 3 or at least one macrophage-specific marker.
45. The antibody cocktail of embodiment 44, wherein the at least one cell-type or cancer-type specific marker protein in Table 3 includes: at least one of UCHL, SFTPb, MUC4, WDR72, RAGE, or EGFR (*lung markers*); at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*); at least one of HER2, ER, and AR (*breast cancer markers*); at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*); at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*); at least one of GFAP and Nestin (*glioblastoma markers*); at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and/or at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).
46. The antibody cocktail of embodiment 45, including at least nine antibodies or antigen-binding fragments thereof, which individually specifically bind to: at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*); at least one of UCHL, SFTPb, MUC4, WDR72, RAGE, or EGFR (*lung markers*); at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*); at least one of HER2, ER, and AR (*breast cancer markers*); at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*); at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*); at least one of GFAP and Nestin (*glioblastoma markers*); at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).
47. Use of the assay device of any one of embodiments 31-35, or the kit of any one of embodiments 36-40, or the antibody cocktail of any one of embodiments 41-46 to detect one or more CHCs in a sample from a subject.

48. A method of diagnosing or grading/stratifying a patient or a sample from a patient, essentially as described herein.
49. A pathology or disease grading/stratifying array or antibody panel essentially as described herein.
50. A method of treatment essentially as described herein.

[0228] Example 1: Cell fusion potentiates tumor heterogeneity and reveals circulating hybrid cells that correlate with stage and survival

[0229] This example describes the discovery that fusion of neoplastic cells with leukocytes (for example, macrophages) contributes to tumor heterogeneity, resulting in cells exhibiting increased metastatic behavior. Fusion hybrids (cells harboring both hematopoietic and epithelial properties) are readily detectable in cell culture and tumor-bearing mice. Further, hybrids enumerated in peripheral blood of human cancer patients correlate with disease stage and predict overall survival. This unique population of neoplastic cells provides a novel biomarker for tumor staging, as well as a therapeutic target for intervention. At least some of the research described in this Example was published as Gast *et al.* (*Sci. Adv.* 4:3aa67828, 1-15, 12 September 2018).

[0230] Methods

[0231] *Fluorescence in-situ hybridization and immunohistochemical analyses of human solid tumors and peripheral blood cells: Analyses of human solid tumors.* Presence of cell fusion between Y-chromosome containing blood cells and host tumor epithelium was evaluated by dual FISH and immunohistochemical analyses. X- and Y-chromosome FISH probes were hybridized to 5 µm formalin-fixed paraffin embedded primary human tumor sections using CEP X (DXZ1 locus) and Y (DYZ1 locus) probes (Abbott Molecular, Des Plaines, IL) following manufacturer's protocols. Briefly, tissue was treated with Retrieagen A solutions (BD Biosciences, San Jose, CA), Tissue Digestion Kit II reagents (Kreatech, Amsterdam, Netherlands) then hybridized with probe at 80 °C for 5 mins and 37 °C for 12 hr. Tissue sections were permeabilized with graded detergent washes at 24 °C, then processed for immunohistochemical staining. Tissue was incubated with antibodies to pan-cytokeratin (Fitzgerald, Acton, MA) and counterstained with Hoechst dye (1 µg/mL). Two slides were analyzed for each tumor section. Slides were digitally scanned and quantified by two independent investigators. Areas with Y-chromosome positivity were analyzed by confocal microscopy. Hematoxylin and eosin stain was conducted on adjacent sections.

[0232] *In situ analyses of human peripheral blood analyses:* Patient peripheral blood was collected in heparinized vacutainer tubes (BD), then lymphocytes and peripheral mononuclear cells were isolated using density centrifugation and LeucoSep™ Centrifuge Tubes (Greiner Bio-One, Kremsmünster, Austria) according to manufacturer's protocol. Cells were then prepared for antibody staining. Briefly, cells were adhered to Poly-D-Lysine-coated slides, fixed, and permeabilized prior to staining for CD45

and cytokeratin expression using antibodies to CD45 (eBioscience, San Diego, CA) and human pan-cytokeratin (Fitzgerald). Phenotypes of circulating hybrid cells were evaluated with additional antibody staining, including to CD66b (BDPharmingen, San Jose, CA), CD68 (Abcam, Cambridge, United Kingdom), CD163 (Neomarkers, Portsmouth, NH), CSF1R (Abcam), and EPCAM (1:200, US Biological, Salem, MA). Tissue was developed with appropriate fluorescent-conjugated secondary antibodies (anti-mouse Cy3 (Jackson ImmunoResearch, West Grove, PA), goat anti-guinea pig 488 (Invitrogen), goat anti-guinea pig 555 (Invitrogen), anti-rabbit A647 (1:500, Thermo Fisher, Waltham, MA), anti-mouse Cy5 (1:500, Jackson ImmunoResearch), then was stained with Hoechst (1 μ g/mL). Slides were digitally scanned with a Leica DM6000 B microscope, or Zeiss AxioObserverZ1 microscope and analyzed using ARIOL® or Zeiss ZENBLUE® software.

[0233] To determine if circulating CD45+/CK+ or CD45+/EPCAM+ cells were cell fusion products, patient peripheral blood was subjected to FISH/immunohistochemical analyses as described for solid tissues, above. Processed peripheral blood was interrogated with Y-chromosome FISH (DYZ1 locus) probes (Abbott Molecular) following manufacturer's protocols. Briefly, cells were hybridized for with probe at 42 °C for 16-20 h, then subjected to graded detergent washes. Cells were then subjected to antibody staining with anti-CD45 conjugated to FITC (1:100, Biolegend, San Diego, CA), EPCAM (1:100, US Biological), and processed with anti-rabbit AF647 secondary antibodies (1:250; Jackson ImmunoResearch). Cells were imaged on a Zeiss AxioObserverZ1 microscope. Images were post-processed to rule out non-specific staining. Briefly, CZI files were opened using ZEN 2.3 Lite (Blue Edition) and saved as Single-Channel TIF files (four channels per CZI: EPCAM [white], Y-chromosome [red], CD45 [green], and DAPI [blue]). Single-Channel TIF files were loaded into MatLab as UINT8 matrices containing RGB information at each pixel. To create binary images, pixel intensity thresholds were set for each channel image separately: any pixel with a value above the threshold was turned ON (*i.e.* maximum intensity) and the remaining pixels were turned OFF (*i.e.* zero intensity). Two binary channel images were reassigned colors (EPCAM: white → yellow, Y-chromosome: red → white); all binary channel images were then overlaid.

[0234] *Quantification of CHCs in patient blood:* Manual quantification by three independent investigators of randomly selected regions containing 2,000 cells evaluated CD45 and cytokeratin status of Hoescht+ cells. Percentages of circulating hybrid cells (CHCs) in the buffy coat correlate with disease stage with significance determined by overall ANOVA post-test, $p < 6.3 \times 10^{-8}$, (p-values: no nodal-met (0.00035), nodal-met (0.05), no nodal-nodal (0.15), while none of the conventional circulating tumor cells (CTC; *i.e.* CD45-) comparisons across stage were statically significant, p-values for no nodal-met (0.31), nodal-met (0.9). Survival analysis was conducted on 18/20 pancreatic patients (two were lost to follow-up, 9 patients with high levels and 9 patients with low levels) to correlate CHCs or CTCs with time to death using Kaplan-Meier curve and log rank test using dichotomized biomarkers based on median value. High CK+/CD45+ (> 0.808 , median) was associated with a statistically

significant increased risk of death (log rank test, $p = 0.0029$) with a hazard ratio of 8.31, but high CK+/CD45- (> 0.101 , median) did not have a statistically significant effect on time to death (log rank test, $p = 0.95$).

[0235] *Flow cytometric analyses of fusion hybrids in peripheral blood:* For flow cytometric analysis, patient blood was collected as described above. RBC lysis was performed by a 1 minute incubation in 0.2% NaCl followed by addition of the equivalent volume of 1.6% NaCl. Cells were washed and resuspended in FACS Buffer (PBS, 1.0 mM EDTA, 5% FBS). Cells were incubated in PBS containing Live Dead Aqua (1:500, Invitrogen) with Fc Receptor Binding Inhibitor (1:200, eBioscience). Cells were then incubated in FACS buffer for 30 min with CD45-APC (1:25, Thermo Fisher), CD11c-APCeF780 (1:100, Thermo Fisher), CD14-BV785 (1:100, Biolegend), CD163-PECy7 (1:100, Biolegend), EPCAM-FITC (1:100, Abcam), or Cytokeratin-PE (1:500, Abcam). BD Fortessa FACS machine was used for analyses. Gating scheme established with single color controls is provided in FIGs. 4A-4B). Data reflects analyses from $n=3$ patients with PDAC.

[0236] *Mice:* All mouse experiments were performed in accordance to the guidelines issued by the Animal Care and Use Committee at Oregon Health & Science University or the Fred Hutchinson Cancer Center, using approved protocols. Mice were housed in a specific pathogen-free environment under strictly controlled light cycle conditions, fed a standard rodent Lab Chow (#5001 PMI Nutrition International, St. Louis, MO), and provided water *ad libitum*. The following strains were used in the described studies: C57BL/6J (JAX #000664), Gt(ROSA)26Sortm(EYFP)Cos/J (R26R-stop-YFP; JAX#006148)21, Tg(act-EGFP)Y01Osb (Act-GFP; JAX #006567)20, B6.129P2-Lyz2tm1(cre)Lfo/J (LysM-Cre; JAX#004781) (Clausen *et al.*, *Transgenic Res.* 8(4):265-77, 1999). Mice of both genders were randomized and analyzed at 8-10 weeks of age. When possible, controls were littermates housed in the same cage as experimental animals.

[0237] *Cell culture:* MC38 mouse intestinal epithelial cancer cells were kindly provided by Jeffrey Schlom, (NCI, MD) and B16F10 mouse melanoma cells were obtained from the ATCC. Validation of cell lines were confirmed by PCR and by functional metastasis assay for the later. Cell lines, both derived from C57BL/6J mice, were cultured in DMEM + 10% serum (Life Technologies, NY). Stable cancer cell lines, MC38(H2B-RFP), MC38(H2B-RFP/Cre, B16F10(H2B-RFP), and B16F10(H2B-RFP/Cre), were generated by retroviral transduction using pBABE-based retroviruses, and polyclonal populations were selected by antibiotic resistance and flow-sorted for bright fluorescence as appropriate. B16F10(fl-dsRed-fl-eGFP) cells were generated by stably expressing a pMSCV-LoxP-dsRed-LoxP-eGFP-PURO construct (Addgene #32702) into the parental B16F10 cells. Primary MΦ derivation was conducted from the bone marrow of R26R-stop-YFP or Act-GFP mice. To elicit MΦs, cells were cultured for six days in DMEM + 15% serum supplemented with sodium pyruvate, non-essential amino acids (Life Technologies, Carlsbad, CA) and 25 ng/ml Csf1 (Peprotech, Rocky Hill, NJ).

[0238] Cell fusion hybrid generating co-cultures were established in MΦ-derivation media without Csf1 for four days. MC38 or B16F10 cells and MΦs were co-seeded at a 1:2 ratio at low density. Hybrid cells were FACS-isolated for appropriate fusion markers on a Becton Dickinson InFlux™ or FACSVantage™ SE cell sorters (BD Biosciences). FACS plots are representative of at least 20 independent MC38 or B16F10 hybrid isolates (technical replicates). Low passage hybrid isolates were established; functional experiments were conducted on passage 8-20 hybrid isolates. Live-imaging of co-cultured cells were performed using an INCUCYTE® Zoom automated microscope system and associated software (Essen Bioscience, Ann Arbor, MI). Technical triplicates generated 36 movies that covered 77.4 mm² and were screened for hybrid generation and division. Movie represents fusion event captured in one of 21 movies containing hybrids.

[0239] *EdU-labeling and karyotype analysis during hybrid generation:* Cultured cells were fixed in 4% formaldehyde in PBS and processed for immunohistochemical analyses with antibodies against GFP (1:500; Life Technologies, NY) or RFP (1:1000; Allele Biotechnology, San Diego, CA). 5-ethynyl-2'-deoxyuridine (EdU) labeling and detection was performed according to manufacturer directions (Life Technologies). Briefly, MΦ DNA was labeled with 10 μM EdU supplemented in media for 24 h prior to hybrid generation co-culture. 10 μM EdU was also used for determination of S-phase indices. N=6 biologic and technical replicates were conducted and screened for bi-parental hybrids.

[0240] *Karyotype analyses:* Chromosome spreads from cells in S-phase were prepared using standard protocols, from cells treated for >12 hours with 100 ng/ml colcemid (Life Technologies) to induce mitotic arrest. DNA was visualized by staining with DAPI; X- and Y- chromosomes were identified using fluorescently labeled nucleotide probes (ID Labs, Ontario, Canada) as directed by the manufacturer. Images of stained fixed cells and chromosome spreads were acquired using a 40x1.35 UApo oil objective on a DeltaVision-modified inverted microscope (IX70; Olympus) using SoftWorx software (Applied Precision, LLC), and represent maximum intensity projections of deconvolved z-stacks unless otherwise indicated. Experiments were replicated 8 times. Each biologic replicate was analyzed in an independent experiment. A minimum of n=20 cells were analyzed in each experiment. Chromosomes were counted manually by two independent investigators.

[0241] *Gene expression analysis:* Microarray analysis was performed with Mouse 430.2 gene chips (Affymetrix, Santa Clara, CA) at the OHSU Gene Profiling Shared Resource and data were analyzed using GeneSifter software (Geospiza, Seattle, WA) to identify relative expression differences between cell types (Replicates: MΦ, n=3; MC38, n=3; hybrids, n=5 independent isolates) and produce Gene Ontology analyses. Gene ontology category enrichment was calculated using the GOstats R package (Falcon, *Bioinformatics*. 23(2):257-8, 2007) and visualized using functions from the GOplot R package (Walter *et al.*, *Bioinformatics*. 31(17):2912-4, 2015).

[0242] *Code availability:* Source code used to generate figures and corresponding tables is available for download from a public repository (Burkhart, *Cell-fusion-potentiated-acquisition-of-macrophage-like-behavior-in-cancer-cells-and-its-contribution*. Oregon Health & Science University, 2016).

[0243] *Polymerase Chain Reaction:* DNA was extracted from frozen formalin fixed melanoma primary tumor and lymph node sections by 40 min incubation in lysis buffer (25 mM NaOH, 0.2 mM EDTA pH 12) at 95 °C followed by neutralization with equal volumes of neutralization buffer (40 mM Tris-HCl pH 5). RFP primers: fwd 5'-CAGTTCCAGTACGGCTCCAAG-3' (SEQ ID NO: 1) and rev 5'-CCTCGGGGTACATCCGCTC-3' (SEQ ID NO: 2). Actin primers: fwd 5'-GAAGTACCCCATTTGAACATGGC-3' (SEQ ID NO: 3) and rev 5'-GACACCGTCCCCAGAATCC-3' (SEQ ID NO: 4). Reactions were run with a 60 °C annealing temperature.

[0244] *Microenvironment arrays:* Recombinant proteins (R&D Systems, Minneapolis, MN) (Millipore, Burlington, MA) were diluted to desired concentrations in print buffer (ArrayIt, Sunnyvale, CA) and pair-wise 32 combinations of extracellular matrix proteins and growth factors or cytokines were made in a 384 well plate. A Q-Array Mini microarray printer (Genetix, Sunnyvale, CA) was used to draw from the 384 well plate and print protein combinations onto Nunc 8-well chambered cell culture plates (Thermo Scientific, Waltham, MA). Each combination was printed in quintuplicate in each array, and arrays were dried at room temperature. Printed MEMAs were blocked for 5 mins using 0.25% w/v PLURONIC® F108 copolymer (Sigma-Aldrich, St. Louis, MO) in PBS, and then rinsed with PBS and media prior to plating cells. Cells were trypsinized, filtered to exclude cell clumps and counted; 105 cells were plated on each array in 2 ml of DMEM + 2.5% serum and incubated for 30 minutes in a humidified tissue culture incubator. Unbound cells were gently removed, and fresh media added; after 12 hours, arrays were fixed with 4% formaldehyde in PBS for 10 mins and stained with DAPI. Adhesion was measured as relative cellular preference: the number of cells occupying a given microenvironment condition relative to the average cell number over all occupied microenvironmental spots across the entire MEMA for each sample. Five replicate samples each for MC38 cells and MΦ, and five independent MC38-derived hybrid isolates were analyzed. Standard two tailed t-tests were performed with $p < 0.05$ reported as significant. Error bars represent S.E.M.

[0245] *In vitro-derived hybrid proliferation:* For phenotypic profiling growth responsiveness to cytokines and soluble factors, 95 different cytokines or soluble signaling molecules were distributed at high, medium and low concentrations in 384 well plates, in 25 µl of RPMI (Life Technologies) supplemented with 1% FBS; and 25 µl of a 1.2×10^4 cells/ml suspension of hybrid or MC-38 cells in DMEM + 4% FBS was added to each well. 99 wells of each plate were left cytokine-free and no cells were added to two of these wells, which served to provide measurements of background signal. Plates were cultured in a humidified incubator for 72 hours, after which 5 µl of MTS reagent was added to each well. Two hours later, absorbance at 490 nm was read with a 384-well plate reader. For each plate, absorbance values for each cytokine-treated well were normalized to the mean absorbance of

the cytokine-free wells on that plate, and expressed in terms of standard deviations from the cytokine-free mean. Three independent hybrid isolates and three MC38 replicates were analyzed. Cytokines or factors that showed a potential differential effect on growth of MC38 and hybrid cells were re-tested in 96-well plates. In these experiments, 2.5×10^4 hybrid or MC38 cells were plated in the presence of three different concentrations for each soluble factor, or in media alone (DMEM + 2.5% FBS), in triplicate for each condition. Plates were imaged every two hours for 90 hours, and then cell viability was assessed.

[0246] *Chemotaxis assay:* Chemotaxis assays were performed using IncuCyte™ Chemotaxis Cell Migration Assay (Essen Biosciences) with at least three technical replicates of triplicate samples. Briefly, 1000 cancer cells were plated in the top wells in DMEM + 0.2% FBS after incubation in serum-free media for 20 h. Csf1 or Sdf1 ligand (25 ng/mL) was added to the bottom well and cells were incubated at 37 °C for at least 36 hours with live-imaging. The neutralizing antibodies to the Csf1R (eBioscience), Cxcr4 (Biolegend) and isotype control antibody were added to the top and bottom well (2.5 ng/μL). Migration was quantified by measuring phase contrast area of the top and bottom wells for each timepoint using IncuCyte™ ZOOM® software. Triplicates of each condition were performed, and the means and standard deviations were calculated. $p < 0.02$ for hybrids treated with Csf1 or Sdf1 relative to hybrids without Csf1 or Sdf1 by unpaired t-test. Two independent hybrid isolates were analyzed. Technical octuplicates (MC38) or sextuplicates (B16F10) with biologic quadruplicates or triplicates were analyzed. For inhibitor studies technical duplicates with biologic triplicates were analyzed.

[0247] *Migration Analysis:* From INCUCYTE® live imaging of co-cultured MΦs and cancer cells, 24 to 48 h image series containing a cancer-MΦ fusion event was cropped and exported as two separate uncompressed Audio Video Interleave (AVI) files: one containing only the red channel for TrackMate analysis and another containing both red and green channels with a sizing legend. Red channel AVI files were imported into FIJI and converted to 8-bit image series with a mean filter of 1.5 pixels applied. TrackMate analysis was then performed on nuclei with an estimated diameter of 10 pixels and a tolerance of 17.5. Using the Linear Assignment Problem (LAP) Tracker, settings for tracking nuclei were as follows: 75.0 pixel frame to frame linking, 25.0 pixel and 2 frame gap track segment gap closing. Tracks segments were not allowed to split or merge. Using the analysis function in TrackMate, track statistics were exported to an excel file and tracks containing 11 or fewer frames were excluded from analysis. A total of 9 hybrid cells and 536 unfused cells were analyzed with a $p < 1.1 \times 10^{-9}$ by unpaired t-test. Error bars represent s.d.

[0248] *Boyden chamber invasion assay:* *In vitro* invasion assay was performed as described previously (Seals *et al.*, *Cancer Cell*. 7(2):155-65, 2005). Briefly, cellular invasion was measured in a growth factor reduced Matrigel invasion chamber with 8 μm pores (#354483, Corning, Corning, NY). 3×10^5 cells in media containing 0.1% FBS were placed into each Boyden chamber. The media

containing 10% FBS was placed in the lower chamber to facilitate chemotaxis. Invasion assays were run for 15 hr, then cells which passed through the Matrigel membrane were stained with 0.09% crystal violet/10% ethanol. After extraction by elution buffer (1 part acetate buffer, pH 4.5 : 2 parts ethanol : 1 part deionized water), the stain was measured at 560 nm. Representative image of invaded cells were taken by Axio Zoom.V16 microscope (Zeiss). Assay was run in triplicate, in biologic replicate.

[0249] *In vivo analyses of in vitro-derived cell fusion hybrids:* For tumor growth, 8-12 week old C57BL/6J mice (Jackson, Bar Harbor, ME) were injected with 5×10^4 cells (MC38, MC38-derived hybrids) or 5×10^5 cells (B16F10, B16F10-derived hybrids) subcutaneously or intradermally, respectively. Length (L) and width (W) of palpable tumors were measured three times weekly with calipers until tumors reached a maximum diameter of 2 cm. Tumors were surgically removed in survival surgery or animals were sacrificed during tumor removal in accordance with OHSU IACUC guidelines. Animals were observed for at least six months for detection of tumor growth. For each tumor, volume (V) was calculated by the formula $V = \frac{1}{2}(L \times W^2)$; volume doubling time for each tumor was extracted from a curve fit to a plot of log tumor volume over time. Curves with R² values of less than 0.8 were excluded from analysis, as were tumors with six or less dimension measurements; these exclusion criteria were established in response to the unanticipated early ulceration of some tumors, which precluded accurate measurements of length and width, $p < 0.05$, by Mann-Whitney U test. For growth of tumor at metastatic sites, 1×10^6 MC38 cells were injected into the spleen. Livers were analyzed 3 weeks later for tumor burden by Hematoxylin and Eosin stain. Hybrids formed metastatic foci more readily with a $p < 0.008$ by Mann-Whitney U Test. N=17 (MC38) and n=13 (MC38-derived hybrids) were injected in four different technical replicate experiments. For B16F10 cells, 2.5×10^5 cells were retro-orbitally injected and lungs were analyzed 16 days post-injection. Melanin marked tumor metastasis were visualized. Tumor burden was analyzed on paraffin-embedded tissue sections located every 100 μ m apart through the entire lung (n=5 tissue sections/lung). Metastatic foci areas was measured using an Aperio ImageScope V12.3.0.5056 to outline metastatic tumors and quantify area. A non-parametric t-test was performed. Duplicate studies of B16F10 and B16F10-derived hybrids (n=12 mice) were analyzed.

[0250] *In vivo-derived cell fusion hybrids:* For isolation of *in vivo*-derived hybrids or assessment of circulating tumor cells, 5×10^5 B16F10 (H2B-RFP with or without Cre) cells were injected intradermally into R26R-YFP or Actin-GFP mice respectively. Once tumors reached 1-2 cm³ in diameter, it was surgically removed for immunohistochemical analyses or for FACS/flow analyses.

For demonstration that tumor cells can fuse with myeloid cells, 5×10^5 B16F10(fl-dsRed-fl-eGFP) cells were injected intradermally into 6-8 week old LysM-Cre transgenic mice. When tumors reached 1 cm³, primary tumors and lungs were removed for immunohistochemical analyses.

[0251] *Immunohistochemical analysis of in vivo-derived tumors:* B16F10(H2B-RFP, Cre) primary tumors in Act-GFP or R26R-stop-YFP mice were fixed in 10% buffered formalin, frozen in OCT and

5 μ m sections were obtained. Tumors from R26R-stop-YFP mice were incubated with antibodies for GFP (1:500; Life Technologies) followed by detection with fluorescent secondary antibody (1:500, Alexa488, Jackson ImmunoResearch). Nuclei were counterstained with Hoechst (1 μ g/mL). Slides were digitally scanned with a Leica DM6000 B microscope and analyzed using Ariol® software. Confocal images were acquired with a FluoView™ FV1000 confocal microscope (Olympus).

[0252] B16F10(fl-dsRed-fl-eGFP) primary tumors and lungs from LysM-Cre mice were fixed in 4% paraformaldehyde for 2 h at 20 °C, washed and cryopreserved in 30% sucrose for 16 h at 4 °C, then embedded in OCT. Tissue sections were cut to 8 μ m thickness, baked for 30 min at 37 °C then subjected to antigen retrieval under standard conditions (R&D Systems, CTS016), blocked with DAKO Serum Free block (Agilent, X090930-2), and incubated for 16 h at 4 °C with primary antibodies (anti-MITF, 1:500, Abcam, ab12039; anti-dsRed, 1:250, Clontech, 632496; and anti-GFP, 1:1000, Abcam, ab13970) in background-reducing antibody diluent (Agilent, S302281-2). Fluorescent-tagged secondary antibodies were applied, then sections were mounted in Prolong Gold antifade reagent (Molecular Probes, P36934). Antibody specificity was determined by immunostaining healthy lungs of non-tumor-bearing mice, and performing secondary antibody only controls.

[0253] *FACS-isolation and flow cytometric analyses of fusion hybrids:* Tumors were diced, and digested for 30 minutes at 37 °C in DMEM + 2 mg/mL Collagenase A (Roche, Basel, Switzerland) + DNase (Roche) under stirring conditions. Digested tumor was filtered through a 40 μ m filter and washed with PBS. For FACS-isolation, hybrid and unfused cells were isolated by direct fluorescence on a Becton Dickinson InFlux sorter. For flow cytometric analysis, blood was collected retro-orbitally using heparinized micro-hematocrit capillary tubes (Fisher, Hampton, NH) into K2-EDTA-coated tubes (BD). RBC lysis was performed as described above. Cells were washed and resuspended in FACS Buffer (PBS, 1.0 mM EDTA, 5% FBS). Cells were incubated in PBS containing Live Dead Aqua (1:500, Invitrogen) with Fc Receptor Binding Inhibitor (1:200, eBioscience). Cells were then incubated in FACS buffer for 30 min with CD45-PeCy7 (1:8000, Biolegend), CSF1R-BV711 (1:200, Biolegend), F4/80-APC (1:400 Biolegend), CD11b-AF700 (1:200, eBioscience). BD Fortessa FACS machine was used for analyses. Statistical significance of $p < 2.2 \times 10^{-6}$ by unpaired t-test was determined for CD45+ hybrid CTCs relative to CD45- hybrid, CD45+ unfused, and CD45- unfused CTCs. Technical duplicates of $n=5$ or 6 mice were analyzed.

[0254] *Tumorigenic analyses of FACS-isolated in vivo-derived hybrids:* A total of 100 or 3,000 FACS-isolated hybrids and unfused B16F10 cells were injected intradermally into C57BL/6J mice. For experiments with 100 cells, technical octuplicates with biologic duplicates, triplicates or quadruplicates were performed, dependent upon the number of hybrids isolated from the primary tumor, for a total of $n=16$ mice analyzed. For experiments with 3,000 cells injected, technical triplicates were performed.

[0255] *Statistical analyses and graphical displays:* Dotplots, bar charts and line charts were generated in GraphPad Prism or Excel, which was also used for statistical analyses of these data, including

ensuring that data met assumptions of the tests used and comparisons of variance between groups when appropriate. Microsoft Excel was used to perform 2-tailed t-tests. A three-dimensional scatterplot was generated in R using the rgl package. Flow cytometry data were prepared for display using FlowJo software. Microarray gene expression data were displayed as a heatmap prepared using GeneSifter software. Heatmap of MEMA data was generated in R using the standard heatmap function and default parameters.

[0256] Results

[0257] *In vitro*-derived MΦ-neoplastic cell fusion hybrids display bi-parental lineage: Based upon previous findings that MΦs are the prominent fusogenic bone marrow-derived cell partner for epithelial cells (Powell *et al.*, *Cancer Res.* 71(4):1497-505, 2011), *in vitro* validation and analyses of MΦ-cancer cell fusion hybrids was used to examine contributions of the neoplastic cell and MΦ to the identity of the resulting hybrid cell. To generate *in vitro*-derived hybrids, two murine cancer cell lines, colon adenocarcinoma (MC38) and melanoma (B16F10), were engineered to stably express Cre recombinase and histone 2B fused to red fluorescent protein (H2B-RFP). In co-cultures, engineered MC38 and B16F10 cancer cells spontaneously fused with bone marrow-derived MΦs isolated from transgenic mice expressing either actin-GFP (Okabe *et al.*, *FEBS Lett.* 407(3):313-9, 1997) or a YFP Cre reporter (Srinivas *et al.*, *BMC Dev Biol.* 1:4, 2001). This resulted in MΦ-cancer fusion hybrids identified by co-expression of nuclear RFP, and cytoplasmic GFP or YFP. YFP-expression enabled subsequent FACS-isolation of hybrid cells and downstream validation of their identity using immunoblot analyses and YFP expression. Notably, in control experiments where conditioned media from GFP-expressing MΦs incubated on MC38s or conditioned media from Cre-expressing MC38s incubated on YFP Cre reporter MΦs, fusion hybrids were not detected.

[0258] To demonstrate the bi-parental lineage of hybrid cells, three discrete approaches were employed. First, MΦs labeled with 5-ethynyl-2'-deoxyuridine (EdU) prior to co-culture with neoplastic cells produced MΦ-cancer cell fusion hybrids that initially harbored two nuclei, one labelled with EdU (MΦ origin), and the other expressing H2B-RFP (neoplastic cell origin). Upon the first mitotic division, bi-nucleated hybrids underwent nuclear fusion, yielding a single nucleus containing both EdU-labelled and H2B-RFP-labelled DNA.

[0259] A second approach, using karyotype analysis of sex-chromosomes from male-isolated MΦs (XY) fused to neoplastic cells (XO), revealed that fusion hybrids contained three sex chromosomes (XXY), consistent with a fusion event. Chromosome enumeration revealed that hybrids clustered as a unique cell population defined by their total chromosome number and sex-chromosome content. Loss of chromosomes was observed in hybrid clones with *in vitro* passage and karyotype analysis of single hybrid cells revealed variable chromosome numbers, indicating that cell fusion contributes to tumor cell heterogeneity.

[0260] Finally, transcriptome analyses revealed that MΦ-cancer cell hybrids predominantly exhibited neoplastic cell transcriptional identity but, notably, retained MΦ gene expression signatures (see also Table S1, available online at advances.sciencemag.org/content/suppl/2018/09/10/4.9.eaat7828.DC1) that clustered into GO Biologic functions attributed to MΦ behavior (Table S2, available on line at advances.sciencemag.org/content/suppl/2018/09/10/4.9.eaat7828.DC1). Of the five independently analyzed hybrid clones, each displayed a high degree of heterogeneity with respect to their MΦ gene expression. Together, these findings support the tenet that cell fusion between MΦs and neoplastic cells produces heterogeneous hybrid cells sharing characteristics of both parental predecessors and having their own characteristics.

[0261] *Fusion hybrids acquire differential response to the microenvironment:* Despite acquiring MΦ gene expression profiles, MΦ-cancer cell fusion hybrids retained *in vitro* proliferative capacity similar to unfused neoplastic cells, as opposed to MΦs. However, with prolonged culture—past confluence—unfused neoplastic cells formed cellular aggregates, whereas MΦ-cancer fusion hybrids remained sheet-like with mesenchymal histologic features suggesting a renewed contact inhibition. This data indicates that although hybrids have similar division rates, they gain differential growth properties as compared to unfused cancer cells. To determine if these *in vitro* differences were recapitulated when cells were grown *in vivo*, *in vitro*-derived hybrids from MC38 or B16F10 cells were respectively injected subcutaneously into the flank, or intradermally into syngeneic immune-competent mice. Hybrids retained tumorigenic potential, with MC38 hybrids displaying shorter doubling times as compared to unfused parental cancer cells, indicating hybrids gain growth advantage in an *in vivo* microenvironment.

[0262] To determine if hybrid cells acquired enhanced ability to seed and/or proliferate in ectopic microenvironments, experimental metastases assays were conducted. MC38-derived hybrids injected into spleens readily trafficked to the liver and resulted in increased metastatic foci per area compared to unfused parental cancer cells indicating that cancer fusion hybrids gained enhanced properties required for trafficking to metastatic sites, seeding, and growing in a new microenvironment. Likewise, B16F10-derived fusions injected intravenously resulted in greater metastatic lung tumor area relative to unfused B16F10, indicating they could have trafficked, adhered, or proliferated more efficiently within the lung. These acquired phenotypic behaviors aligned with data from gene expression analyses that identified increased fusion-associated expression of GO pathway genes implicated in metastatic spread (Table S2, available online at advances.sciencemag.org/content/suppl/2018/09/10/4.9.eaat7828.DC1). In particular those pathways contributing to tumor invasion (attachment, matrix dissolution and migration) as well as pathways involving response to specific microenvironmental cues (Bissell & Hines, *Nat Med.* 17(3):320-9, 2011; Hoshino *et al.*, *J Cell Sci.* 126(Pt 14):2979-89, 2013; Massague, *Cell.* 134(2):215-30, 2008) were upregulated in hybrids relative to unfused tumor cells.

[0263] *In vitro-derived fusion hybrid characterization.* Proliferation analysis of MC38 cells and MC38-derived hybrids injected into flanks of immune competent syngeneic mice (n = 13 mice, each from two different hybrid isolates). Each data point reflects tumor growth in a single mouse. Analysis of metastatic seeding potential of hybrids and MC38 cells injected into spleens, and area analyzed in hematoxylin and eosin-stained tissue sections of the liver (n = 15 mice injected with MC38 cells (three different hybrid clones), n = 17 mice injected with hybrids, with each data point reflecting metastatic tumors analyzed in the liver). Static portrayal of migration tracks were generated from unfused MC38s and a MC38-derived fusion hybrid generated from live-imaged co-cultures. Mean speed of hybrids relative to MC38s was statistically significant, $*p < 1.1 \times 10^{-9}$. *In vitro* invasion assay of MC38 cells and MC38-derived hybrids in Matrigel invasion chambers, stained with crystal violet after 15h. Data reflects the average of triplicate samples in biologic replicates. Hybrid cell chemotaxis towards CSF1 and SDF1 is statistically significant relative to unfused MC38 cells after 24 hr ($p < 0.05$). Three independent experiments of triplicate or quadruplicates were conducted for each ligand. Multiple hybrid clones were assessed. Cells incubated with blocking antibodies to CSF1R and CXCR4 reduce migration of hybrids towards ligands. $p < 0.05$ and 0.01 respectively.

[0264] *B16F10 in vivo-derived fusion hybrids.* B16F10 (H2B-RFP) cells (5×10^4 cells) intradermally injected into GFP-expressing mice (n = 12, two hybrid clones) were harvested at ~1.0 cm at study end point. Fluorescence analyses of tumor sections for RFP (red) and GFP (green) reveal double-positive hybrids and phagocytosed cancer cells with different nuclear morphology. Bar = 25 μ m. B16F10 (H2B-RFP/Cre) cells injected (5×10^4 cells) into R26R-stop-YFP transgenic mice (n=8). Representative FACS-plot of hybrid and unfused cancer cells from a dissociated tumor, e.g., hybrids (red box) and unfused (black box) cancer cells. (n=6 single tumor analyses, n=2 pooled tumor analyses; n=13 mice). 300 FACS-isolated cells injected into wildtype secondary recipient mice (n=19 unfused, n=19 hybrids) analyzed for tumor growth at 40 days, and 3000 FACS-isolated cells injected into syngeneic recipient mice (n=3 MC38 injected mice; black lines, n=3 hybrid injected mice; red lines) and temporally monitored for growth. B16F10 (H2B-RFP) or M Φ -B16F10-derived hybrid cells tail vein injected into wild type mice (n=12 mice). Macroscopic view of lungs and H&E of a tissue section. Quantification of tumor area. Flow analyses of *in vivo*-derived B16F10 fusion hybrids from a primary tumor. RFP/GFP co-expressing cells analyzed for cell surface M Φ identity. All boxes represent hybrid populations. Open box are hybrids that have lost CD45-expression, (n = 6 mice each). B16F10 (fl-dsRed-fl-eGFP) cells intradermally injected into LysM-Cre mice (n=4) were harvested at ~1 cm. Primary tumor or metastatic lung tumors stained with antibodies to GFP, and the tumor protein MITF.

[0265] These results led to asking whether tumor hybrids gain selective advantages in different microenvironments that may reflect primary tumor or metastatic sites. To directly test distinct microenvironmental interactions, adhesion phenotypes and cytokine-dependent growth responsiveness of MC38-derived fusion hybrids were evaluated versus unfused tumor cells using a

microenvironment microarray (MEMA) platform (Lin *et al.*, *J Vis Exp.* (68) doi:10.3791/4152, 2012). This high throughput assay specifically measures cellular behavior in distinct engineered microenvironments containing variable extracellular matrix (ECM) molecules, growth factors, and chemokines, spotted combinatorically in rows and columns, thus permitting comparison of adhesion phenotypes among unfused cancer cells, MΦs, and hybrids. Analysis of microenvironment-specific adhesion revealed that MC38 cells harbor distinct growth factor-independent adhesive preferences for select ECM molecules, most notably, fibronectin. MΦs, by contrast, exhibited enhanced adhesion to collagen XXIII, vitronectin (the ECM component), and more uniform adhesion across all MEMA conditions relative to unfused cancer cells. Interestingly, fusion hybrids exhibited a combination of adhesion biases, reflecting properties of both parental cells, potentially providing a broader adhesive affinity in different microenvironments. Further analysis, using hierarchical clustering, distinguished hybrids from unfused cancer cells with respect to adhesion on independent microenvironments.

[0266] To extend these observations, and to determine whether MΦ fusion provided cancer cells with a selective proliferative or survival advantage, effects of >90 different cytokines, chemokines and soluble factors on unfused MC38s and hybrids were directly analyzed. A number of growth factors induced differential influence on MC38 cells as compared to hybrids, including transforming growth factor (Tgfβ1-3), which induced dose-dependent suppression of MC38 proliferation but showed no effect on hybrids. Likewise, a moderate, dose-dependent growth-suppressing effect of hepatocyte growth factor (Hgf) was apparent on MC38 cells but not on hybrids. More strikingly, hybrids were resistant to tumor necrosis factor (Tnf)-α that profoundly inhibited proliferation of MC38 cells. Resistance of hybrids to cytokine concentrations that suppressed MC38 growth indicates MΦ fusion influences selective cellular phenotypes, and altered cancer cell responses to microenvironmental factors to yield adhesive, proliferative, and potentially survival advantages.

[0267] *Fusion hybrids acquire MΦ-associated phenotypes:* To determine whether cell fusion provides a mechanism by which neoplastic cells acquire MΦ phenotypes consistent with tumor promotion, MΦ attributes upregulated in hybrids identified by GO pathway analysis were evaluated (Table S2 available online at advances.sciencemag.org/content/suppl/2018/09/10/4.9.eaat7828.DC1); behaviors shared by both MΦ and fusion hybrids included migration, invasion and response to paracrine stimuli (Table S3 available online at advances.sciencemag.org/content/suppl/2018/09/10/4.9.eaat7828.DC1). To determine whether hybrids harbored enhanced functional motility, *in vitro*-derived MC38-derived fusion hybrids and unfused MC38 cells were evaluated for migratory capacity in live imaged MΦ and neoplastic cell co-cultures. Using TrackMate analysis to calculate mean speed of cells, hybrid clones exhibited increased motility compared to nearby unfused MC38 cells. Moreover, when evaluated for invasive properties using a Boyden invasion assay, MC38-derived hybrids exhibited enhanced migration and invasion activity, relative to unfused cancer cells; these results were consistent with invasive properties displayed by B16F10-derived hybrid. Of note, two independent hybrid isolates

were evaluated for each cell type; these displayed varying degrees of invasion, supporting the hypothesis that cell fusion can yield heterogeneous clonal outgrowths.

[0268] GO genes involved in “response to stimulus” expressed at high levels in MΦs were also upregulated in MΦ-cancer fusion hybrids. In particular, fusion hybrids harbored elevated expression of the MΦ-associated gene colony stimulating factor 1 receptor (CSF1R), a significant recruitment, differentiation and survival molecule for MΦs (Sherr & Rettenmier, *Cancer Surv.* 5(2):221-32, 1986) implicated in regulating pro-metastatic macrophage effector functions (DeNardo *et al.*, *Cancer Discov.* 1(1):54-67, 2011). To determine whether acquisition of MΦ-associated receptor gene expression translated to functional ligand-mediated migration, hybrids and unfused cancer cells were analyzed in transwell chemotaxis assays coupled to live-imaging (Incucyte Chemotaxis, Essen). Under these conditions, fusion hybrids migrated towards the ligands Csf1 or Sdf1 at multiple concentrations (shown 25 ng/ml) whereas unfused MC38 cancer cells were incapable of responding to either chemoattractants; in contrast, B16F10 cancer cell hybrids exhibited decreased migratory responses. Notably, presence of ligand did not change proliferative dynamics of either fusion hybrids or unfused cancer cells (not shown); however, incubation with blocking antibodies to CSF1R or Cxcr4, reduced chemotactic responses of fusion hybrids. Interestingly, some hybrid clones expressed both CSF1R as well as its main ligand, Csf1.

[0269] *In vivo* generation of tumor cell fusion hybrids: While *in vitro*-derived fusion hybrids allowed for in-depth functional analyses of acquired MΦ behaviors and FISH analysis of human tumors indicated that cell fusion occurs *in vivo*, these studies provided only partial insight into the physiologic relevance of fusion hybrids in human cancer. Therefore, to extend the relevance and functional significance of fusion in enhancing tumor heterogeneity, cell fusion *in vivo* was investigated. MC38 cancer cells were injected into the flanks of R26R-YFP Cre reporter mice; fusion hybrids were identified as RFP+YFP+ cells detected among unfused tumor cells (RFP+) by immunohistochemical analyses of primary tumors.

[0270] Orthotopic injection of MC38 cancer cells into cecum resulted in pervasive peritoneal seeding, limiting the utility of this model. B16F10 melanoma cells injected intradermally into recipient mice readily developed 1.0 cm tumors. Fluorescence analysis of primary B16F10 tumors revealed presence of RFP+/GFP+ fusion hybrids in actin-GFP recipient mice and RFP+/YFP+ fusion hybrids in R26R-YFP Cre reporter mice. Using the latter system, the extent of cell fusion was determined by FACS; primary tumors were dissociated and YFP+/RFP+ fusion hybrids quantified. Within primary tumors, hybrids represented a rare neoplastic cell population (representative experiment, hybrids = 0.48% of RFP+ cells, but overall hybrids range 0.03-0.69% of RFP+ cells from n=6 individually analyzed tumors). To determine if hybrids retained tumorigenicity, 300 FACS-isolated *in vivo*-derived hybrid cells were injected intradermally into secondary recipient mice (n=19) where retention of tumorigenicity was observed. To assess tumor heterogeneity, 3,000 *in vivo*-derived fusion hybrids were subsequently

isolated and injected to facilitate temporal analyses of robust tumor growth properties in n=3 mice. *In vivo*-derived fusion hybrids displayed different rates of tumor growth, indicating that hybrid cells have heterogeneous growth capacity and resulted in different rates of tumor growth.

[0271] Hybrid cell metastatic potential was evaluated using an experimental metastases model. *In vitro*-derived hybrids were introduced into circulation by tail vein injection. Tumor cells that trafficked to the lungs and grew as metastatic foci were identified macroscopically by their pigmented appearance and microscopically on tissue section by H&E staining. Metastatic tumor area was quantified; hybrid cells showed markedly greater metastatic burden than injected unfused tumor cells.

[0272] To determine if *in vivo*-derived fusion was generated by MΦ fusion partners, primary tumors were dissociated into single cells, and fusion hybrids co-expressing RFP and GFP were analyzed for cell surface MΦ antigen expression. Identification of discrete populations of MΦ-associated surface identity indicates a MΦ fusion partner with the cancer cell. To further establish that cancer cells can fuse with MΦs, B16F10 cells harboring fl-dsRed-fl-eGFP were orthotopically injected into LysM-Cre transgenic mice. Primary tumor and lung metastases contained Cre-mediated GFP-expression in tumor cells (positive for tumor marker MITF) consistent with the presence of MΦ-tumor cell fusion. Collectively, these data indicate hybrid cells develop spontaneously *in vivo*, retain tumorigenic capacity, and exhibit accelerated tumor progression properties.

[0273] *MΦ-tumor cell fusion hybrids are enriched in circulation:* Detectable fusion hybrids in both primary and metastatic sites supported the possibility that fused neoplastic cells readily disseminate from primary to distant sites. To explore this, blood from GFP+ mice with established isogenic RFP+ B16F10 tumors was collected. Peripheral blood was subjected to flow cytometry for quantification of circulating tumor cells (CTCs) as defined by RFP expression. RFP+/GFP+ fusion hybrids (or circulating hybrid cells, CHCs) were easily detectable, representing 90.1% of the tumor cells in circulation, dramatically out-numbering unfused RFP+/GFP-ve CTCs. Imaging of individual CHCs confirmed their fusion identity and morphologically distinguished them from MΦs that had phagocytosed or adhered to cancer cells.

[0274] *Murine circulating tumor cells.* B16F10 (H2B-RFP) cells (5×10^4 cells) intradermally injected into a syngeneic GFP-expressing recipient mouse. Blood collected at time of tumor resection and analyzed by flow cytometry for GFP and RFP expression. RFP+GFP+ cells were detectable in pre-sorted cell preparations by immunofluorescence. GFP-expressing blood analyzed by flow cytometry as a negative control for. Percentages of fusion hybrids (RFP+/GFP+) and unfused CTCs (RFP+/GFP-) expressing the leukocyte antigen CD45 were significantly different, * $p < 0.000002$.

[0275] Importantly, the classical definition of CTCs in human cancer is a circulating cell expressing a tumor antigen (typically EPCAM, or cytokeratin for epithelial cancers) and not expressing the pan-leukocyte antigen CD45 (Fehm *et al.*, *Clin Cancer Res.* 8(7):2073-84, 2002; Racila *et al.*, *Proc Natl Acad Sci U S A.* 95(8):4589-94, 1998). MΦs normally express CD45, therefore, it was reasoned that

MΦ-cancer cell fusion hybrids would also express this cell surface epitope. Indeed, the majority of RFP+/GFP+ fusion hybrids expressed CD45, while unfused RFP+ cancer cells largely did not. Based on the classical isolation of CTCs, the novel CHC population is excluded from routine analyses. The diversity of cells derived from fusions. Presence of CD45-expressing CHCs in mouse models prompted investigation of the presence of this unique hybrid population in human cancer patients.

[0276] *Hybrids in humans correlate with disease stage and patient survival:* To evaluate the biological significance of hybrids in humans, first it was determined whether hybrids between blood cells and epithelial-derived cancer cells were detectible. To accomplish this, a disease scenario that supports identification of hybrids harboring properties of peripheral mononuclear blood cells and epithelial cells was exploited (Silk *et al.*, *PLoS One*. 8(1):e55572, 2013) -specifically, the analysis of tumor biopsies from female cancer patients who had previously received a sex-mismatched bone marrow transplant (BMT) and subsequently developed a secondary solid tumor. In these patients, only the male donor-transplanted hematopoietic cells should contain a Y-chromosome, therefore identification of Y-chromosome-positive nuclei in cytokeratin-positive cells within the tumor biopsy could indicate fusion between a peripheral mononuclear blood cell and an epithelial tumor cell. Tumor epithelia was identified by pathologic review on hematoxylin and eosin-stained tumor biopsies. Tissue sections were probed with pan-cytokeratin antibodies and interrogated with Y-chromosome fluorescence in-situ hybridization (FISH) probes to identify cellular products consistent with fusion between neoplastic cells and transplanted male hematopoietic cells (FIGs. 1A-1E, FIGs. 3A-3B). In a biopsy from a female patient with pancreatic ductal adenocarcinoma (PDAC), neoplastic cell nuclei containing a Y-chromosome were readily detectible throughout regions of the tumor (FIGs. 1A-1E, FIGs. 3A-3B), as well as in pre-malignant regions of pancreatic intraepithelial neoplasia (PanIN; FIG. 3C). Confocal microscopy confirmed that Y-chromosomes were located in nuclei of cytokeratin-positive epithelial tumor cells (see higher magnifications in FIG. 1). For these studies, tumor specimens from 7 patients were examined, and all contained evidence of fusion by these criteria. Y-chromosome-positive epithelial tumor cells were not unique to PDAC, as fusion hybrids were detected in other solid tumors from female recipients of sex-mismatched transplantation, including renal cell carcinoma, head and neck squamous cell carcinoma, and lung adenocarcinoma (FIGs. 3D-3F). Control tissue staining from female and male tissue were carried as controls for Y-chromosome detection (FIGs. 4A, 4B). These observations were consistent with previous case reports of cell fusion in human cancer using a variety of other detection methods (Heppner *Cancer Res.* 44(6):2259-65, 1984; LaBerge *et al.*, *PLoS One*. 12(2):e0168581, 2017; Lorico *et al.*, *Biomed Res Int.* 2015:289567, 2015; Lazova *et al.*, *Adv Exp Med Biol.* 714:151-72, 2011).

[0277] *Cell fusion in human tumors.* Solid tumors from women (N = 7) with previous sex-mismatched bone marrow transplantation (BMT) permits analysis of cell fusion. (FIG. 1A) Pancreatic ductal adenocarcinoma tumor section with cytokeratin (CK), the Y-chromosome (Ychr) and Hoechst

detection revealed areas of cytokeratin-positive cells with Y-chromosome-positive nuclei, white arrowhead. Representative areas boxed enlarged in FIGs. 1B-1E. Bar = 25 μ m. To examine hybrid cells in circulation from human patients, peripheral blood from a sex-mismatched bone marrow transplanted female cancer patient was analyzed. Circulating hybrid cells (CHCs) that co-expressed CD45, a pan-leukocyte marker, and EPCAM, an epithelial marker, were detectable (FIG. 2A). Both these CHCs and leukocytes expressed the Y-chromosome (FIG. 2A, FIG. 4B). To determine whether CHCs expressed M Φ markers, analogous to fusion hybrids detected in the murine tumor model, M Φ epitope expression by immunohistochemical (FIG. 2B) and by flow cytometric analyses (FIG. 2C, FIG. 5) was performed. A variety of M Φ epitopes were expressed on CK+/CD45+ CHCs, including CD163, CD68, CSFR1 and CD66b (FIG. 2B). Similarly, flow-cytometric analyses revealed that CHCs from three different PDAC patients expressed M Φ epitopes, including CD14, CD16, CD11c, and CD163 (FIG. 2C). CTCs analyzed from the same patients had low expression levels of CD16. These results suggest that M Φ -tumor cell hybrids are the predominant tumor cell in circulation, even though other leukocyte-tumor cell hybrids with discrete M Φ surface antigen expression most likely exists.

[0278] To explore the presence of CHCs in pancreatic cancer patients diagnosed at various tumor stages, node-negative, node-positive, or metastatic, peripheral blood was collected and *in situ* antibody staining (CD45, CK) performed on isolated cells. Digital image analyses allowed validation of double-positive expression of CD45 and CK on CHCs (FIG. 2D) while excluding doublets or clusters of cells registering as double-positive cells by flow cytometry. The number of CHCs expressing CD45+/CK+ significantly correlated with advanced disease (FIG. 2E) were determined and, notably, this provided a prognostic indicator of overall survival, regardless of disease stage (FIG. 2F). Conventionally defined CTCs (CD45-ve/CK+) did not correlate with stage or survival (FIGs. 2E, 2G) and were detected at quantities an order of magnitude lower than CHCs in metastatic disease. These findings identify a novel population of tumor cells in circulation, a population previously overlooked and excluded from routine analyses, which has biologic function and correlation to clinically relevant disease status in human cancer patients.

[0279] *Human circulating tumor cells.* (FIG. 2A) Sex-mismatched bone marrow transplanted (BMT) patient who acquired a solid tumor (pancreatic ductal adenocarcinoma, PDAC). Peripheral blood analyzed for the presence of cell fusion.

[0280] Two panels displaying cell fusion hybrids (arrowheads) that co-stain for EPCAM, CD45, and have Y-chromosome (white dot) in their nuclei. Arrow denotes leukocytes. (FIG. 2B) Circulating hybrid cells (CHCs) and circulating tumor cells (CTCs) analyzed from n=4 patients with PDAC. CHCs (CK+/CD45+) also express M Φ proteins (cocktail = CD68, CD163, CD66b, CSF1R), while CTCs (CK+/CD45-ve) do not. CHCs also express the tumor specific protein MUC4. (FIG. 2C) CHCs and CTCs analyzed by flow cytometry for CD14, CD16, CD11c and CD163 expression, or the cancer specific protein MUC4. (n=4 patients). (FIG. 2D) Human pancreatic cancer patient peripheral blood

analyzed for cytokeratin+ and CD45+ expression using *in situ* analyses and digital scanning. (FIG. 2E) CK+/CD45+ and CK+/CD45- cells quantified in patient blood across cancer stage, *ANOVA $p < 0.023$. (FIGs. 2F, 2G) Kaplan-Meier Curve of dichotomized biomarkers based on median value (CHC and CTC) was associated with statistically significant increased risk of death for CHC ($p = 0.0029$) but not for CTCs ($p = 0.95$).

[0281] Discussion

[0282] Cell fusion between immune and neoplastic cells initiating tumorigenesis and impacting progression is an untested, century-old hypothesis (Carter, *J Natl Cancer Inst.* 100(18):1279-81, 2008; Pawelek, *Lancet Oncol.* 6(12):988-93, 2005) that has been only circumstantially examined (Pawelek, *Lancet Oncol.* 6(12):988-93, 2005; Powell *et al.*, *Cancer Res.* 71(4):1497-505, 2011; LaBerge *et al.*, *PLoS One.* 12(2):e0168581, 2017; Lorico *et al.*, *Biomed Res Int.* 2015:289567, 2015; Halaban *et al.*, *Somatic Cell Genet.* 6(1):29-44, 198; Kerbel *et al.*, *Mol Cell Biol.* 3(4):523-38, 1983; Lizier *et al.*, *Oncotarget.* 7(38):60793-806, 2016; Lu & Kang, *Cancer Res.* 69(22):8536-9, 2009; Shabo *et al.*, *BMC Cancer.* 15:922, 2015). Reports of cells located in tumors containing components of both immune and neoplastic cells are increasingly frequent (Heppner, *Cancer Res.* 44(6):2259-65, 1984; Pawelek, *Lancet Oncol.* 6(12):988-93, 2005; Dittmar & Zanker, *Int J Mol Sci.* 16(12):30362-81, 2015; Powell *et al.*, *Cancer Res.* 71(4):1497-505, 2011; Lazova *et al.*, *PLoS One.* 8(6):e66731, 2013; Grimm *et al.*, *Oral Surg Oral Med Oral Pathol Oral Radiol.* 121(3):301-6, 2016; LaBerge *et al.*, *PLoS One.* 12(2):e0168581, 2017; Yilmaz *et al.*, *Bone Marrow Transplant.* 35(10):1021-4, 2005; Lizier *et al.*, *Oncotarget.* 7(38):60793-806, 2016; Patsialou *et al.*, *Oncogene.* 34(21):2721-31, 2015; Mo *et al.*, *Cancer Res* 73, Abstract A26, 2013; Sheng *et al.*, *Lab Chip.* 14(1):89-98, 2014; Clawson *et al.*, *PLoS One.* 7(7):e41052, 2012; Clawson *et al.*, *PLoS One.* 12(9):e0184451, 2017; Rappa *et al.*, *Am J Pathol.* 180(6):2504-15, 2012; Xu *et al.*, *PLoS One.* 9(2):e87893, 2014), though without strong evidence of etiologic mechanism or physiologic relevance. Early *in vitro* studies revealed cell fusion hybrids displayed reduced cell doubling times relative to their genetic burden (McGill *et al.*, *Cell Prolif.* 25(4):345-55, 1992). More recently, it was suggested tumor cell fusions gain shorter cell cycling times compared to either of their parental cells (Islam *et al.*, *Stem Cells Dev.* 15(6):905-19, 2006; Xue *et al.*, *BMC Cancer.* 15:793, 2015). These divergent views add to the controversy of whether cell fusion provides a selective advantage to evolving tumors. Moreover, description of macrophage (MΦ) gene expression in metastatic cancer cells as evidence fusion propagates aggressive metastatic spread of cancer (Lorico *et al.*, *Biomed Res Int.* 2015:289567, 2015; Clawson *et al.*, *PLoS One.* 12(9):e0184451, 2017) is presented without substantial proof that these cells arise from a fusion event, further diminishing enthusiasm for this mechanism.

[0283] Despite this, neoplastic cell-MΦ fusion does provide an intriguing mechanism for how neoplastic cells rapidly gain discrete cellular behaviors to facilitate metastases and to propagate

intratumoral heterogeneity. Prior to this study, experimental results demonstrating *in vivo* tumor cell fusion with MΦs, or investigating a function role for cell fusion in tumor progression was undetermined. Here, it is demonstrate cell fusion occurs spontaneously in a number of systems. Cell fusion can contribute to the generation of diverse neoplastic clones with altered phenotypes, implicating it as a mechanism for gain of intratumoral heterogeneity. This finding may reveal insight into diverse tumor cell pathophysiology that underlies treatment resistance, progression, and post-treatment tumor recurrence in human cancer.

[0284] Present herein is a systematic analysis of MΦ-cancer cell fusion, along with evidence that hybrids impart physiologically relevant and functionally significant aspects contributing to tumor evolution. Together, these *in vitro* and *in vivo* murine data indicate neoplastic cells fuse spontaneously with leukocytes and myeloid (*i.e.* MΦs, neutrophils, or dendritic cells) produce heterogeneous cancer hybrid clones. Despite their diversity, hybrid clones retain MΦ genotypes with functional phenotypes, thereby bestowing MΦ-like behaviors on neoplastic cells. Fusion hybrids express functional levels of CSF1R, which is relevant to cancer progression exemplified by the association of CSF1 overexpression in lung cancer with increased tumor cell proliferation and invasion (Hung *et al.*, *Lab Invest.* 94(4):371-81, 2014), by the inhibition of CSF1R with decreased tumor metastasis (Mitchem *et al.*, *Cancer Res.* 73(3):1128-41, 2013), and by late stage metastatic breast carcinomas frequently acquiring CSF1R expression (Patsialou *et al.*, *Oncogene.* 34(21):2721-31, 2015). In human tumors, the mechanism by which tumor cells gain chemotactic responsive receptor expression remains unclear; multiple mechanisms likely underlie transcriptional changes. Our data indicates cell fusion could play a role in the acquisition of migratory/chemotactic functional behavior, and may have important clinical implications considering hybrids' potential response to clinically relevant CSF1R inhibitors (DeNardo *et al.*, *Cancer Discov.* 1(1):54-67, 2011; Ngiow *et al.*, *Oncoimmunology.* 5(3):e1089381, 2016; Ruffell & Coussens, *Cancer Cell.* 27(4):462-72, 2015). Data presented here indicate MΦ-cancer cell hybrids are differentially responsive to microenvironment-derived regulatory forces. Specific extracellular conditions provide a selective adhesive and/or growth advantage to hybrids but not to unfused neoplastic cells. Given that genotypic and phenotypic diversity provides selective advantages to the fittest neoplastic clones, continued cell fusion may underlie adaptation, survival, and growth of dominant neoplastic clones within the evolving tumor microenvironment during tumor progression. Considering this, cell fusion provides a previously underappreciated mechanism by which phenotypic diversity is gained by neoplastic cells, increasing the opportunity for highly fit sub-clones to overcome selection pressure and drive tumor progression.

[0285] These data indicate tumor-initiating hybrid populations are able to acquire behaviors allowing for navigation of the metastatic cascade - from the primary tumor, to survival in circulation, to seed ectopic sites, and to propagate metastatic foci. *In vivo*-derived hybrid cells were readily detected in peripheral blood. Circulating hybrid cells (CHCs) outnumbered conventionally-isolated CTCs in both

mice and humans. Moreover, in patients with pancreatic cancer, CHCs directly correlated with tumor stage, and inversely correlated with overall survival - highlighting an exciting prognostic opportunity for development of this novel cell population as a liquid biomarker for disease status in discrete cancers where conventional biomarkers (e.g. CTCs, cell-free DNA, exosomes, proteins) have not demonstrated efficacy.

[0286] Unlike in murine models, the etiology of human CHCs, while consistent with cell fusion, cannot be conclusively determined. It is possible that tumor cells can gain expression of leukocyte and MΦ-associated proteins by an undetermined mechanism, or that CD45-expressing blood cells transdifferentiate into epithelial-cancer cells. Despite these unexplored caveats, the CHC is a population of tumor cells that is overlooked and under studied. Initial investigations indicate that this novel cell population has exciting potential.

[0287] Identification of functionally significant properties of this unique tumor population, a chimera of MΦs and neoplastic cells, offers opportunities for understanding the dynamic interaction between neoplastic cells and diverse infiltrating immune cell populations. Elevated CHCs relative to CTCs in peripheral blood might suggest hybrids are immune privileged—a trait bestowed by their leukocyte identity. This scenario could have implications on immune-mediated therapeutic strategies for cancer treatment. Therefore, understanding how hybrids respond to immune therapies, such as inhibitors or agonists to co-stimulatory and/or co-inhibitory receptors, offers an important area of future investigation. Acquisition of functional myelomonocytic receptors on hybrids indicates they may be vulnerable to targeted therapies such as CSF1/CSF1R blockade, now being investigated in clinical trials (Ruffell & Coussens, *Cancer Cell*. 27(4):462-72, 2015). Alternatively, these therapies may inhibit MΦ-neoplastic cell fusion. The presence of tumor cells with acquired MΦ phenotypes supports a cell fusion mechanism in the propagation of intratumoral heterogeneity, introduces a functionally significant aspect of tumor progression and evolution, identifies an unappreciated circulating hybrid cell population, and uncovers a new area of tumor cell biology.

[0288] Example 2: Representative Cell Separation Techniques

[0289] This example provides descriptions of representative cell separation techniques, which for instance can be used to separate, and optionally quantify, circulating hybrid cells.

[0290] One primary technique for identifying CHCs is fluorescence microscopy (e.g., based on antibody-mediated binding to molecular marker(s); fluorescent immunostaining); however, spreading millions of cells on a glass microscope slide poses challenges for detecting CHCs. Cells tend to aggregate on the surface of glass microscope slides, making it difficult to identify cellular boundaries. Poorly defined cellular boundaries yield convoluted fluorescence signatures; sources of fluorescence signal can become indiscernible. Issues involving aggregation, coupled with the fact that CHCs are

present at relatively low concentrations in the blood, complicate fluorescence detection of CHCs using conventional hardware.

[0291] Spatially separating individual cells from the blood can de-convolute fluorescence signatures, making CHC detection more feasible. Spatial separation can be achieved by allowing a suspension of cells to settle via gravity or centrifugation into wells that have been patterned in an optically transparent, polymeric microscope slide. Each microscope slide can contain hundreds of thousands or millions of cylindrical wells with diameter, depth, and inter-well spacing on the micrometer scale (0.1 – 100 μm). Identifying and patterning appropriate cylinder dimensions can minimize the number of wells containing multiple cells and maximize cell boundary separation. Cells can be stained either before or after settling into wells on the microscope slide. Using existing slide scanning technology, millions of wells can be examined in an automated way. Custom software can analyze fluorescence images produced from slide scanning to detect CHCs and provide quantitative information about CHC concentration.

[0292] *Representative Cell Separation Methods:* A number of methods may be employed to separate rare cell populations from blood. Some methods use physical or biophysical separation criteria while others use affinity-based methods. Among the former, separating cells by density gradient centrifugation is common but the process is time consuming and recovery of small cell populations is difficult. A microfluidic version using paramagnetic medium to separate cells in flow by buoyant density has been described (Utkan *et al.*, *PNAS* 112(28):E3661-E3668, 2015) for CHCs. This method works well for cell populations that do not have high overlap with the unwanted cells, *i.e.* red blood cells (rbc) vs. white blood cells (wbc), but is less effective for populations that have high overlap in density, *i.e.* monocytes vs lymphocytes.

[0293] CHCs are larger than wbcs so they have been isolated using track etched Nucleopore membranes, in planar well arrays, in microfluidic channels with crenulated channel profiles, field flow fractionation, flow sorting by size, dielectrophoresis, and directed diffusion “pachinko effect.” Multiple parameters can be used simultaneously or sequentially to increase yield and/or purity.

[0294] In one version, which may be referred to as a “Cell-in-Well” process (FIG. 6), white blood cells (WBCs or wbcs) are placed in a uniform manner on a microscope slide as single cells in a monolayer that can be processed with immunofluorescence (IF) and fit into a slide scanner. Only one cell per well location on one focal plane allows for easy use of a slide scanner to analyze every cell. As many cells as possible (in some embodiments > 1,000,000) are placed per slide in a nonselective manner (*i.e.*, almost any cell can fit in a well). A polymer, such as polydimethylsiloxane (PDMS) in some embodiments, is used to create the well; the polymer needs to not attract the cell and be compatible with IF analysis.

[0295] By way of example, for an initial master photolithography is used to etch different sized, cylindrical shapes to the characteristics required to load a single cell per well while having as many

well occupied as possible. The initial master plate can, for instance, be made with a 4 cm plate; then the PDMS will be diced (separated into individual flow cell units) to be similar size of microscope glass slides.

[0296] Cells can be covered by Matrigel for growing to 3D spheroids. One can differentiate CHCs from other CTC or WBC by the capability of cell proliferation.

[0297] A second, two-step cell separation embodiment provides:

- (A) A polymer (such as PDMS) with through-hole well(s) is placed on top of a diced Indium Tin Oxide (ITO) coated glasses which allows user to either (1) apply positive charge and attract the cells to the wells, and/or (2) immersing the whole chip in cell suspended media and applying an AC field which creates dielectrophoresis force to attract the cells. The electrodes in such a system must also be compatible with IF analysis. Cells will embed themselves in a layer of Matrigel (or another matrix) between the top of the PDMS well and the Pt electrode to form a 3-dimensional matrix which will allow CHC to proliferate into organoids. In an alternative approach, the electrode and agarose (or another matrix) could be replaced by a layer of Poly-L-Lysine on the slide, subsequently fixed with formaldehyde. Finally, a removable reservoir is made around the entire slide, to permit antibody staining. The reservoir can also be used to divide the slide into multiple (e.g., 10) regions for running multiple tests or controls on the same slide. (For more details see designs below.)
- (B) The PDMS slides and/or ITO coated glasses substrate is compatible with slide readers. This compatibility provide tremendous speed to examine multiple slides in a short period of time.

[0298] In a third version (FIG. 7), a polymer (such as PDMS) with through-hole well(s) is placed on top of a water permeable membrane to create > 1,000,000 transwell arrays. This chip is placed in a 3D-printed fluidic chamber followed by pipetting cell suspended media on top. By applying negative pressure, cell settle individually into each well by hydrodynamic force in a very short time. This fluidic chamber is then used to carry out following staining and washing processes.

[0299] Example 3: Detection/Determination by Secretion Assays

[0300] In typical cell-in-well applications, immunostaining with cell specific markers (such as CHC-specific markers, or cell-type specific markers) is used to identify the target population for enumeration or downstream assays, such as sequencing. Unique cell markers are not always readily available so it is advantageous in some embodiments to identify the target cells using other techniques. These methods can be used independently, or combined with immunostaining to provide a more robust signature for diagnostic or therapeutic applications.

[0301] One approach uses the cell secretome to differentiate CHCs from other cells, such as CAMLs, CTCs, WBCs, etc. Cancer cells secrete proteases, cytokines, exosomes, etc. that can be used as a proxy for the cell type. In one embodiment, beads containing quenched protease substrates and/or

cytokine binders can be loaded into the wells before/after the cells are loaded. If the correct protease(s) is secreted, then it will cleave the substrate, releasing a detectable signal, such as fluorescence. Similarly, if a particular cytokine in bound it would alter the conformation of the affinity agent and unquench the reporter molecule. Imaging of the well array would identify target cells. Each bead can have multiple substrates and binders and can be multiplexed by color. Use of many smaller beads can increase surface area and ensure that every well is loaded.

[0302] Exosomes can be detected in similar manner by using CD63, CD9, and/or CD81 binders that are capable of conformational change upon ligand binding. Conformational change results in reporter signal.

[0303] Signal-noise ratio would be dependent on how dark or well quenched the initial state is. DNA molecular beacons have good On/Off ratios and can be multiplexed readily using the same stem sequences. Peptide substrates could use DNA sequences to control the distance between quencher and reporter and be common to all enzyme substrates.

[0304] Example 4: Dielectrophoretic Cell Sorting

[0305] Dielectric particles (including cells) exposed to non-uniform electric fields are subjected to external forces that can modify/control their trajectory. The magnitude of these forces depends, among others, on the electrical properties of the particles and the medium, the shape and size of the particles and the frequency of the electric field. This frequency can be tuned to manipulate particles/cells with great selectivity, allowing to sort and collect specific cell types from a general population in a label-free manner. This opens the door to the design of a variety of devices that make use of electrokinetic technology to sort and collect cells.

[0306] For example, a continuous cell sorter for the isolation of CHCs can be fabricated by combining a microfluidic channel and a Ti/Pt electrode layer on a glass/silicon substrate (see schematic). The sample containing CHCs and other cell types is injected in the device with a specific flow rate. A sheath flow is injected from another inlet. Controlling the voltage and frequency of the AC field applied to the electrodes allows to control the sign and magnitude of the force exerted to the CHCs. The direction of the force and thus, the trajectory of the CHCs will depend on the direction of the electric field, that is, it will depend on the shape of the electrodes. A suitable electrode design will allow to progressively move the CHCs toward a specific outlet in the device and thus, isolate the CHCs from other cell types.

[0307] Multiple electrode batches/devices (with particular voltages and frequencies) can be disposed in series to isolate multiple cell types simultaneously or refine/improve the initial sorting (see schematic). The distance between electrodes can be modified to change the magnitude of the electric gradient and thus, the DEP force. Smaller distances will induce stronger forces on the cells.

[0308] At the end of the batches of devices, arrays of DEP electrodes perpendicular to the flow will be used to capture CHC from targeted channel which allows concentration and enrichment the CHC

population. These enriched CHCs can be released to the outlet by infusing small amount of fluid after pausing the DEP force which allows user to concentrate the cells number per volume.

[0309] The chip has excellent optical transparency, therefore a real-time monitoring camera combined with a hardware based cell counting algorithm can be embedded to give number of CHCs passing the targeted channel, the percentage of CHCs out of the amount of WBCs can be acquired. A quick screening results can be achieved by running through large portion of samples by using these real-time monitoring system.

[0310] Dielectrophoresis (DEP)-responsive particles can be used to label CHCs, potentially improving contrast between dielectric properties of CHCs and those of surrounding cells, bioparticles, and fluids. Enhanced contrast of dielectric properties via DEP-responsive particle can sequentially enable identification, isolation, and purification of CHCs from biosamples. CHCs can be isolated using the DEP flow-sorter technology previously described in this document by treating a biosample with DEP-responsive tags functionalized to target a type of biomarker that is common to all subclasses of CHCs. Depending on the achievable degree of contrast, subclasses of CHCs could be separated from one another as well.

[0311] After retrieving CHCs using the DEP flow-sorter and concentrating the suspension, biomarkers specific to each subclass of CHCs may be targeted using respective types of functionalized DEP-responsive particles. CHCs can be flowed into a chamber where a given AC signal ("AC signal #1") prompts a positive DEP response in particles ("particle type #1") bound to a given CHC subclass ("CHC subclass #1") that enables collection. A different subclass of CHCs ("CHC subclass #2") bound to another set of particles ("particle type #2") may either respond via negative DEP or not respond. CHC subclass #2 can be removed from the chamber, while CHC subclass #1 is held via DEP within the chamber, during a subsequent wash. The result is two purified suspensions, each containing an isolated subclass of CHCs. Applying a different AC signal ("AC signal #2") to initiate a DEP response in particle type #2 enables collection of CHC subclass #2 via positive DEP within the static flow chamber while prompting a negative or minimal DEP response in CHC subclass #1.

[0312] DEP responsive particles can include, but are not limited to: several geometries and morphologies (spheres, cubes, polyhedral, Janus, core-shell, fibrous, cylindrical, etc.), functionalized with a variety of capping molecules (organic, polymeric, biochemical, etc.), composed of at least one material or metamaterial (ferroelectric, metallic, metal/metal-oxide, polymeric, etc.), over a range of sizes (nanoscale between 0.1 to 100 nm; microscale between 0.1 to 100 μ m).

[0313] The markers could be intra- or extracellular and could be used individually or in combination to fine tune DEP separations.

[0314] Example 5

[0315] Circulating cells in peripheral blood that express epithelial and/or tumor gene/protein expression, plus CD45 and/or macrophage gene/protein expression are termed circulating hybrid cells (CHCs). This example provides representative functional utilities of these CHCs in patient samples.

[0316] *Specific CHC subtypes correlate with disease pathology across the cancer disease continuum*

[0317] FIG. 9A-9C. CHCs are detected in multiple different cancer organ sites. Using an in situ platform, by which peripheral blood mononuclear cells are first isolated, then spread onto glass slides, CHCs are identified as cytokeratin+ (CK+)/CD45+ double positive for epithelial cancers such as pancreatic ductal adenocarcinoma (PDAC, shown in FIG. 9A), esophageal, lung, breast, head and neck squamous carcinoma, prostate cancer, and colorectal cancer. For non-epithelial cancers such as uveal (or cutaneous) melanoma (shown in FIG. 9A) or glioblastoma (shown in FIG. 9C), CHCs are defined as gp100+/CD45+ in uveal melanoma (shown in FIG. 9B), and GFAP+, Nestin+/CD45+ in glioblastoma (shown in FIG. 9C), respectively. CHCs can also be quantified by flow cytometry and results are visualized in FIG. 9B. In all cases, CHCs outnumber conventionally defined CTCs (CK+/CD45-) cells, shown as black circles or bars. Further, straight enumeration of CHCs within a cancer disease site correlates with disease burden, *i.e.* higher numbers are observed in higher stage disease (see FIG. 9A: comparison of stage I disease with stage IIIa/IV for PDAC and uveal melanoma).

[0318] *CHC levels in early stage cancer patients are detected at levels higher than those found in normal healthy control people*

[0319] FIG. 10 Using in situ detection and quantification of CHCs, CK+/CD45+ CHCs are detected at higher numbers in cancer patients relative to healthy controls. This graph compares CHCs from early and late stage breast cancer patients relative to healthy controls. CHCs in early stage breast cancer are detected 2-3 fold greater numbers than the baseline in healthy normal control blood. A wide range of CHCs are detected in late stage disease.

[0320] FIG. 11 In addition, CHC enumeration in lung cancer revealed an increasing trend of cells correlating with increased stage. Here a patient diagnosed with early stage lung cancer (*i.e.* 1a, designated by asterisk) had levels of CHCs that were consistent with late stage disease. The patient passed away 1 month after diagnoses and it was determined he had metastatic tumors, and thus should have been diagnosed with late stage disease (stage IV). In this experiment, CHCs are defined as CK+/CD45+.

[0321] *CHCs can be subtyped by their protein expression—discrete subtypes of CHCs define different pathologic states within an organ system.*

[0322] FIG. 12A-12C CHCs can be subtyped into different “phenotypes”. Two types of subtyping panels have been developed, one that recognizes cancer-derived CHCs (**cancer-Abs**: MUC4+, Maspin+) and one that recognizes epithelial-derived hybrids generated from inflammation of the

epithelium (**epithelial-Abs**: ECAD+, EpCAM+, CK+). When applied to patient peripheral blood cells, the cancer Abs and Epithelial Abs can stratify patients with chronic inflammation (pancreatitis)—a high risk pathology for cancer, from patients with PDAC. Healthy normal control subject peripheral blood does not harbor cancer-derived CHCs, but does have very low baseline epithelial-derived hybrids. (FIG. 12A) Using an in situ assay, PBMCs are stained with cocktails of cancer antibodies and epithelial antibodies as well as with antibodies to CD45. CHCs in PDAC patient blood co-express CD45, cancer antibodies and epithelial antibodies designated by yellow arrow in top panel. CHCs in patients with chronic pancreatitis co-express CD45 and epithelial antibodies, but not cancer antibodies, designated by the yellow arrow in the bottom panel. (FIG. 12B) together, screening ten patients with PDAC or chronic pancreatitis or healthy normal controls, cancer-derived CHCs and epithelial-derived CHCs are detectable and the different subtypes can differentiate patients with chronic pancreatitis from patients with cancer. Additional cancer epitopes will allow for definition of different subclasses of cancers (*i.e.* breast cancer that is triple positive will harbor CHCs that stain positive for hallmarks of this disease). This discriminatory panel of antibodies will facilitate detection of cancer in high risk groups, such as those with chronic inflammation.

[0323] FIG. 13 Cell signaling pathway activation can be assessed in CHCs. Here activation of the ERK signaling pathway is identified in CHCs derived in cancer and pancreatitis patients. PBMCs were processed and adhered to glass slides, using standard methods (Gast *et al.*, *Sci. Adv.* 4:3aa67828, 1-15, 2018). PBMCs were stained with CK and CD45 antibodies, as well as phospho-ERK (pERK) antibodies. The left panels shows cell populations that are positive for CK+/CD45+ (pink circle) and are therefore considered to be CHCs. Light blue events are from pancreatitis patients, dark blue events are from PDAC patients, and green, red, brown and yellow events are control cells). The right panel depicts cells that are both expressing pERK and CK+ (red box). CHCs from both PDAC and Pancreatitis patients have activated ERK signaling.

[0324] FIG. 13 CHCs analyzed over the course of a patients treatment changes in phenotype. The grids depict CHCs with different protein expression (each column is one CHC), and the change in these phenotypes over time.

[0325] *CHCs have phenotypes that align with phenotypes of the primary tumor, as determined by comparing biopsy and CHC protein expression*

[0326] FIG. 14A-14B Multiple proteins can be analyzed on CHCs that define hormone status, proliferative status, stem status. In the case of this breast cancer patient CHC analyses, CHCs protein expression was consistent with subsets of tumor cells in a tumor biopsy taken at the same time. However, it was determined that CHCs reflect a discrete subset of tumor cells, perhaps those that have the greatest ability to disseminate.

[0327] FIG. 15 CHCs analyzed over the course of a patients treatment changes in phenotype. The green and yellow grids depict CHCs with different protein expression (each column is one CHC), and

the change in these phenotypes over time. The ability to temporally analyze CHCs from cancer patients allows for an assay that can be longitudinally evaluated. This would provide insights into evolution of the tumor in the face of therapeutic treatment and could be useful in defining treatment resistance or identifying new therapeutic targets. In addition, temporal monitoring of CHCs can be used to screen high risk patients for earliest signs of conversion to cancer.

[0328] Example 6: Identifying Aggressive Disease

[0329] This Example describes using the herein-described methods of detecting CHCs in stratifying patients as to disease aggressiveness.

[0330] In standard practice, Head and Neck Squamous Cell Carcinoma patients who are clinically node-negative (cN0) undergo a neck dissection to determine if they have lymph nodes with tumor cells (*i.e.* if they are pathologic node-positive, pN1). This is a morbid surgery with no curative value. It would be beneficial to have a less invasive method for determining the severity of carcinoma in such patients.

[0331] Peripheral blood was acquired at the time of surgery, and analyzed for presence of CHCs (CK+/CD45+). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll gradient, then plated and fixed onto glass slides. PBMCs were stained with antibodies to CK and CD45 and digitally imaged. Manual enumeration of CHCs was conducted and numbers then correlated with patient identification.

[0332] As illustrated in FIG. 17, pre-surgery levels of CHCs strongly correlate with patients that convert to pN1+ (right hand column). Patients that are designated pN0 have a 30% risk of disease recurrence within 2 years after surgery, meaning that they most likely had undetectable positive nodes. All of the pN0 patients in the study are under 2 years post-surgery. The circle indicated with an arrow denotes a patient that has developed recurrent disease. The dashed line designates the value (from the ROC curve) that predicts aggressive disease.

[0333] As will be understood by one of ordinary skill in the art, each embodiment disclosed herein can comprise, consist essentially of or consist of its particular stated element, step, ingredient or component. Thus, the terms “include” or “including” should be interpreted to recite: “comprise, consist of, or consist essentially of.” The transition term “comprise” or “comprises” means includes, but is not limited to, and allows for the inclusion of unspecified elements, steps, ingredients, or components, even in major amounts. The transitional phrase “consisting of” excludes any element, step, ingredient or component not specified. The transition phrase “consisting essentially of” limits the scope of the embodiment to the specified elements, steps, ingredients or components and to those that do not materially affect the embodiment. A material effect, in this context, is any change in method or composition that reduces reliability of detection or characterization or identification of a circulating hybrid cell (CHC), or a type or subtype of CHC.

[0334] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. When further clarity is required, the term “about” has the meaning reasonably ascribed to it by a person skilled in the art when used in conjunction with a stated numerical value or range, *i.e.* denoting somewhat more or somewhat less than the stated value or range, to within a range of $\pm 20\%$ of the stated value; $\pm 19\%$ of the stated value; $\pm 18\%$ of the stated value; $\pm 17\%$ of the stated value; $\pm 16\%$ of the stated value; $\pm 15\%$ of the stated value; $\pm 14\%$ of the stated value; $\pm 13\%$ of the stated value; $\pm 12\%$ of the stated value; $\pm 11\%$ of the stated value; $\pm 10\%$ of the stated value; $\pm 9\%$ of the stated value; $\pm 8\%$ of the stated value; $\pm 7\%$ of the stated value; $\pm 6\%$ of the stated value; $\pm 5\%$ of the stated value; $\pm 4\%$ of the stated value; $\pm 3\%$ of the stated value; $\pm 2\%$ of the stated value; or $\pm 1\%$ of the stated value.

[0335] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0336] The terms “a,” “an,” “the” and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, “such as”) provided herein is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0337] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain

the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0338] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0339] Furthermore, numerous references have been made to patents, printed publications, journal articles and other written text throughout this specification (referenced materials herein). Each of the referenced materials are individually incorporated herein by reference in their entirety for their referenced teaching.

[0340] It is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that may be employed are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention may be utilized in accordance with the teachings herein. Accordingly, the present invention is not limited to that precisely as shown and described.

[0341] The particulars shown herein are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of various embodiments of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for the fundamental understanding of the invention, the description taken with the drawings and/or examples making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

[0342] Definitions and explanations used in the present disclosure are meant and intended to be controlling in any future construction unless clearly and unambiguously modified in the example(s) or when application of the meaning renders any construction meaningless or essentially meaningless. In cases where the construction of the term would render it meaningless or essentially meaningless, the definition should be taken from Webster's Dictionary, 3rd Edition or a dictionary known to those of ordinary skill in the art, such as the Oxford Dictionary of Biochemistry and Molecular Biology (Ed. Anthony Smith, Oxford University Press, Oxford, 2004).

LISTING OF CLAIMS

What is claimed:

1. A method of treating cancer in a human patient, the method comprising:
obtaining a sample from the human patient;
detecting whether Circulating Hybrid Cells (CHCs) are in the sample by
 contacting the sample with an anti-(source of the cancer) antibody;
 contacting the sample with an anti-immune cell antibody;
diagnosing the patient as having cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more Circulating Hybrid Cells; and
administering to the patient in need thereof a pharmaceutically effective amount of an anti-cancer agent.
2. The method of claim 1, wherein the cancer comprises: breast cancer, prostate cancer, head and neck squamous cell carcinoma, lung cancer, pancreatic ductal adenocarcinoma, colorectal cancer, glioblastoma, or melanoma.
3. A method of detecting Circulating Hybrid Cells (CHCs) in a human patient, the method comprising:
 obtaining a sample from the human patient; and
 detecting whether CHCs are in the sample by
 contacting the sample with an anti-source cell antibody that recognizes a marker other than cytokeratin (CK); and
 contacting the sample with an anti-immune cell antibody;
wherein specific binding of both antibodies in the same cell indicates the presence of CHCs.
4. A method of diagnosing a solid tumor in a human patient, the method comprising:
 obtaining a sample from the human patient; and
 detecting whether Circulating Hybrid Cells (CHCs) are in the sample by:
 contacting the sample with an antibody specific for a protein found on cells from a tissue from which the solid tumor is derived;
 contacting the sample with an antibody specific for an immune cell; and
diagnosing the patient as having a solid tumor wherein specific binding of both antibodies in the same cell indicates the presence of one or more CHCs.

5. The method of claim 4, comprising:
 - contacting the sample with at least two different antibodies specific for proteins found on cells from a tissue from which the solid tumor is derived;
 - contacting the sample with at least two different antibodies specific for an immune cell; and
 - diagnosing the patient as having a solid tumor wherein specific binding of antibodies specific for proteins found on cells from a tissue from which the solid tumor is derived and antibodies to immune cells in the same cell indicates the presence of one or more CHCs.
6. The method of claim 4, wherein the solid tumor is a glioblastoma, a melanoma, a head and neck squamous cell carcinoma, a pancreatic ductal adenocarcinoma, a colorectal cancer, a prostate cancer tumor, or a breast cancer tumor
7. A method of diagnosing metastatic cancer in a human patient, the method comprising:
 - obtaining a sample from the human patient; and
 - detecting whether Circulating Hybrid Cells (CHCs) are in the sample by
 - contacting the sample with an anti-(source of cancer) antibody;
 - contacting the sample with an anti-immune cell antibody; and
 - diagnosing the patient as having a metastatic cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more CHCs.
8. A method of differentiating disease status of a human patient, the method comprising:
 - obtaining a sample from the human patient; and
 - typing Circulating Hybrid Cells (CHCs) are in the sample by
 - contacting the sample with at least two panels of antibodies, each panel including at least two antibodies, wherein:
 - a first panel of antibodies comprising at least one anti-immune cell antibody and at least one antibody specific for a first source cell type; and
 - a second panel of antibodies comprising at least one anti-immune cell antibody and at least one antibody specific for a second source cell type,
 - wherein the first source cell type and the second source cell type represent two stages of a disease progression;
 - identifying the disease status of the patient based on detection of circulating cells that exhibit specific binding to both an anti-immune cell antibody and an antibody specific for either the first source cell type or the second source cell type.

9. The method of claim 8, wherein the first source cell type is a cancer cell and the second source cell type is a non-cancerous cell of the same origin as the cancer cell.
10. The method of claim 8, wherein:
 - the first cell type is an epithelial-derived cancer cell and the at least one antibody specific for the first cell type is specific for one of MUC4 or MASPIN; and
 - the second cell type is an epithelial cell and the at least one antibody specific for the first cell type is specific for one of ECAD, EpCAM, or CK.
11. A method of treating metastatic cancer in a human patient, the method comprising:
 - obtaining a sample from the human patient;
 - detecting whether Circulating Hybrid Cells (CHCs) are in the sample by
 - contacting the sample with an anti-(source of the metastatic cancer) antibody;
 - contacting the sample with an anti-immune cell antibody;
 - diagnosing the patient as having metastatic cancer wherein specific binding of both antibodies in the same cell indicates the presence of one or more Circulating Hybrid Cells; and
 - administering to the patient in need thereof a pharmaceutically effective amount of an anti-cancer agent.
12. The method of any one of claims 1-11, wherein at least one of the antibodies is conjugated to a fluorescent label, and the detecting comprises fluorescence activated cell sorting (FACS) analysis
13. The method of any one of claims 1-11, wherein the sample comprises blood, plasma, serum, lymph, another blood fraction, a tumor aspirate, a tumor biopsy, peritoneal fluid, a secretions, urine, or another biological sample that contains or is believed to contain immune cells.
14. The method of any of claims 1-11, wherein the anti-source cell antibody is an epithelial cell antibody that specifically binds to an epitope on a biomarker selected from the group of EpCAM, E-cadherin, cytokeratin, MUC4, MASPIN, and Glypican-1 (GPC1).
15. The method of any of claims 1-10, wherein the anti-source cell antibody is a glioblastoma cell antibody that specifically binds to an epitope on a biomarker selected from the group of GFAP and Nestin.

16. The method of any of claims 1-10, wherein the anti-source cell antibody is a melanoma cell antibody that specifically binds to an epitope on a biomarker selected from the group of gp100, MelanA, TYR, and MAGEA1.

17. The method of any of claims 1-10, wherein detecting whether CHCs are in the sample comprises contacting the sample with two or more, three or more, or four or more anti-epithelial antibodies, each of which specifically binds to an epitope on a biomarker selected from the group of EpCAM, E-cadherin, cytokeratin, MUC4, MASPIN, Glypican-1 (GPC1), GFAP, Nestin, gp100, and MAGEA1.

18. The method of any of claims 1-11, wherein the anti-immune cell antibody specifically binds to an epitope on a biomarker selected from CD45, CD14, CD16, CD11b, CD11c, CD64, CD163, CD68, CD66b, CD71, CD80, CD86, CSF1R, or CCR5.

19. The method of any of claims 1-11, wherein detecting whether CHCs are in the sample comprises contacting the sample with two or more, three or more, or four or more anti-immune cell antibodies, each of which specifically binds to an epitope on a biomarker selected from the group of CD45, CD14, CD16, CD11b, CD11c, CD64, CD163, CD68, CD66b, CD71, CD80, CD86, CSF1R, and CCR5.

20. The method of any of claims 1-11, wherein the anti-immune cell antibody specifically binds to an epitope on a biomarker selected from CD14, CD16, CD45, CD64, CD68, CD71, or CCR5.

21. The method of any of claims 1-11, wherein detecting whether CHCs are in the sample comprises contacting the sample with two or more, three or more, or four or more anti-immune cell antibodies, each of which specifically binds to an epitope on a biomarker selected from CD14, CD16, CD45, CD64, CD68, CD71, or CCR5.

22. The method of claim 11, wherein the anti-cancer agent is selected at least in part based on gene or protein expression in the CHC.

23. The method of claim 11, wherein the anti-cancer agent is a CSF1R inhibitor selected from pexidartinib, PLX7486, LY3022855, MC-CS4, chlauranib, SNDX6352, JNJ-40346527, DCC-3014, linifanib, IMC-CS4, AMG820, BLZ945, TK-1258, dovitinib, vatalinib, sunitinib, ARRY-3882, 5-(3-methoxy-4-((4-methoxybenzyl)oxy)benzyl)pyrimidine-2,4-diamine, CEP-32496, 3-((quinolin-4-

ylmethyl)amino)-N-(4-(trifluoromethoxy)phenyl)thiophene-2-carboxamide; or a pharmaceutically acceptable salt thereof.

24. The method of claim 11, wherein the anti-cancer agent is a CSF1R inhibitor is selected from pexidartinib, chiauranib, linifanib, dovitinib, vatalinib, or sunitinib; or a pharmaceutically acceptable salt thereof.

25. The method of claim 11, wherein the anti-cancer agent is a CSF1R inhibitors is an anti-CSF1R antibody.

26. The method of claim 25, wherein the anti-CSF1R antibody is cabiralizumab or emactuzumab.

27. The method of any of claims 1-11, wherein the anti-(source) antibody is an anti-epithelial antibody that specifically binds to an epitope on a biomarker selected from MUC4, MASPIN, or Glypican-1 (GPC1).

28. A method of diagnosing a subject, comprising:

obtaining a sample from the subject;

characterizing Circulating Hybrid Cells (CHCs) in the sample by:

contacting the sample with an antibody specific for CD45,

contacting the sample with epithelial antibodies specific for ECAD, EpCAM, and CK;

contacting the sample with cancer antibodies specific MUC4 and MASPIN;

identifying the sample as containing:

inflammation-indicative CHCs when the CD45 antibody and epithelial antibodies but not cancer antibodies specifically bind the same cell in the sample;

cancer-indicative CHCs when the CD4 antibody, the epithelia antibodies, and the cancer antibodies specifically bind the same cell in the sample; and

diagnosing the subject as:

having or at risk for cancer when the number of cancer-indicative CHCs is at least twice the number of inflammation indicative CHCs;

having or at risk of chronic inflammation when the number of inflammation-indicative CHCs is at least twice the number of cancer-indicative CHCs; or

having neither cancer nor chronic inflammation when the number of inflammation-indicative CHCs and the number of cancer-indicative CHCs is about equal and fewer than 25 CHCs/20,000 nuclei in the sample.

29. The method of claim 28, wherein at least one of the antibodies is conjugated to a fluorescent label, and the characterizing comprises fluorescence activated cell sorting (FACS) analysis.

30. The method of claim 28, wherein the sample comprises blood, plasma, serum, another blood fraction, lymph, a tumor aspirate, a tumor biopsy, peritoneal fluid, a secretions, urine, or another biological sample that contains or is believed to contain immune cells

31. A chromatographic assay device comprising:
a panel of two or more capture antibodies or antigen-binding fragment thereof, each of which specifically binds a different marker protein in Table 2 or Table 3.

32. The chromatographic assay device of claim 31, wherein the panel comprises two or more capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of:

CD45, CD68, EpCAM, ECAD, CK, MUC4, and MASPIN (*CHC-identity panel*);
CD44, Ki67, pHH3, CD166, Sox9, and PDX1 (*stem/development panel*);
Vimetin, MMP11, and ECAD (*migratory panel*);
KRAS, pKRAS, RAF, pRAF, MEK 1/2, pMEK 1/2, MAPK, pMAPK, ERK 1/2, pERK1/2, PI3K, pPI3K, pS6RP, AKT, pAKT, pSTAT3, TGF β , SMAD4, EGFR, and pEGFR (*cell signalling pathway panel*);
UCHL, SFTPb, MUC4, WDR72, RAGE, and EGFR (*lung panel*);
ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) panel*);
HER2, ER, and AR (*breast cancer panel*);
PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) panel*);
ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial panel*);
GFAP and Nestin (*glioblastoma panel*);
gp100, MageA1, MelanA, and TYR (*melanoma panel*); and/or
CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage panel*).

33. The chromatographic assay device of claim 31, wherein the panel comprises a set of capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of:

at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*);
and

at least one cell-type or cancer-type specific marker protein in Table 3 or at least one macrophage-specific marker.

34. The chromatographic assay device of claim 33, wherein the at least one cell-type or cancer-type specific marker protein in Table 3 comprises:

- at least one of UCHL, SFTPb, MUC4, WDR72, RAGE, or EGFR (*lung markers*);
- at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*);
- at least one of HER2, ER, and AR (*breast cancer markers*);
- at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*);
- at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*);
- at least one of GFAP and Nestin (*glioblastoma markers*);
- at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and/or
- at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).

35. The chromatographic assay device of claim 34, wherein the panel comprises at least nine antibodies or antigen-binding fragments thereof, which individually specifically bind to:

- at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*);
- at least one of UCHL, SFTPb, MUC4, WDR72, RAGE, or EGFR (*lung markers*);
- at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*);
- at least one of HER2, ER, and AR (*breast cancer markers*);
- at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*);
- at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*);
- at least one of GFAP and Nestin (*glioblastoma markers*);
- at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and
- at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).

36. A kit comprising:
a panel of two or more antibodies or antigen-binding fragment thereof, each of which specifically binds a different marker protein in Table 2 or Table 3.
37. The kit of claim 36, wherein the panel comprises two or more capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of:
CD45, CD68, EpCAM, ECAD, CK, MUC4, and MASPIN (*CHC-identity panel*);
CD44, Ki67, pHH3, CD166, Sox9, and PDX1 (*stem/development panel*);
Vimetin, MMP11, and ECAD (*migratory panel*);
KRAS, pKRAS, RAF, pRAF, MEK 1/2, pMEK 1/2, MAPK, pMAPK, ERK 1/2, pERK1/2, PI3K, pPI3K, pS6RP, AKT, pAKT, pSTAT3, TGF β , SMAD4, EGFR, and pEGFR (*cell signalling pathway panel*);
UCHL, SFTPb, MUC4, WDR72, RAGE, and EGFR (*lung panel*);
ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) panel*);
HER2, ER, and AR (*breast cancer panel*);
PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) panel*);
ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial panel*);
GFAP and Nestin (*glioblastoma panel*);
gp100, MageA1, MelanA, and TYR (*melanoma panel*); and/or
CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage panel*).
38. The kit of claim 36, wherein the panel comprises a set of capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of:
at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*);
and
at least one cell-type or cancer-type specific marker protein in Table 3 or at least one macrophage-specific marker.
39. The kit of claim 38, wherein the at least one cell-type or cancer-type specific marker protein in Table 3 comprises:
at least one of UCHL, SFTPb, MUC4, WDR72, RAGE, or EGFR (*lung markers*);

at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNAK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*);

at least one of HER2, ER, and AR (*breast cancer markers*);

at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*);

at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*);

at least one of GFAP and Nestin (*glioblastoma markers*);

at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and/or

at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).

40. The kit of claim 39, wherein the panel comprises at least nine antibodies or antigen-binding fragments thereof, which individually specifically bind to:

at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*);

at least one of UCHL, SFTPb, MUC4, WDR72, RAGE, or EGFR (*lung markers*);

at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNAK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*);

at least one of HER2, ER, and AR (*breast cancer markers*);

at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*);

at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*);

at least one of GFAP and Nestin (*glioblastoma markers*);

at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and

at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).

41. An antibody cocktail that allows for separation of CHCs via a flow based or DEP based assay.

42. The antibody cocktail of claim 41, which comprises: two or more antibodies or antigen-binding fragment thereof, each of which specifically binds a different marker protein in Table 2 or Table 3.

43. The antibody cocktail of claim 42, wherein the cocktail comprises two or more capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of:

CD45, CD68, EpCAM, ECAD, CK, MUC4, and MASPIN (*CHC-identity panel*);
 CD44, Ki67, pHH3, CD166, Sox9, and PDX1 (*stem/development panel*);
 Vimetin, MMP11, and ECAD (*migratory panel*);
 KRAS, pKRAS, RAF, pRAF, MEK 1/2, pMEK 1/2, MAPK, pMAPK, ERK 1/2, pERK1/2, PI3K, pPI3K, pS6RP, AKT, pAKT, pSTAT3, TGF β , SMAD4, EGFR, and pEGFR (*cell signalling pathway panel*);
 UCHL, SFTPb, MUC4, WDR72, RAGE, and EGFR (*lung panel*);
 ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNAK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) panel*);
 HER2, ER, and AR (*breast cancer panel*);
 PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) panel*);
 ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial panel*);
 GFAP and Nestin (*glioblastoma panel*);
 gp100, MageA1, MelanA, and TYR (*melanoma panel*); and/or
 CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage panel*).

44. The antibody cocktail of claim 42, wherein the cocktail comprises a set of capture antibodies or antigen-binding fragments thereof, each of which specifically binds to a marker in the set of:

at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*);
 and

at least one cell-type or cancer-type specific marker protein in Table 3 or at least one macrophage-specific marker.

45. The antibody cocktail of claim 44, wherein the at least one cell-type or cancer-type specific marker protein in Table 3 comprises:

at least one of UCHL, SFTPb, MUC4, WDR72, RAGE, or EGFR (*lung markers*);
 at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNAK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*);

at least one of HER2, ER, and AR (*breast cancer markers*);
 at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*);
 at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*);
 at least one of GFAP and Nestin (*glioblastoma markers*);

at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and/or
 at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).

46. The antibody cocktail of claim 45, comprising at least nine antibodies or antigen-binding fragments thereof, which individually specifically bind to:

at least one of CD45, CD68, EpCAM, ECAD, CK, MUC4, or MASPIN (*CHC-identity markers*);
 at least one of UCHL, SFTPb, MUC4, WDR72, RAGE, or EGFR (*lung markers*);
 at least one of ITGA3, COL17A1, MUC4, MMP11, MUC16, AHNAK2, AGAP1, DAS-1, LEMD1, MSLN, KLCK10, TFF1, PDX1, MASPIN, and GPC1 (*Pancreatic ductal adenocarcinoma (PDAC) markers*);
 at least one of HER2, ER, and AR (*breast cancer markers*);
 at least one of PTPRO, TANGO6, TMEM211, APOBEC1, CST5, PRSS22, DPEP1, PCCA, CHST4, NOX1, CD166, DCAMKL1, and TROP2 (*colorectal cancer (CRC) markers*);
 at least one of ZO1, EpCAM, ECAD, and Cytokeratin (*epithelial markers*);
 at least one of GFAP and Nestin (*glioblastoma markers*);
 at least one of gp100, MageA1, MelanA, and TYR (*melanoma markers*); and
 at least one of CD14, CD33, CD45, CD11b/Mac-1, Ly-71 (F4/80), CSF1R, CD68, CD45, CXCR4, CD16, CD11c, CD64, CD71, CCR5, CD66b, and CD163 (*macrophage markers*).

47. Use of the assay device of any one of claims 31-35, or the kit of any one of claims 36-40, or the antibody cocktail of any one of claims 41-46 to detect one or more CHCs in a sample from a subject.

48. A method of diagnosing or grading/stratifying a patient or a sample from a patient, essentially as described herein.

49. A pathology or disease grading/stratifying array or antibody panel essentially as described herein.

50. A method of treatment essentially as described herein.

Cytokeratin/Ychr/Hoeschst

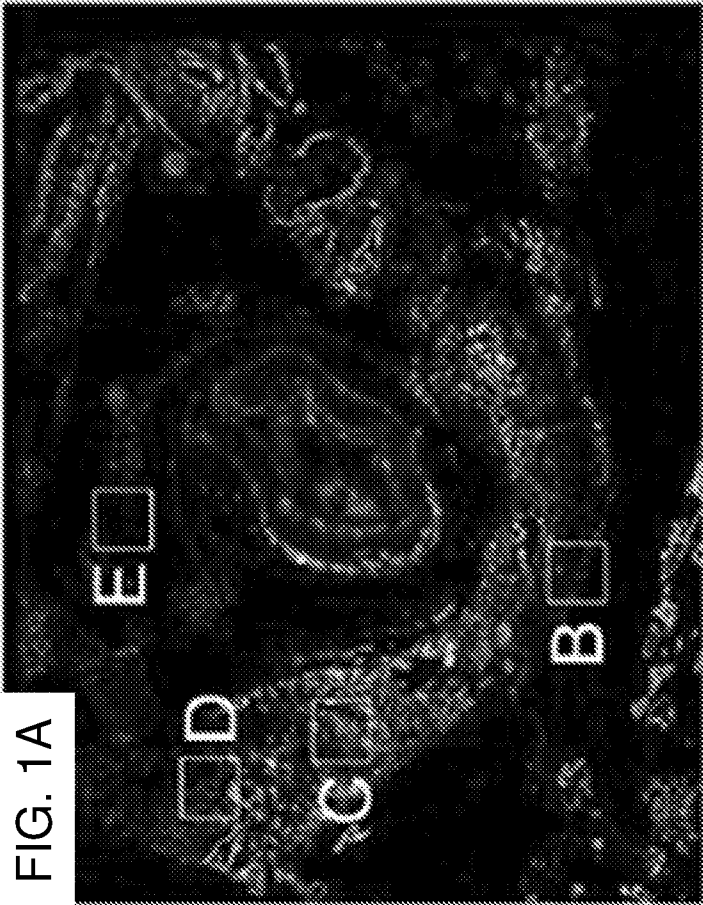


FIG. 1B

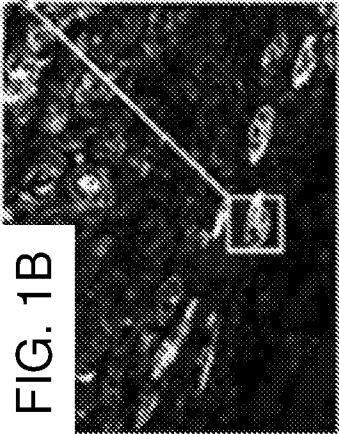


FIG. 1C

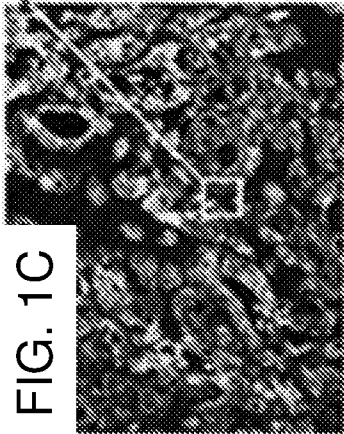


FIG. 1E



FIG. 1D

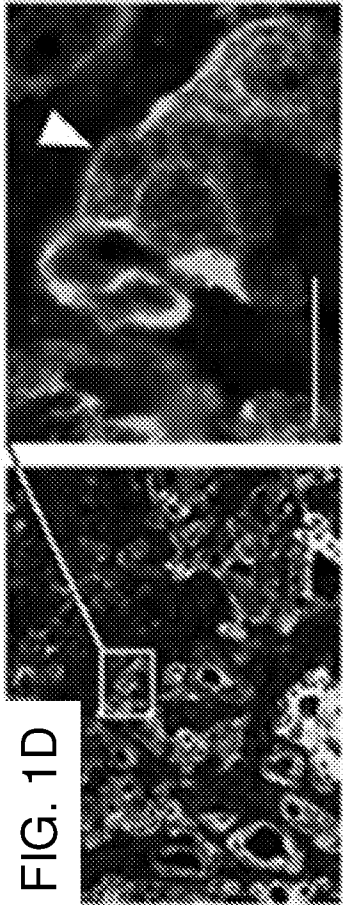


FIG. 2A

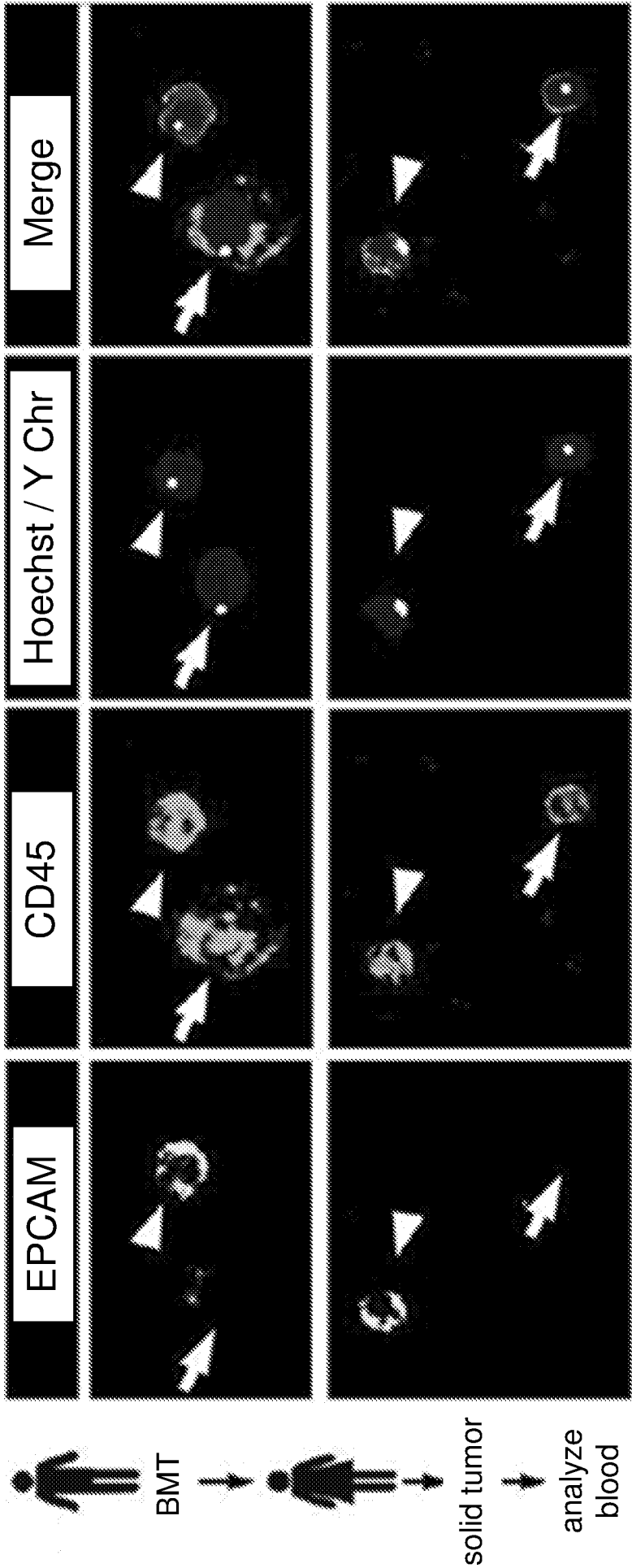


FIG. 2B

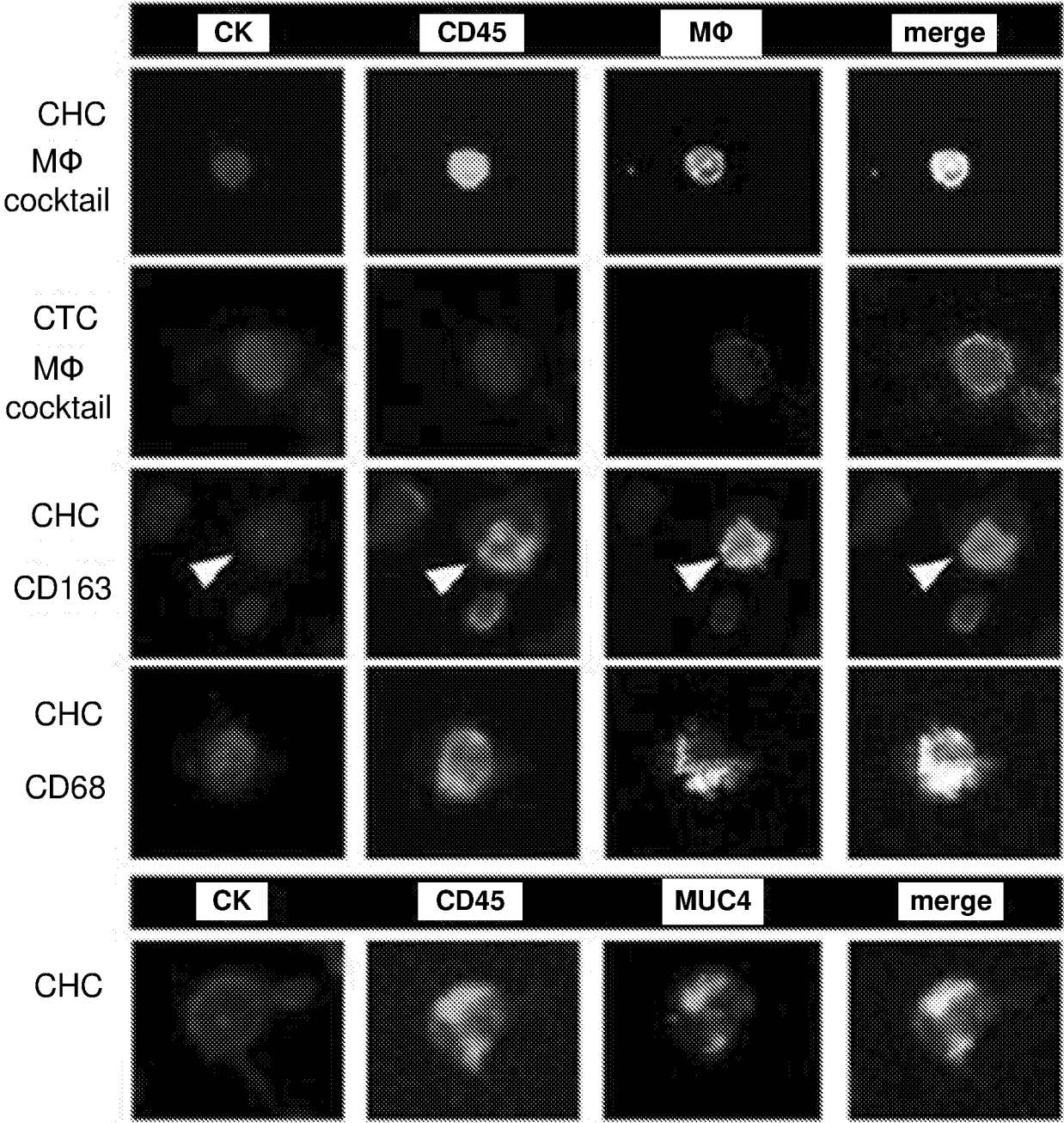


FIG. 2C

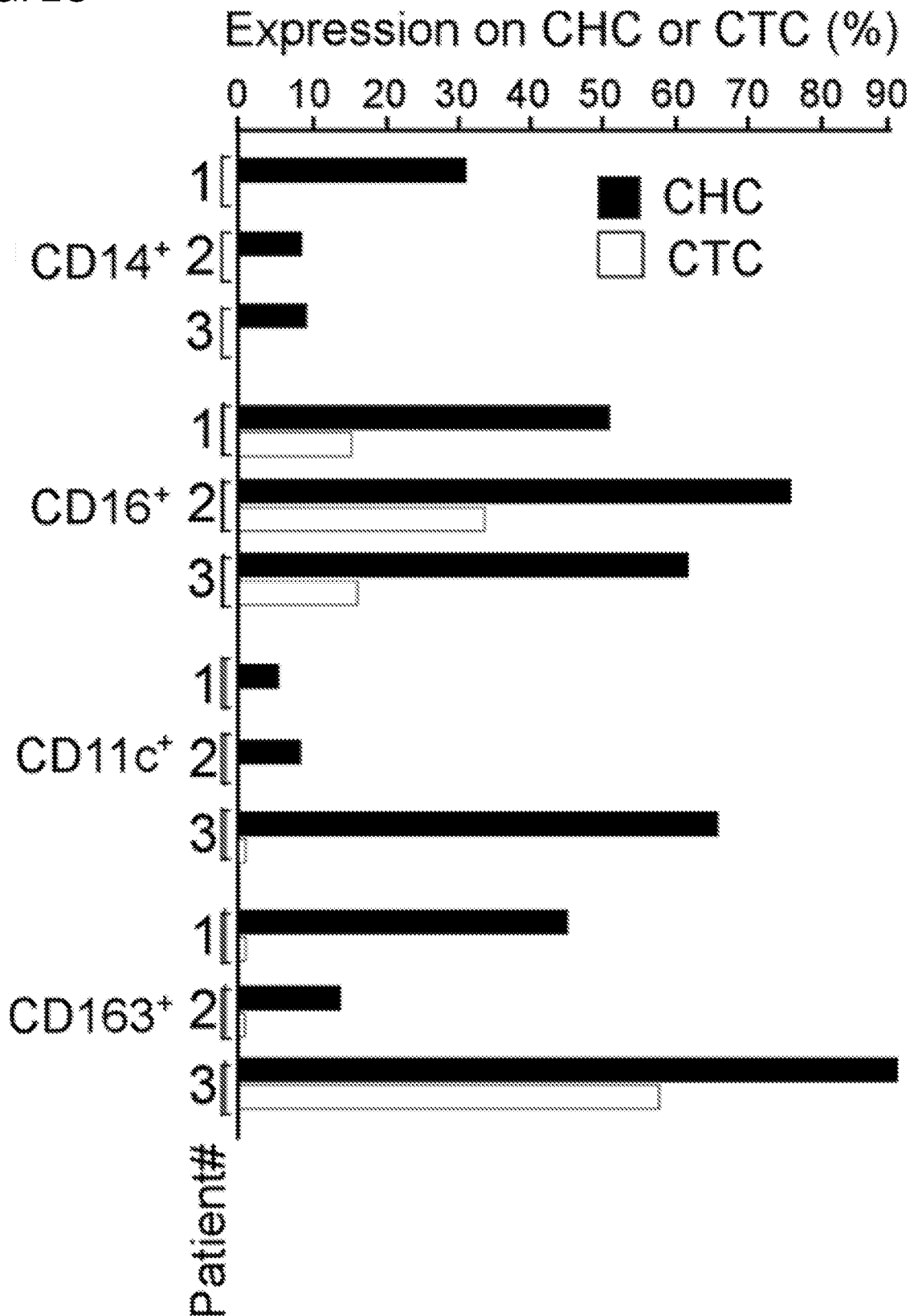


FIG. 2D

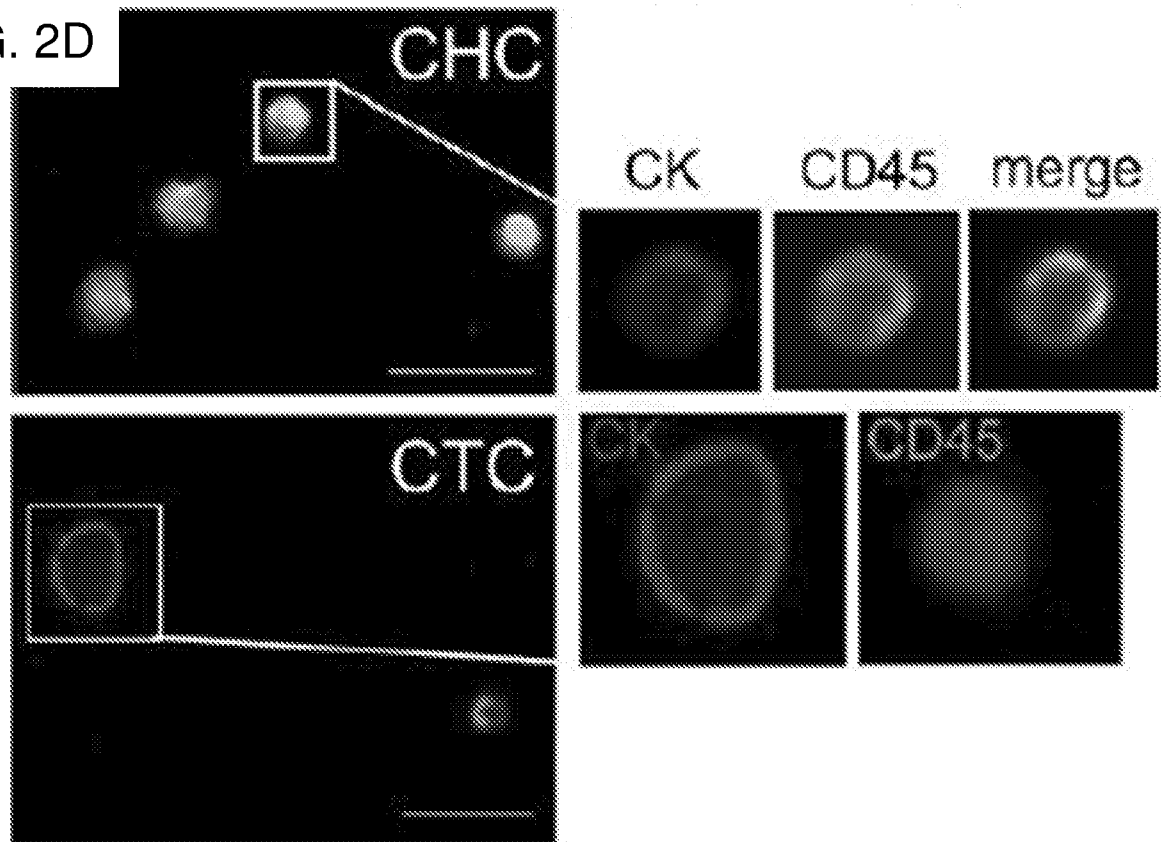


FIG. 2E

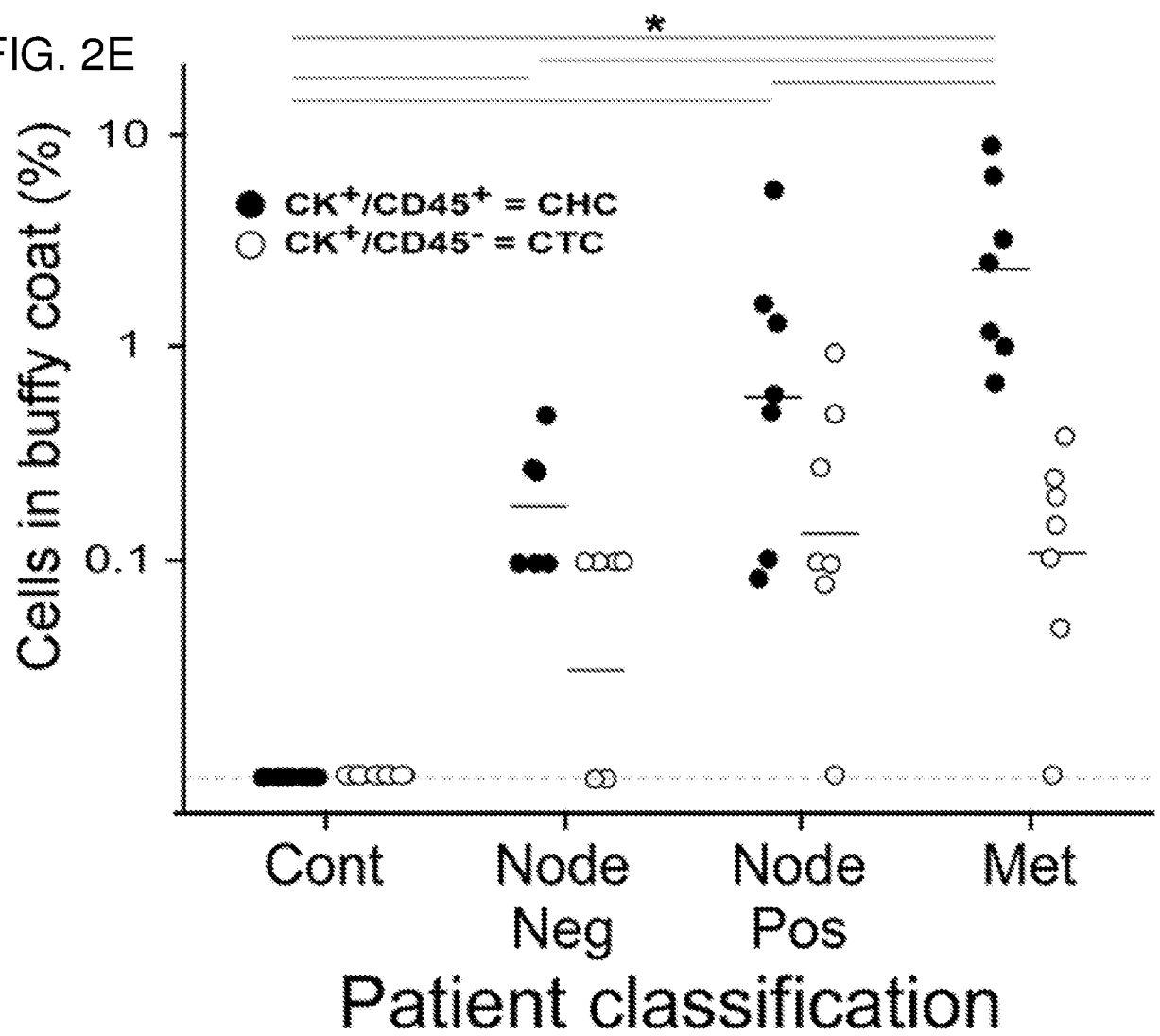


FIG.

2F

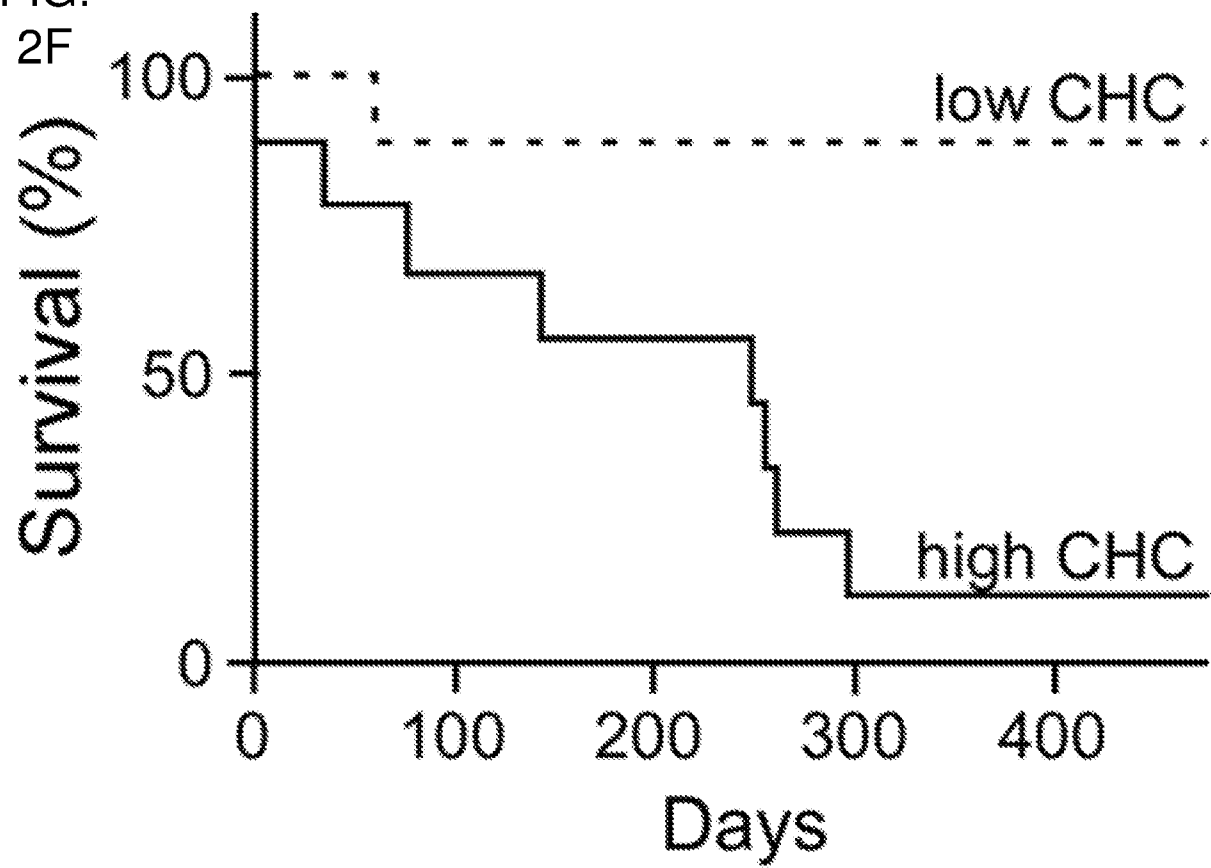
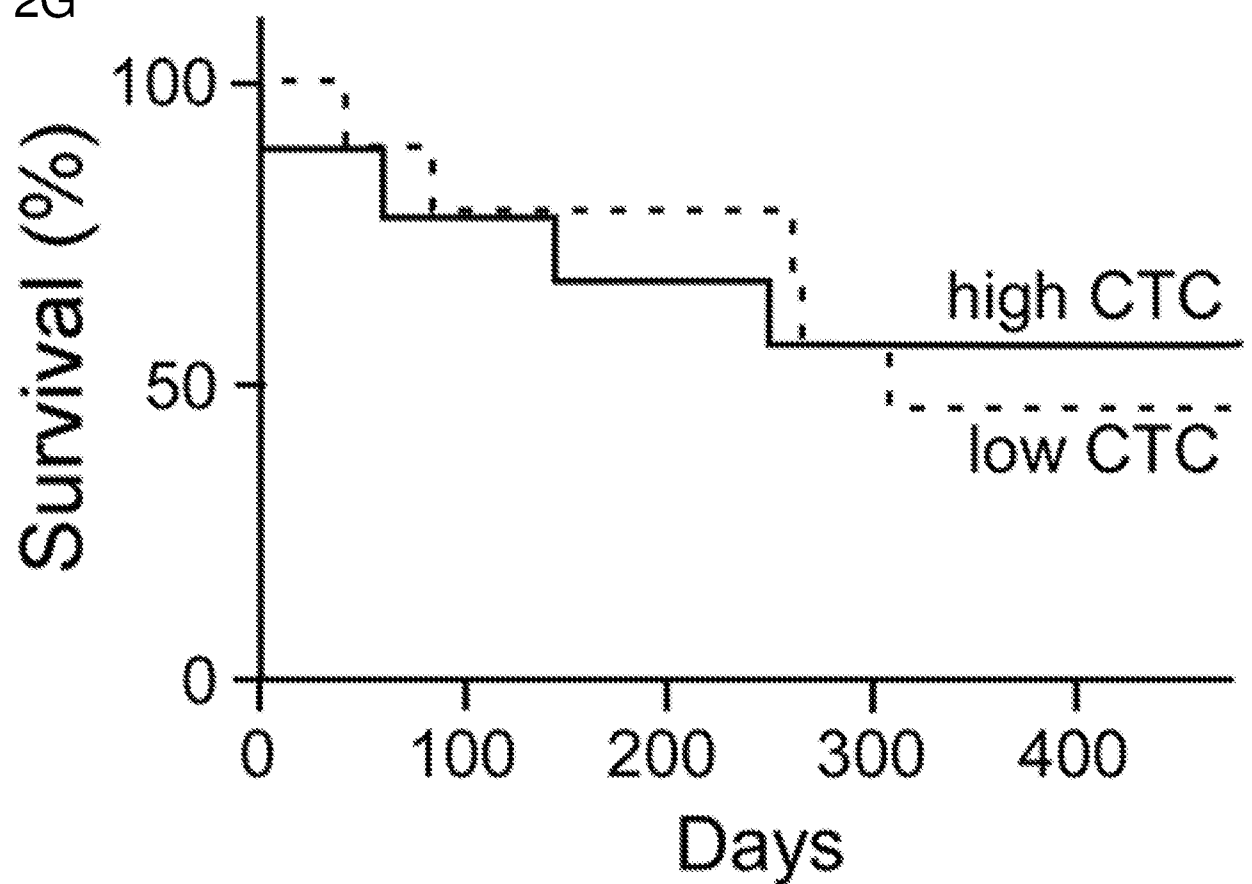


FIG.

2G



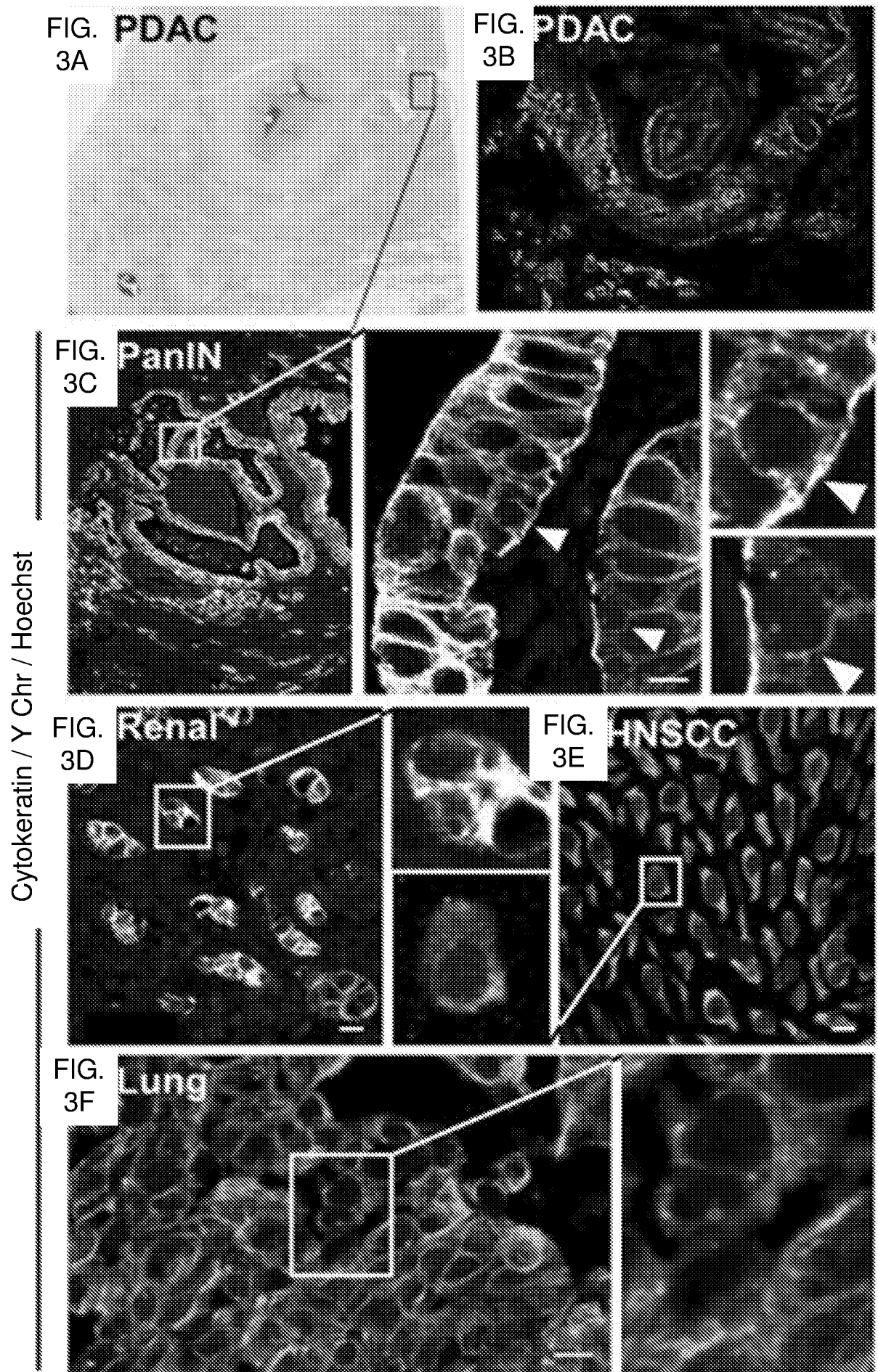


FIG. 4A

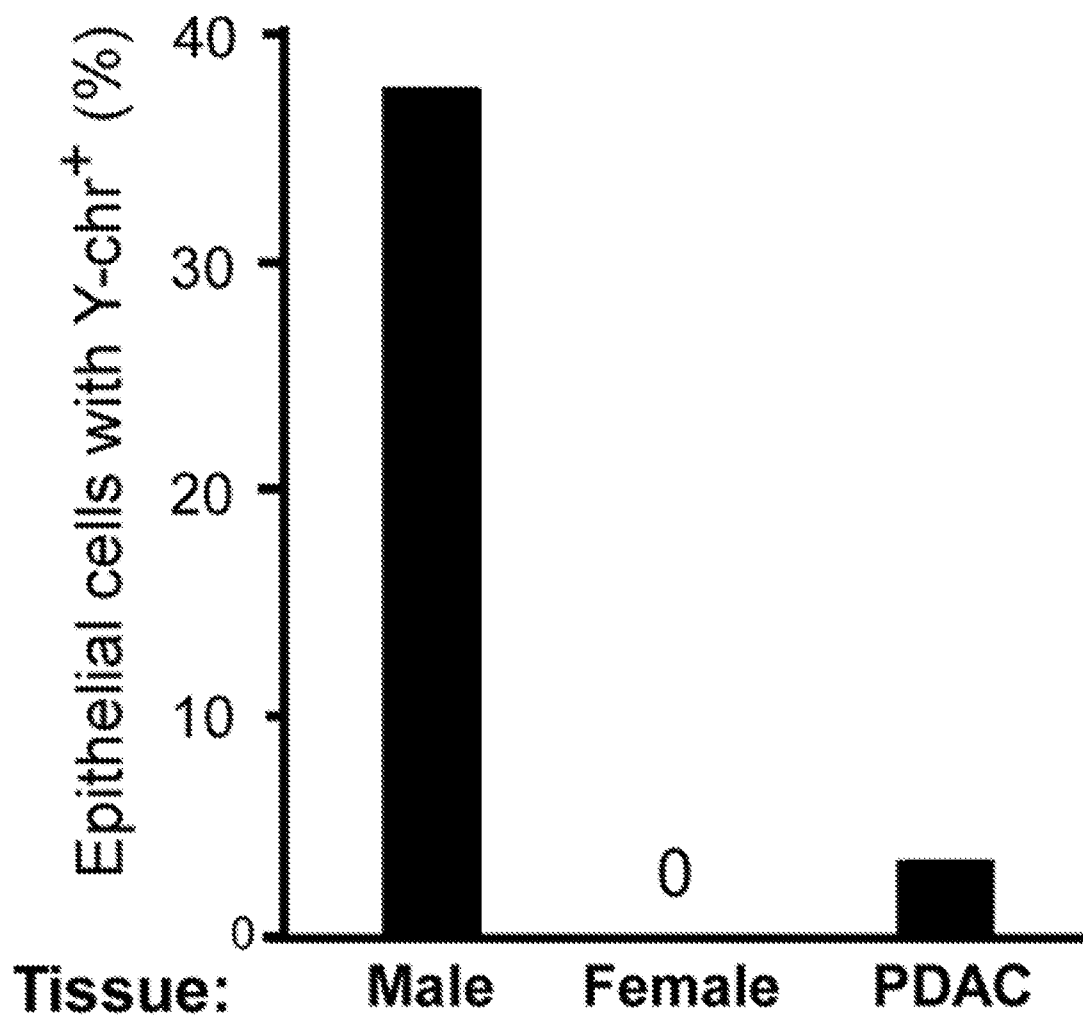
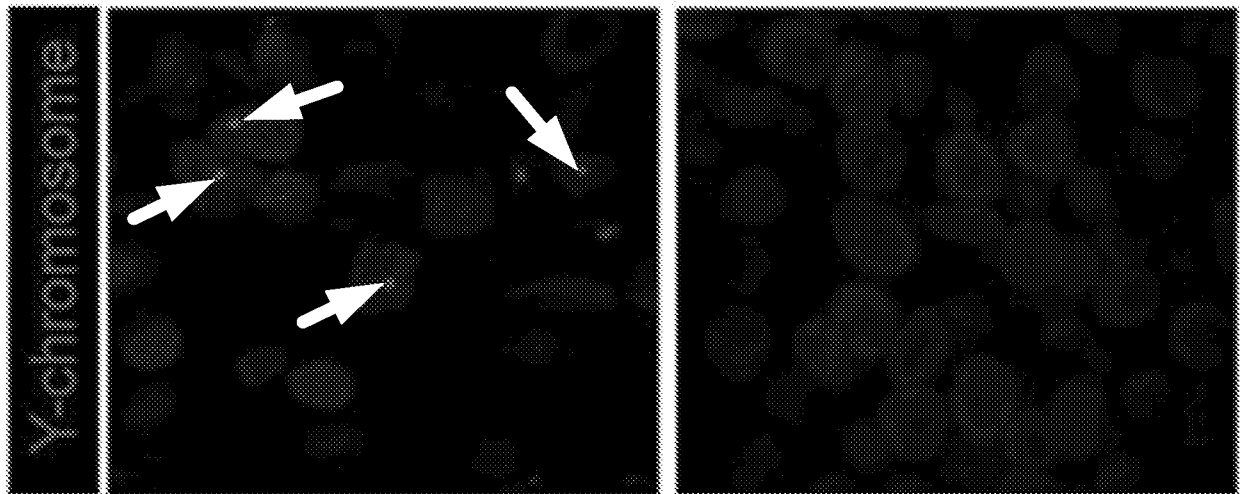
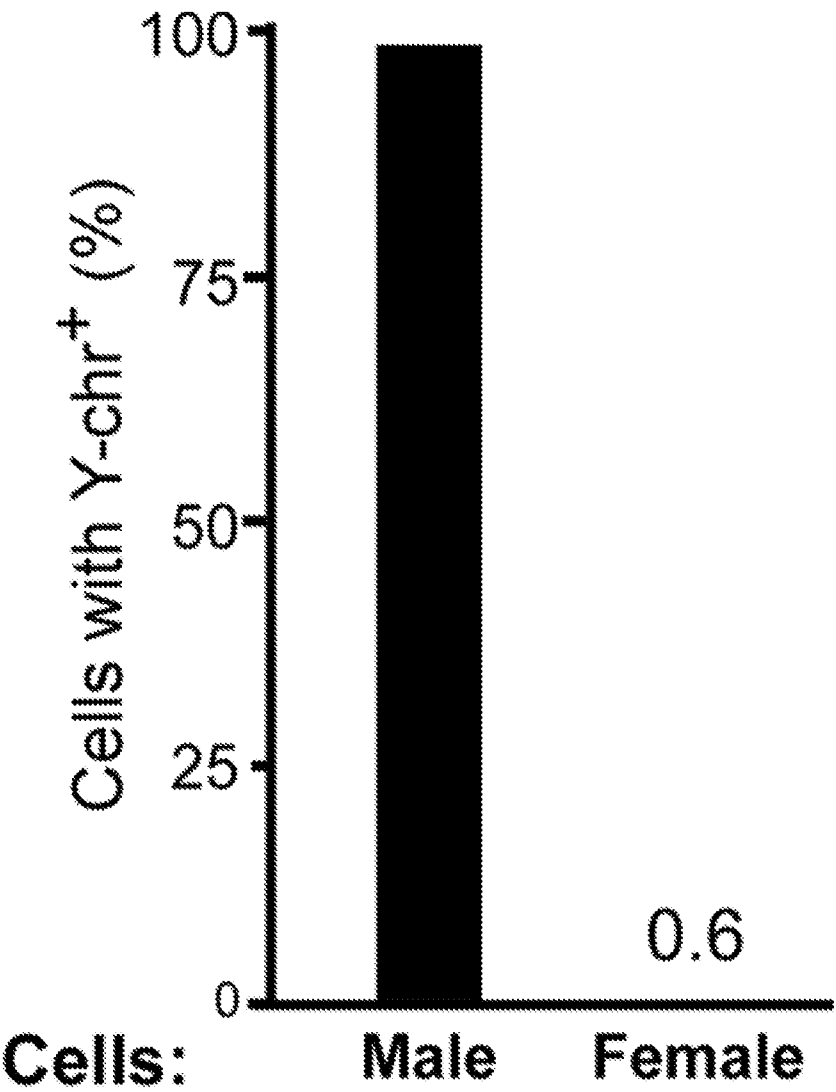
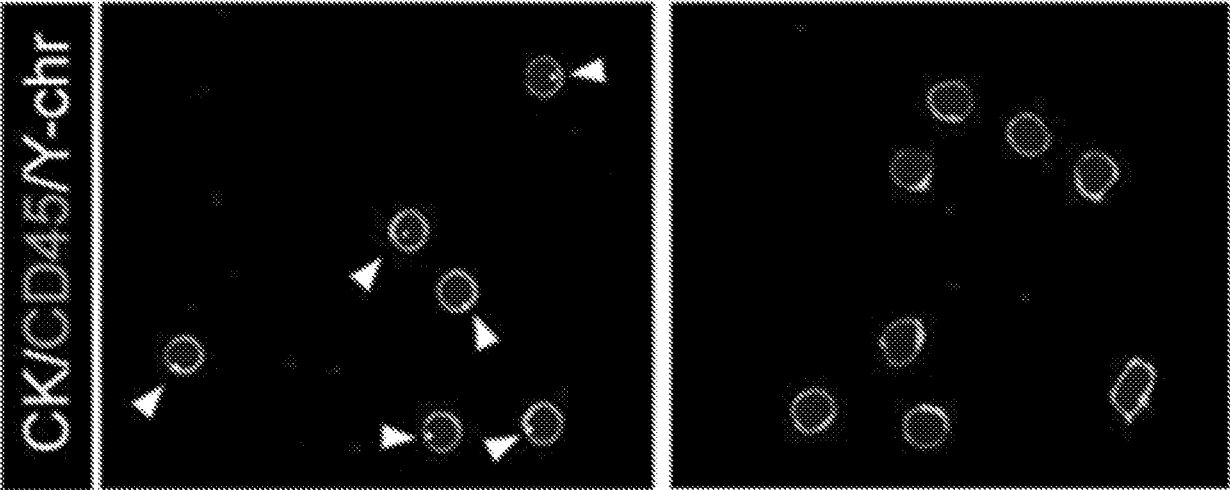
Male**Female**

FIG. 4B

Male

Female



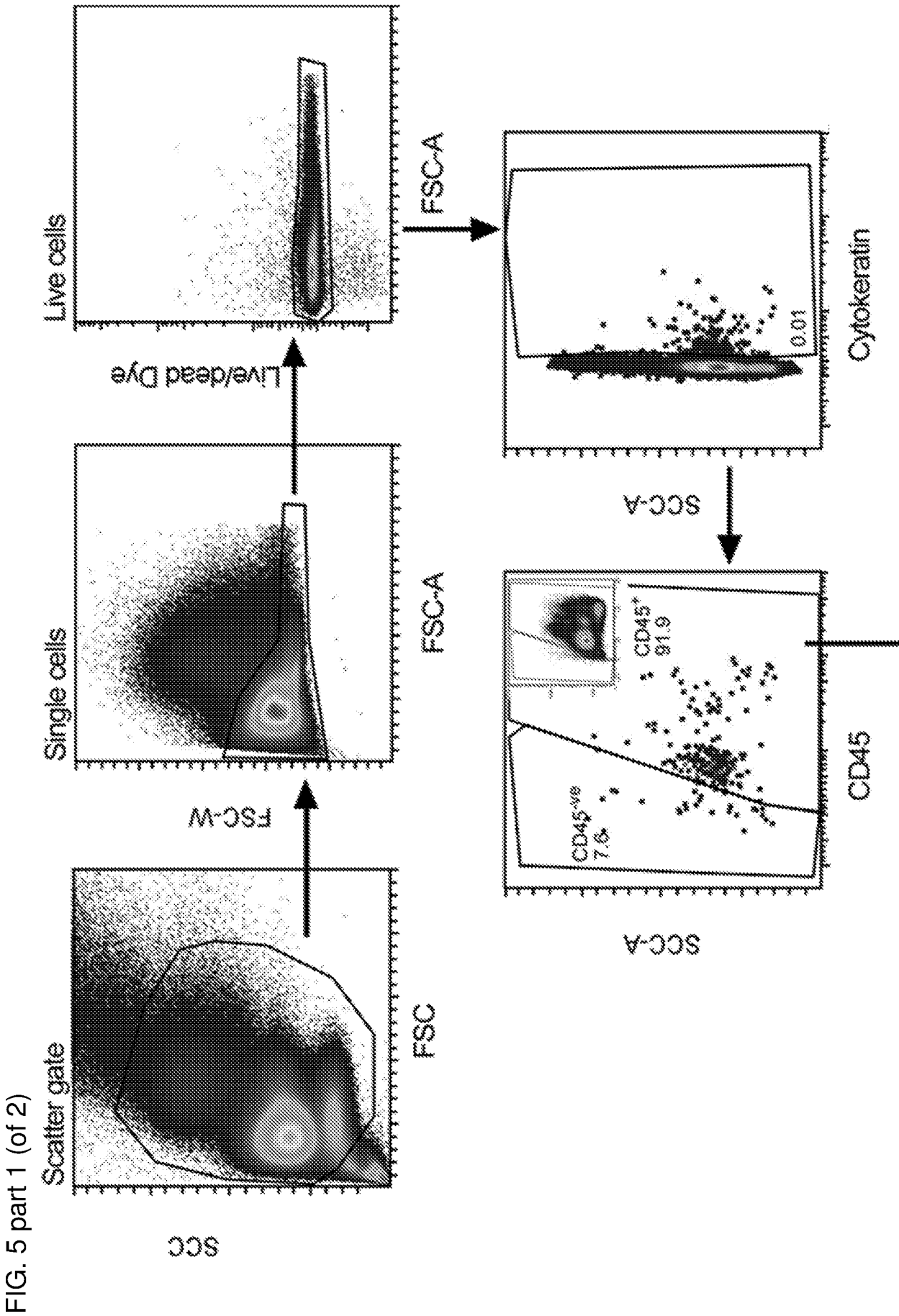


FIG. 5 part 2 (of 2)

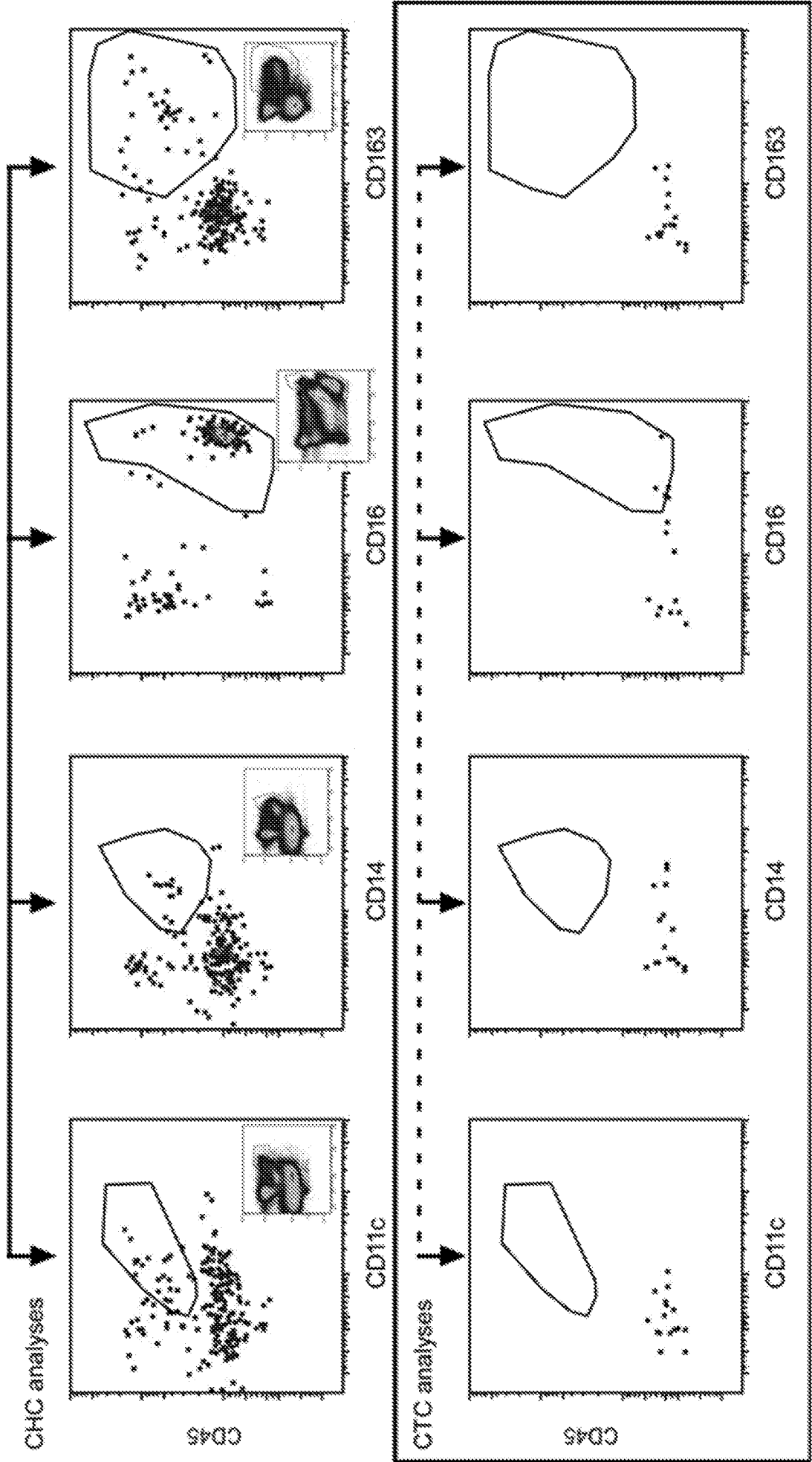


FIG. 6

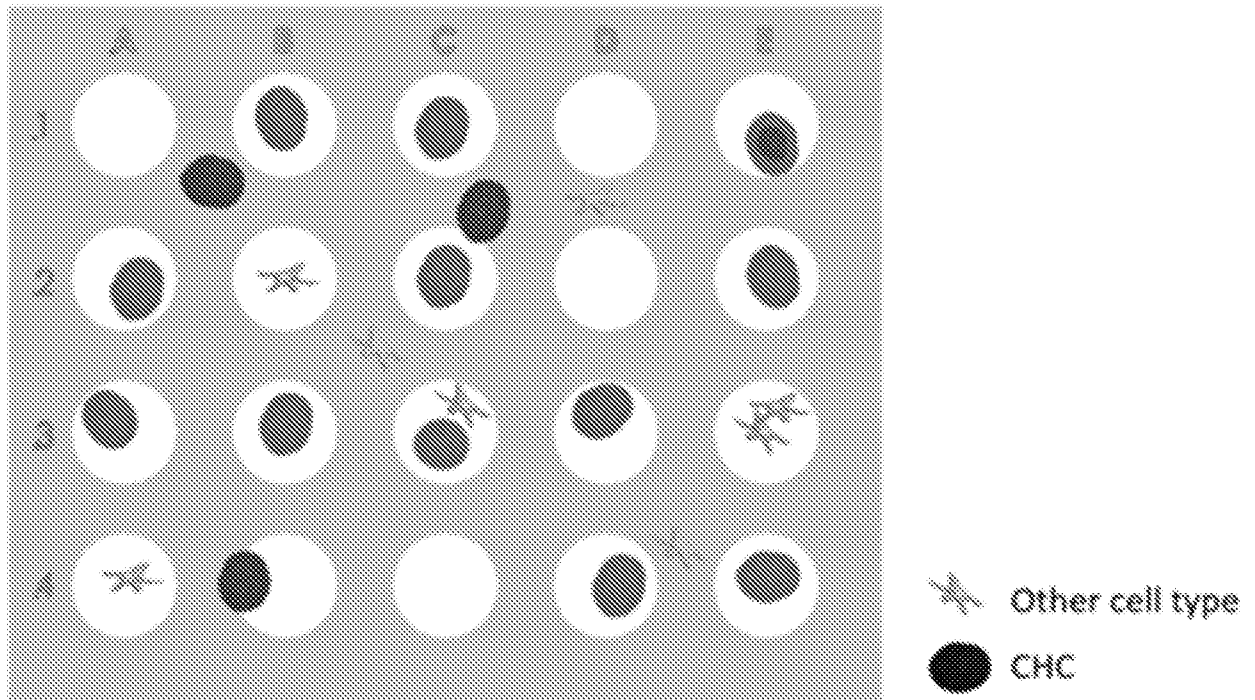
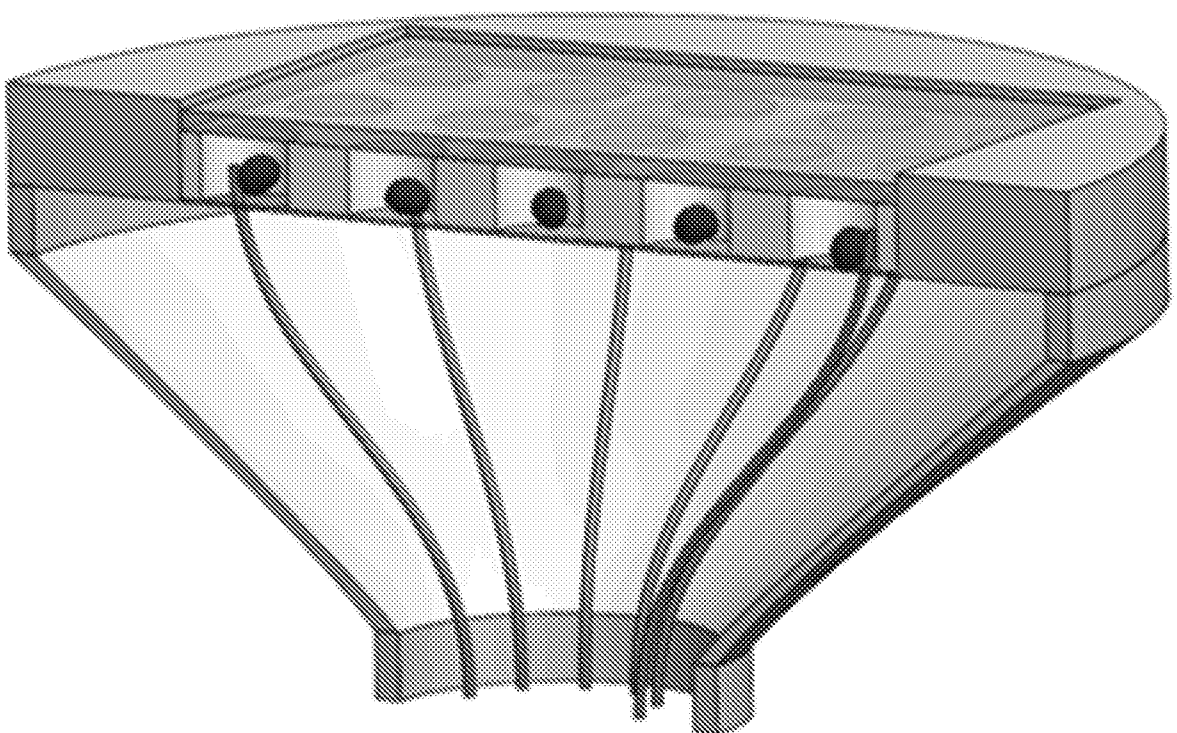


FIG. 7



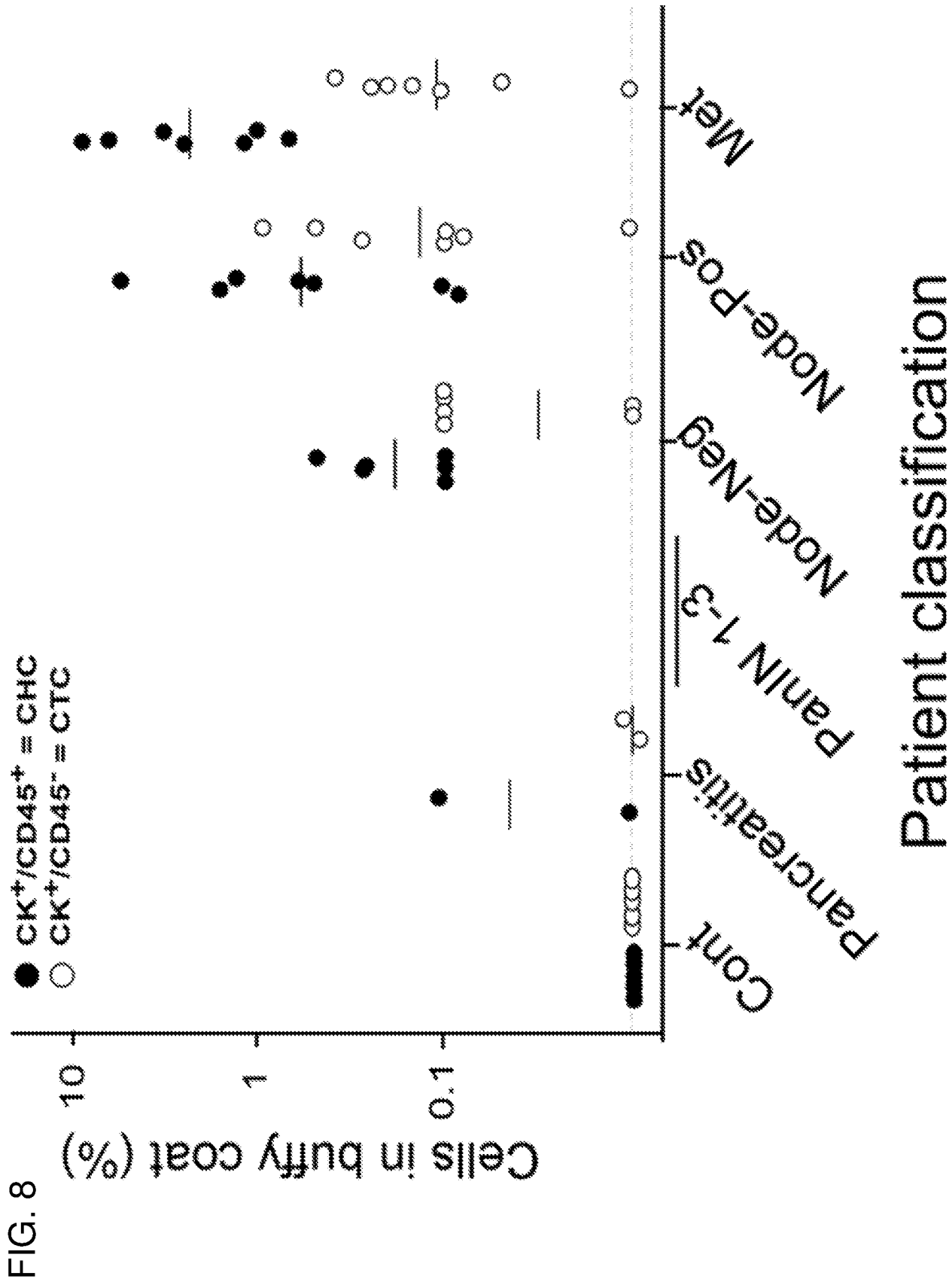
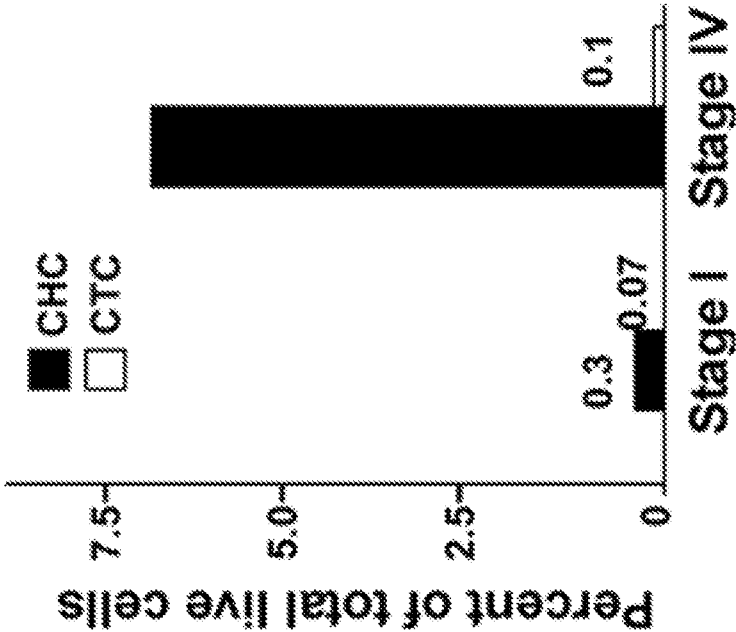


FIG. 9A

PDAC



Uveal Melanoma

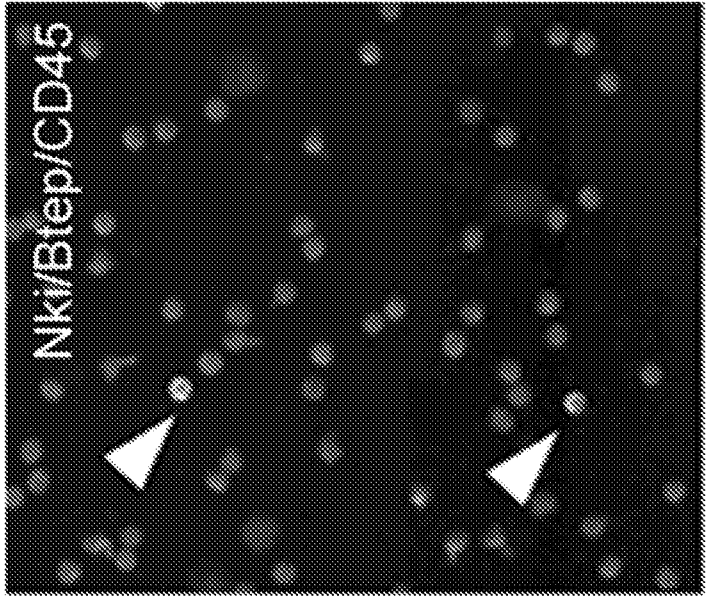
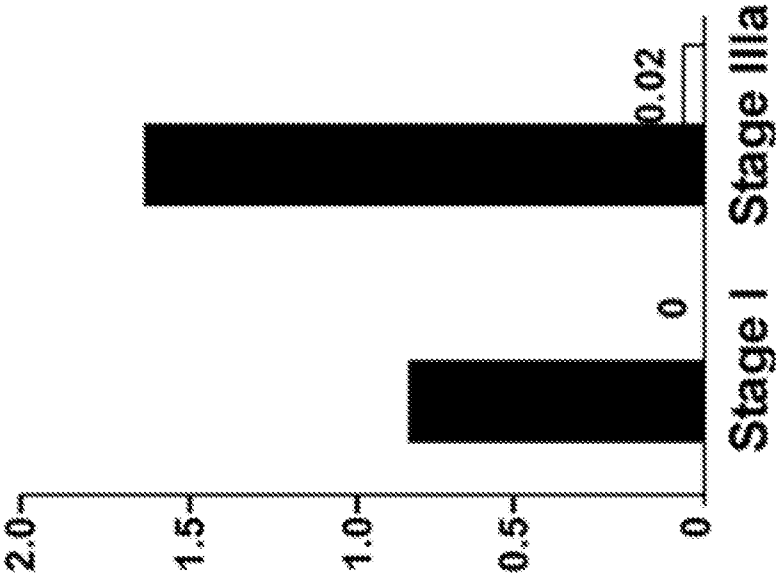


FIG. 9B

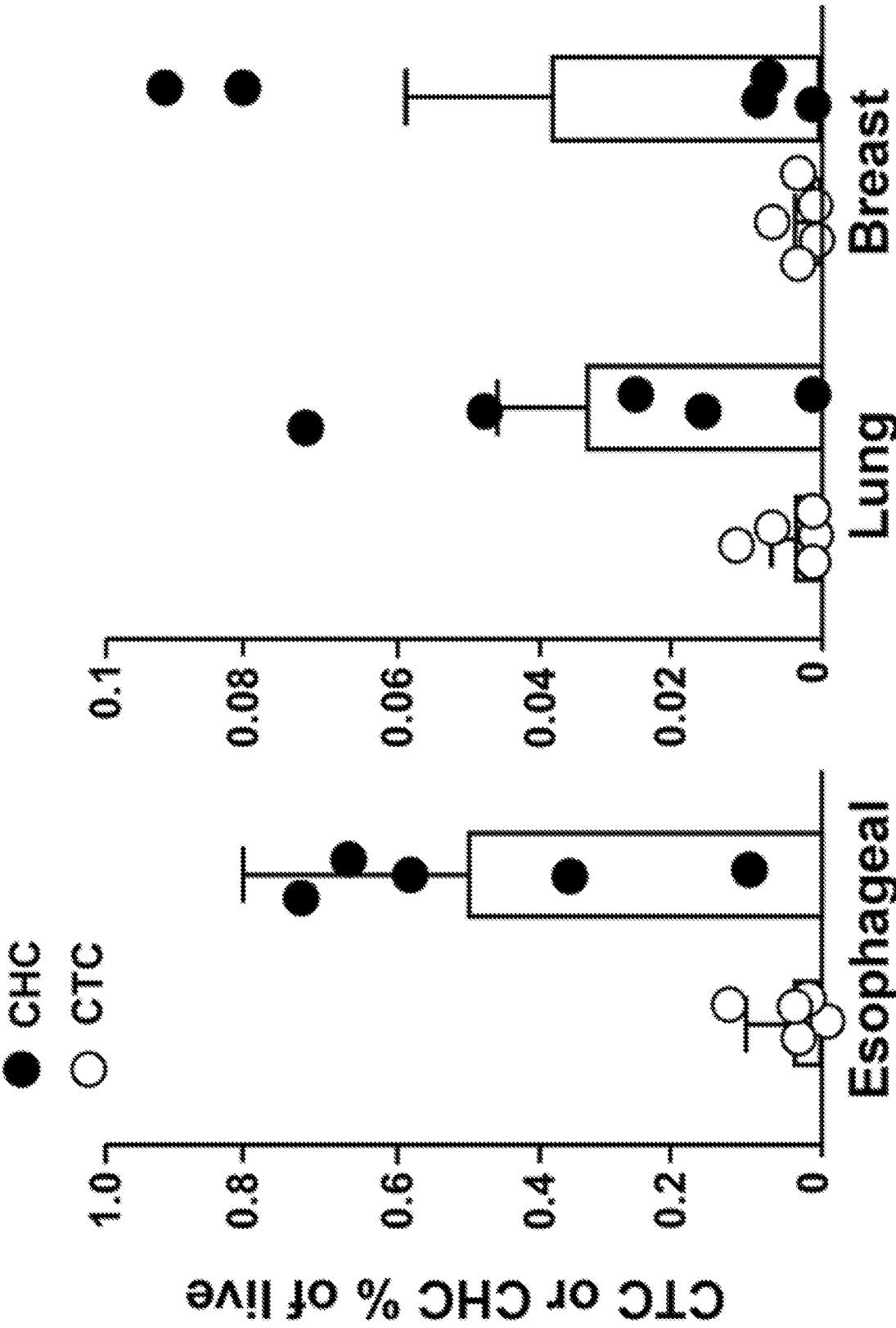


FIG. 9C

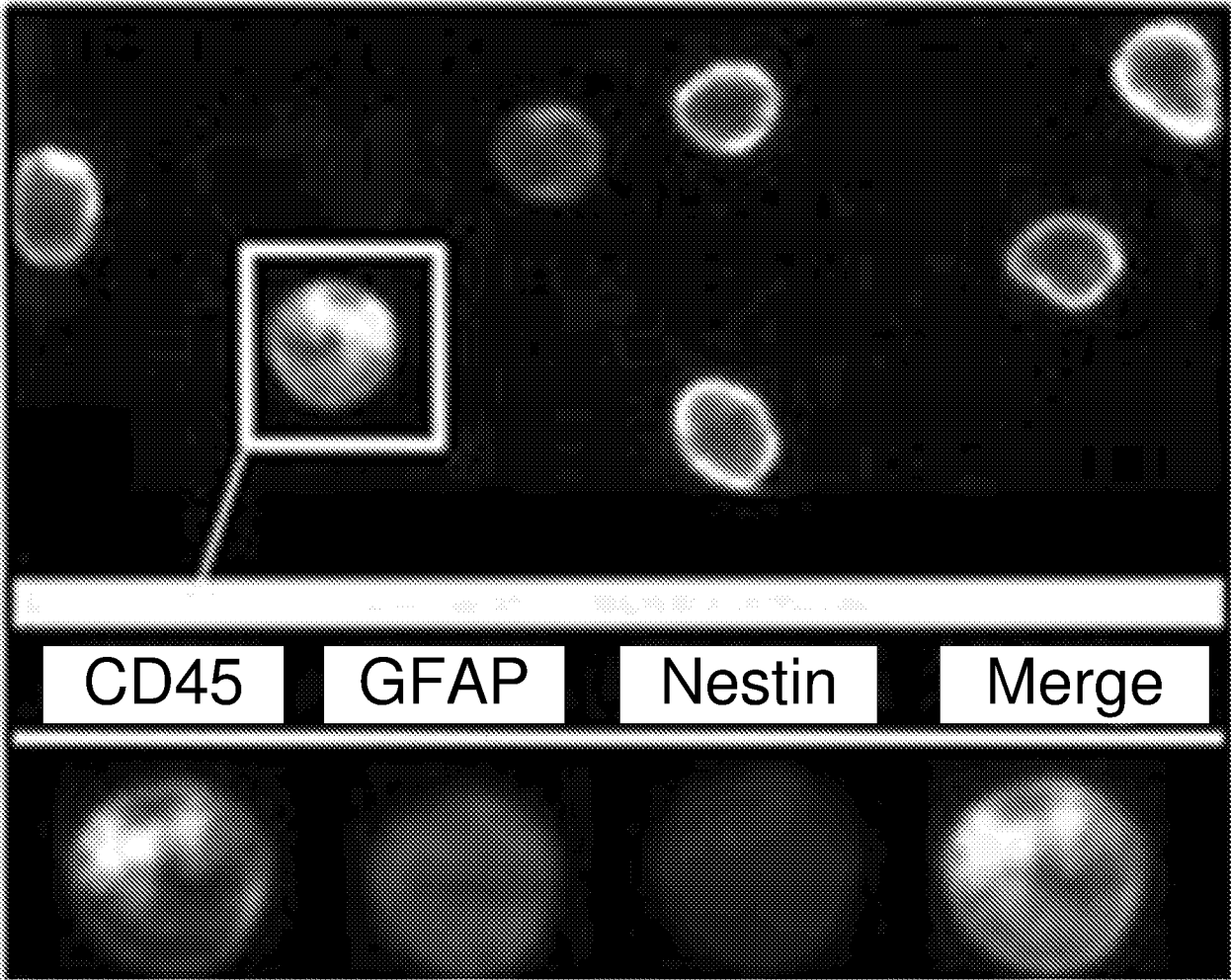
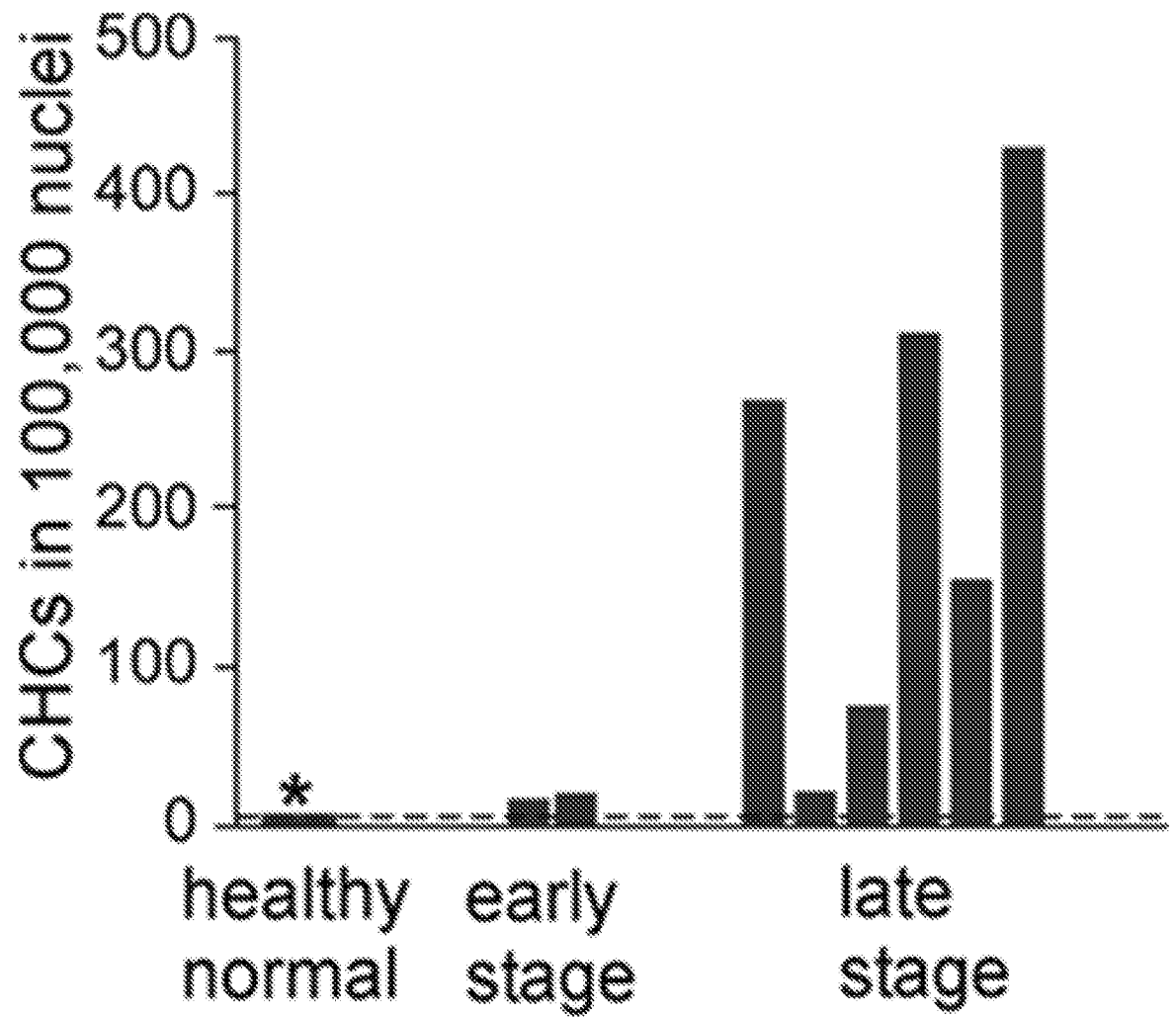
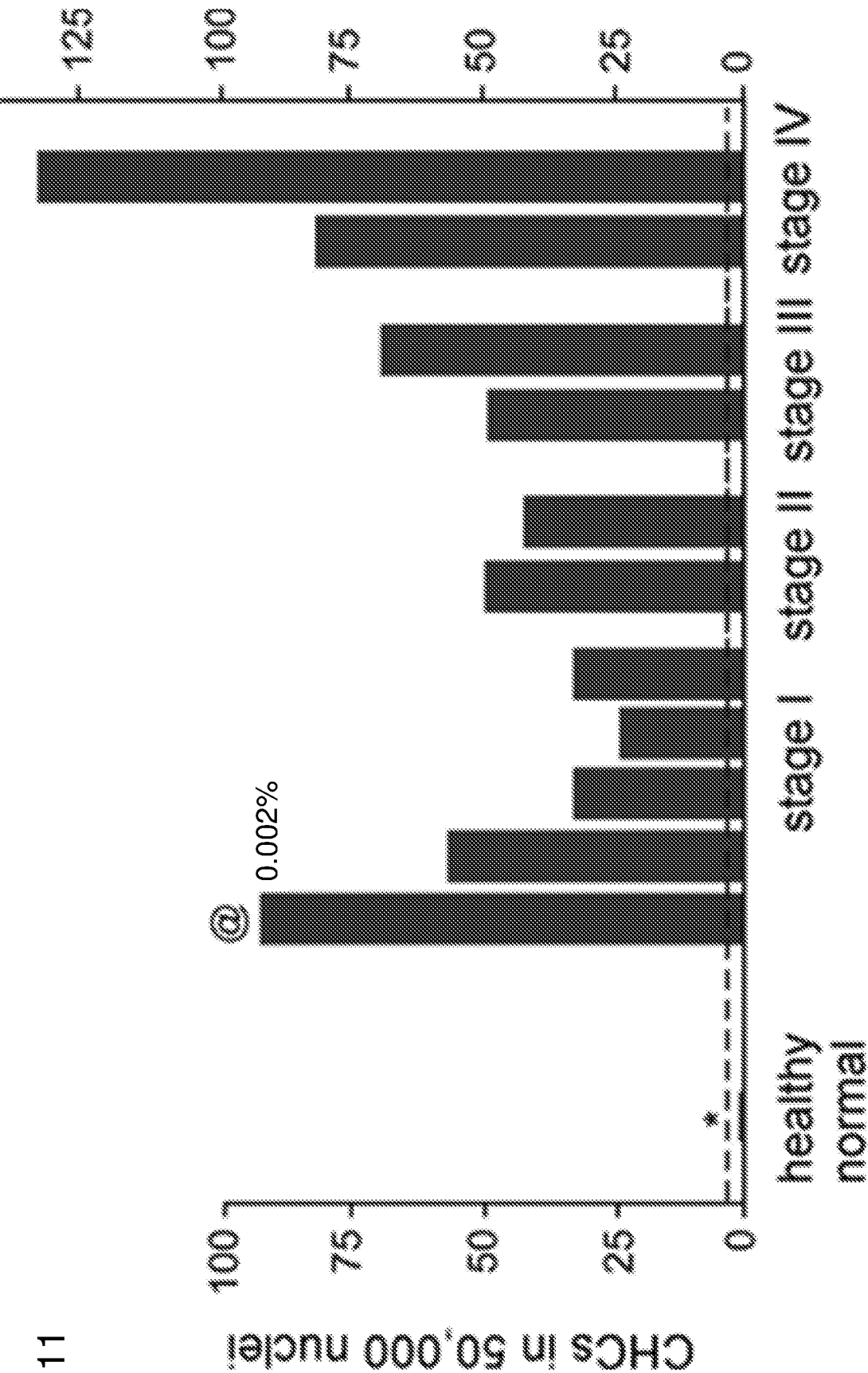


FIG. 10



*n=10, average CHC = 5 +/-0.8

FIG. 11



*n=1-, average CHC = 2.5 +/-0.4
@stage Ia at diagnosis, patient deceased

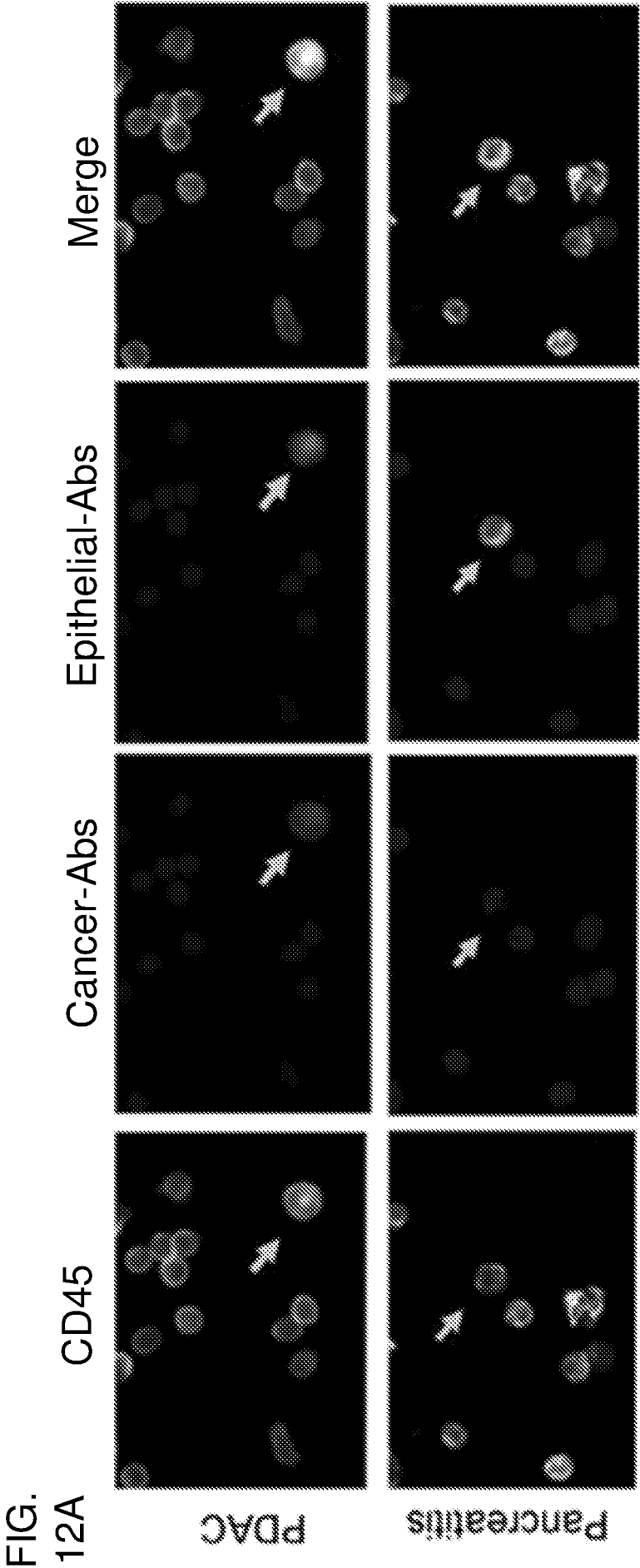


FIG. 12B

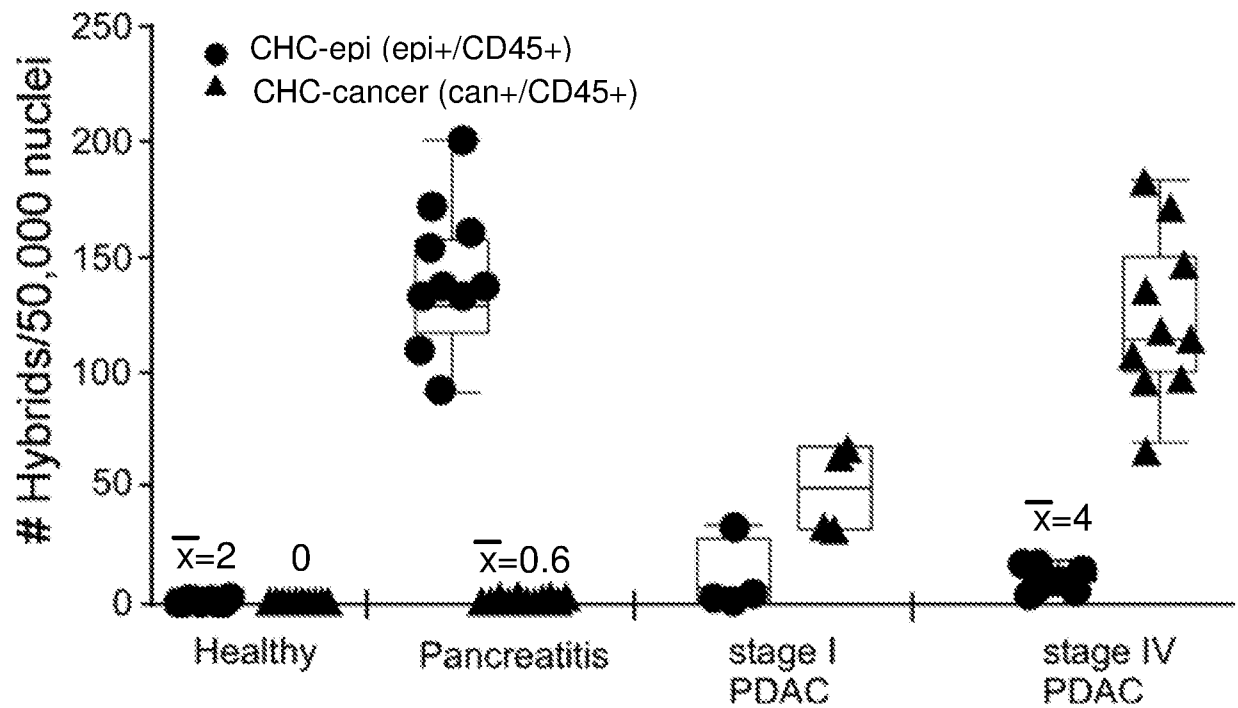


FIG. 12C

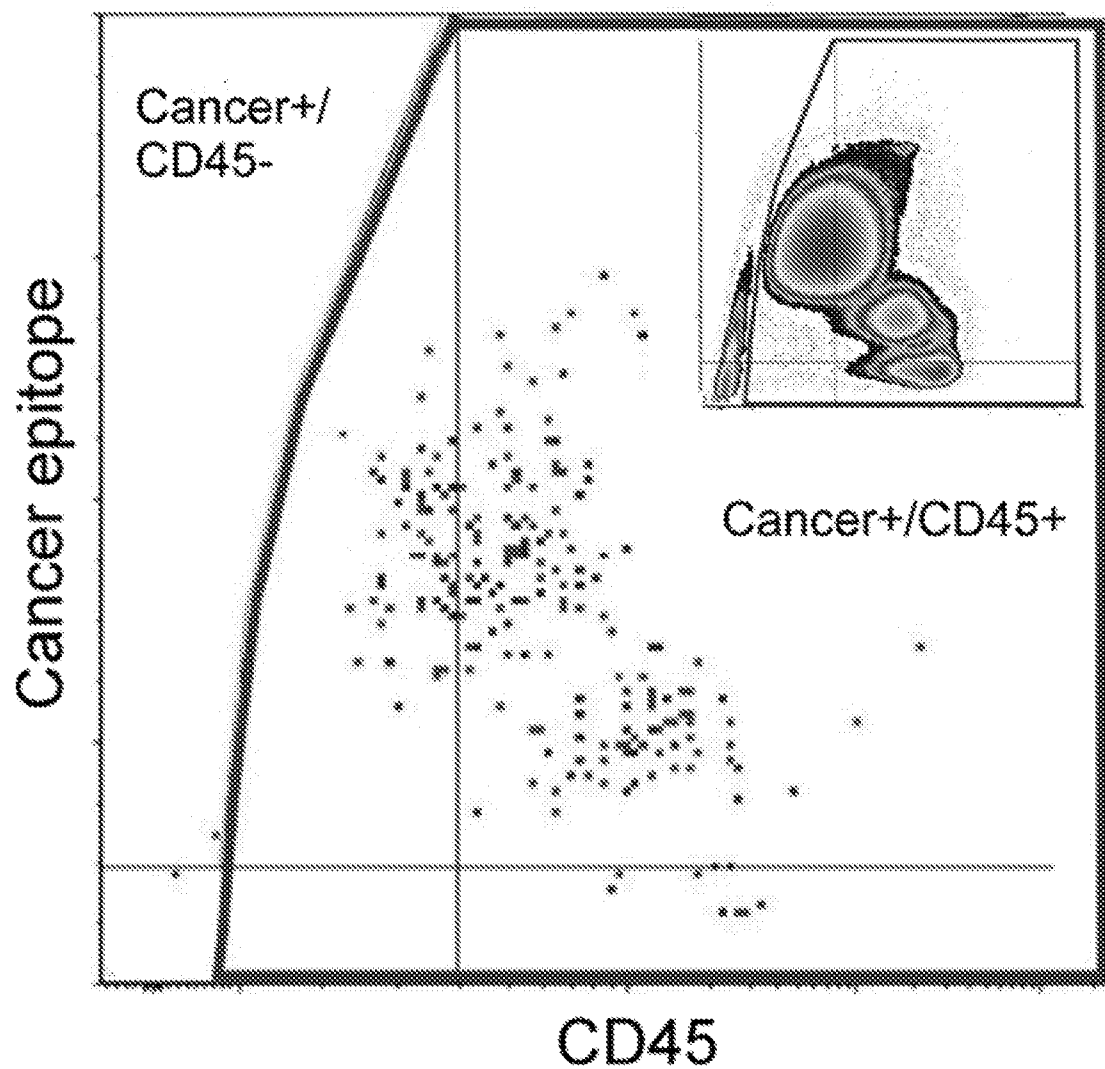


FIG. 13

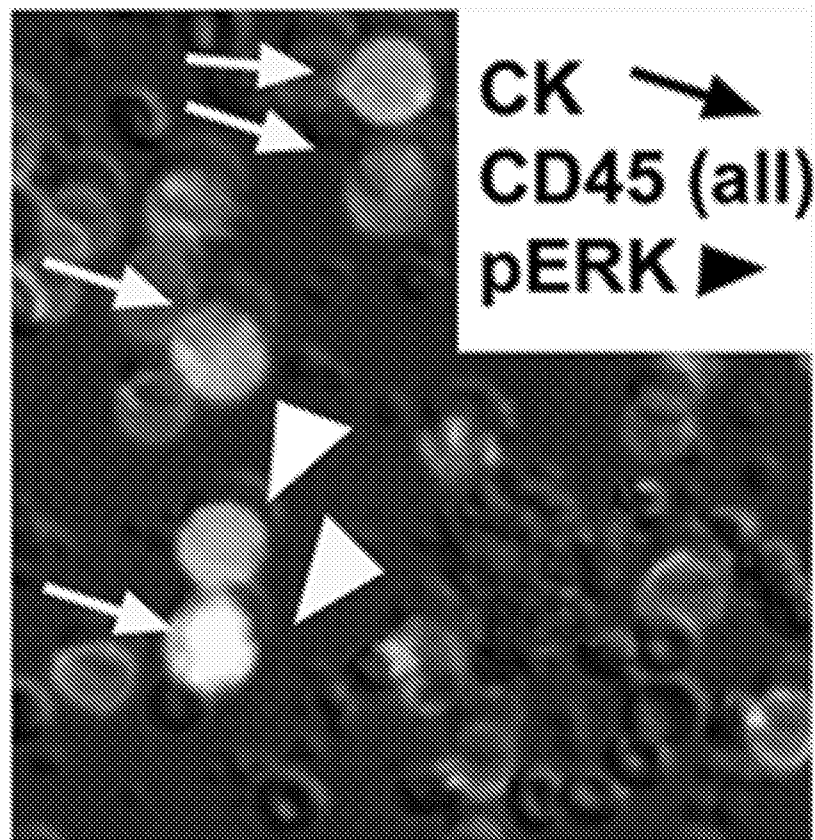
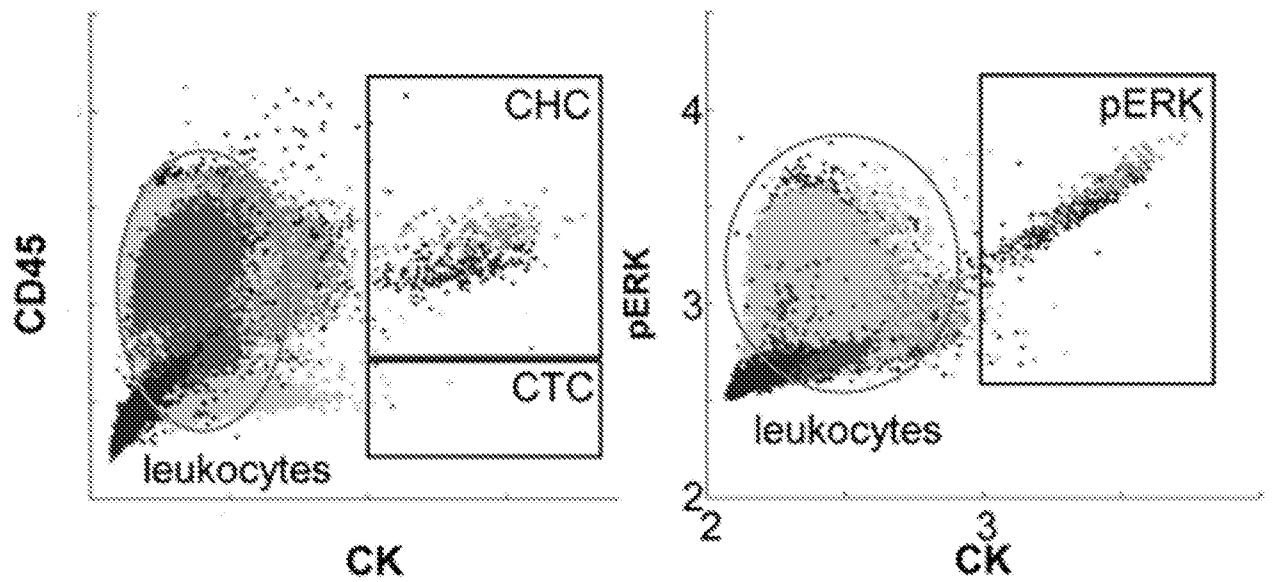
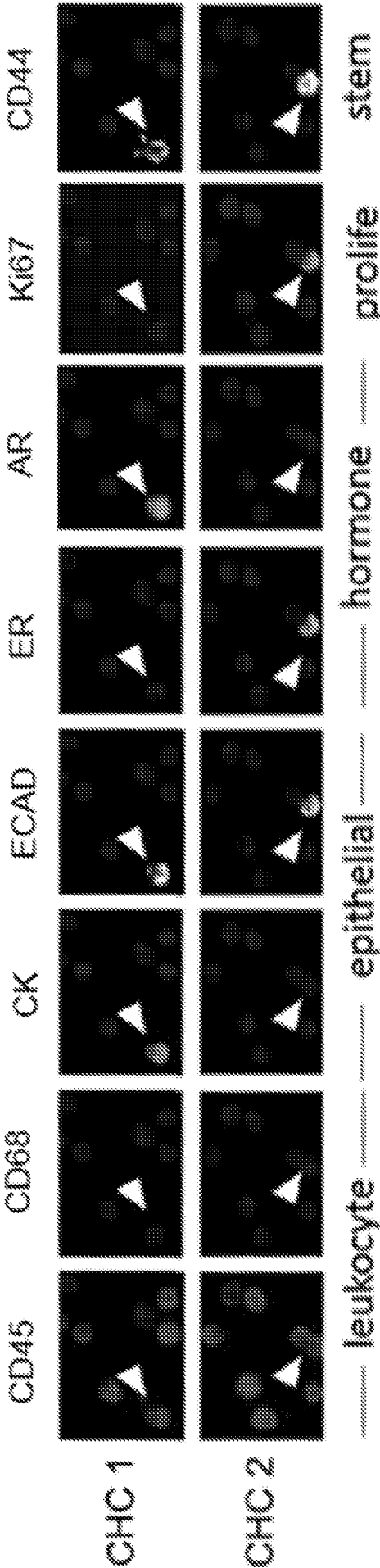


FIG. 14B



CHC1: CD45⁺/CD68⁻/CK⁺/ECAD⁺/ER⁻/AR⁺/Ki67⁻/CD44⁺
CHC2: CD45⁺/CD68⁻/CK⁻/ECAD⁺/ER⁺/AR⁻/Ki67⁺/CD44⁺

FIG. 15

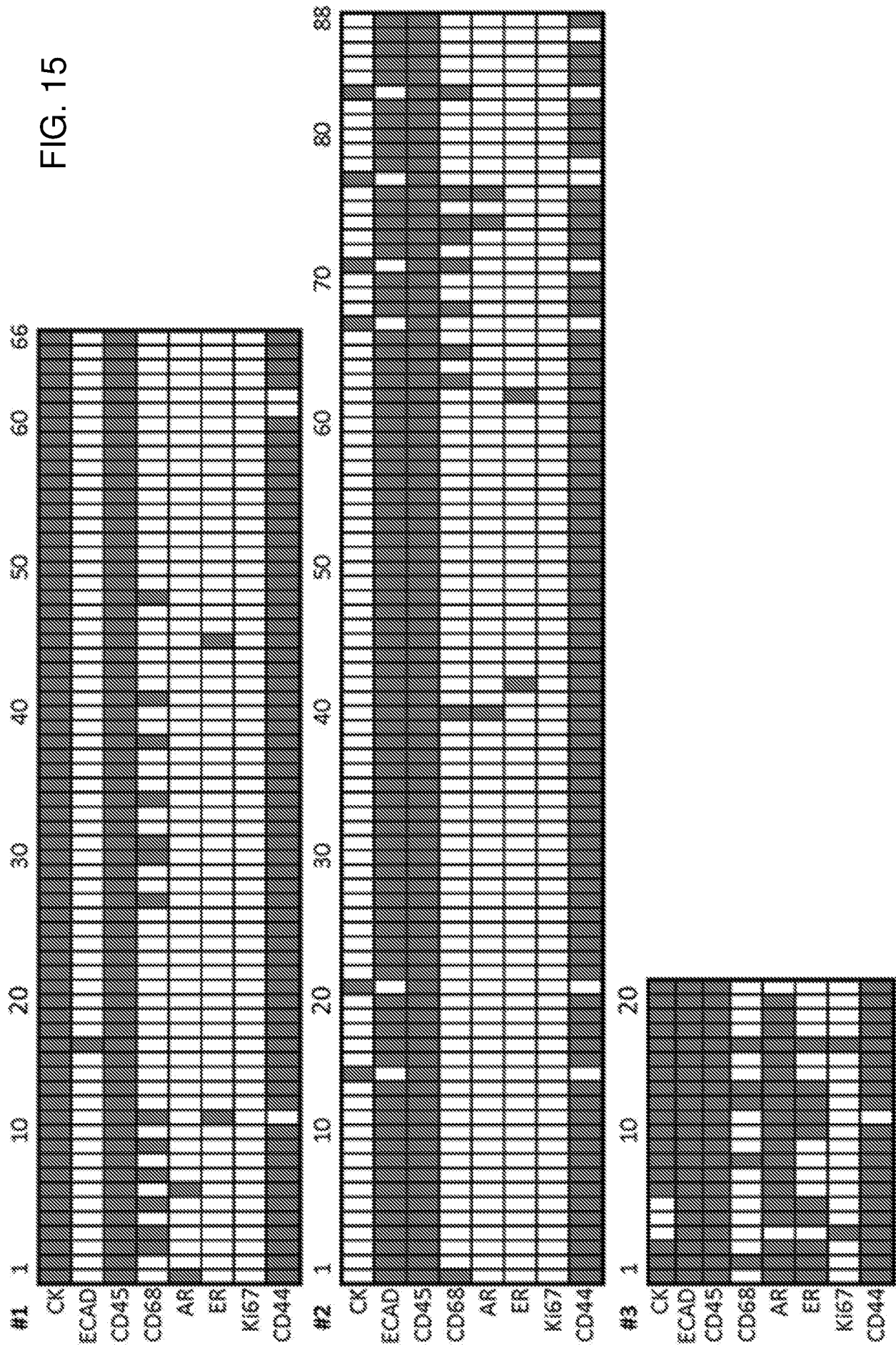


FIG. 16

Longitudinal Assessment of CHCs

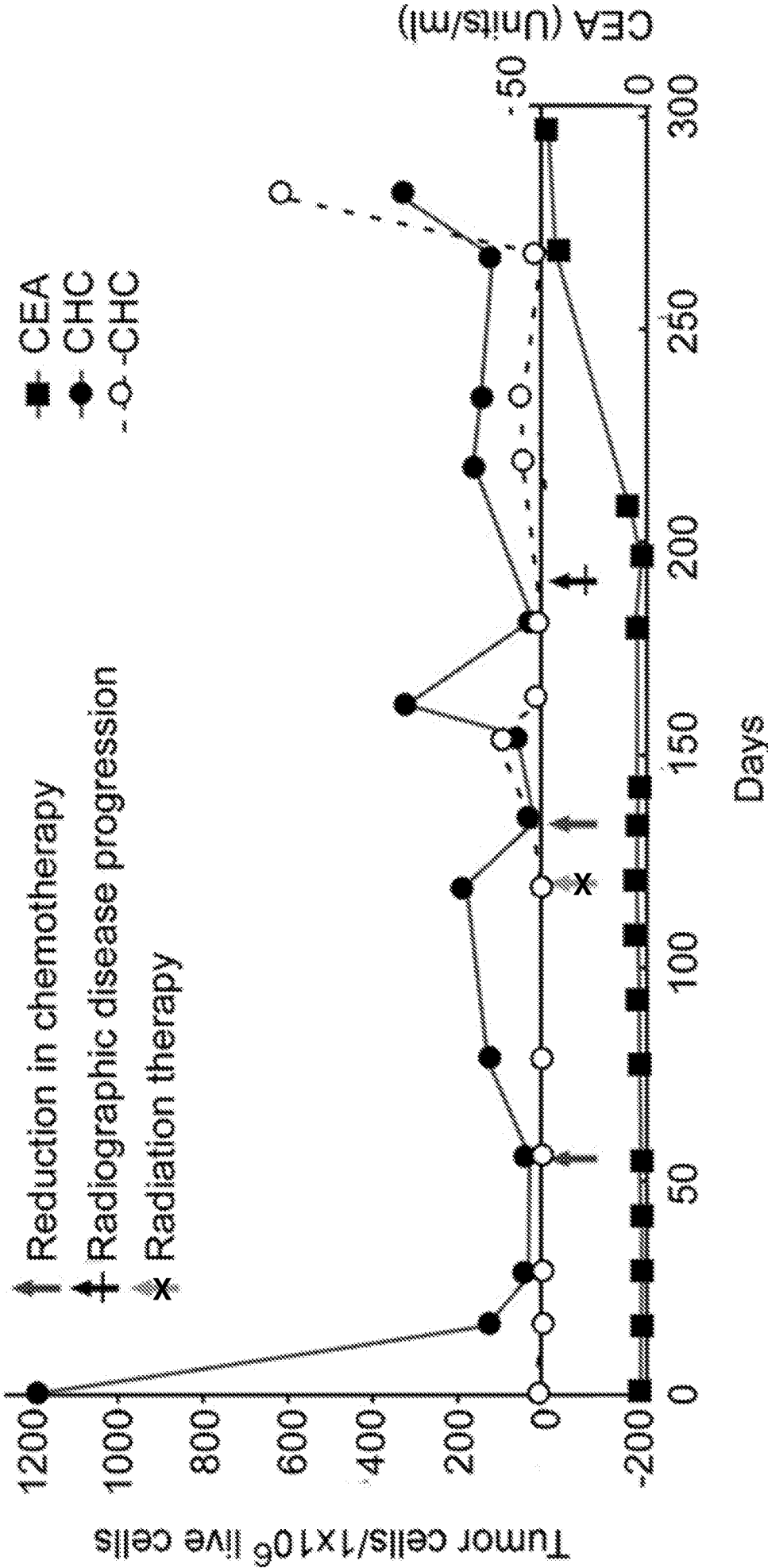
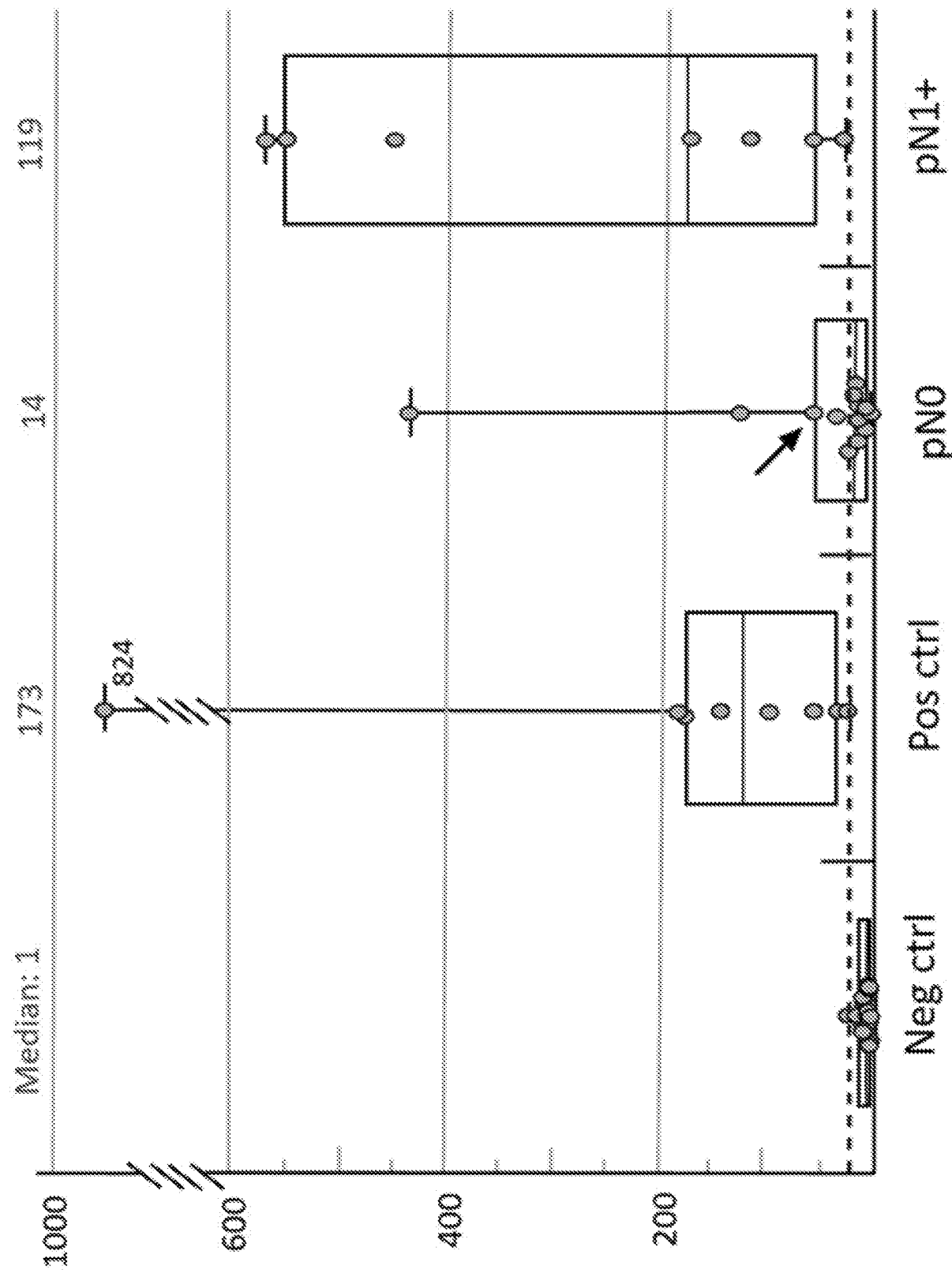


FIG. 17



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/050851

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01K 67/027; A61K 49/00; G01N 33/50; G01N 33/569; G01N 33/574; G01N 33/58 (2019.01)

CPC - A01K 67/0271; A01K 2207/12; A01K 2217/15; A01K 2227/105; A01K 2267/0331; A61K 49/0008; G01N 33/5011; G01N 33/574; G01N 33/57407; G01N 33/57438; G01N 33/57484; G01N 33/582; G01N 2333/4742; G01N 2333/70589 (2019.08)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/0106101 A1 (WONG et al) 20 April 2017 (20.04.2017) entire document	1-9, 11-14, 17-22, 25, 31-34, 41-45, 47
--		
Y		10, 15, 16, 23, 24, 26-30, 35-40, 46
Y	GAST et al. "Cell Fusion Potentiates Tumor Heterogeneity and Reveals Circulating Hybrid Cells that Correlate with Stage and Survival," Science Advances, 01 September 2018 (01.09.2018), Vol. 4, No. 9, Pgs. 1-15. entire document	10, 27-30, 35, 40, 46
Y	US 2011/0027797 A1 (SOMASUNDARAM et al) 03 February 2011 (03.02.2011) entire document	15, 35-40, 46
Y	US 2005/0069982 A1 (SIMARD et al) 31 March 2005 (31.03.2005) entire document	16, 35, 40, 46
Y	WO 2017/137958 A1 (INVICTUS ONCOLOGY PVT. LTD. et al) 17 August 2017 (17.08.2017) entire document	23, 24
Y	WO 2017/186928 A1 (CUREVAC AG) 02 November 2017 (02.11.2017) entire document	26
Y	DAVIES et al. "Inflammation and Proliferation Act Together to Mediate Intestinal Cell Fusion" PLoS One, 06 August 2009 (06.08.2009), Vol. 4, Iss. 8, Pgs. 1-10. entire document	28-30
Y	US 2007/0065846 A1 (BAKER et al) 22 March 2007 (22.03.2007) entire document	28-30, 35, 40, 46

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 November 2019

Date of mailing of the international search report

04 DEC 2019

Name and mailing address of the ISA/US

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Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/050851

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2012/066451 A1 (PFIZER INC. et al) 24 May 2012 (24.05.2012) entire document	35, 40, 46
P, X	SUTTON et al. "Circulating Hybrid Cells Join the Fray of Circulating Cellular Biomarkers," Cellular and Molecular Gastroenterology and Hepatology, 15 July 2019 (15.07.2019), Pgs. 1-13. entire document	1-47
A	CLAWSON et al. "Circulating Tumor Cells in Melanoma Patients," PLoS One, 19 July 2012 (19.07.2012), Vol. 7, Iss. 7, Pgs. 1-12. entire document	1-47
A	US 2007/0099207 A1 (FUCHS et al) 03 May 2007 (03.05.2007) entire document	1-47
A	US 2018/0193454 A1 (IMMUNEXT, INC.) 12 July 2018 (12.07.2018) entire document	1-47
A	SHABO et al. "Macrophage Traits in Cancer Cells are Induced by Macrophage-Cancer Cell Fusion and Cannot be Explained by Cellular Interaction," BMC Cancer, 20 November 2015 (20.11.2015), Vol. 15, Pgs. 1-11. entire document	1-47

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/050851

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1-4 were searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/050851

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 48-50
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 48-50 have been held as omnibus claims, as they refer to an invention "as described herein".

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.