



(51) International Patent Classification:

C12Q 1/6886 (2018.01) A61K 35/17 (2025.01)

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(21) International Application Number:

PCT/US2024/033215

(22) International Filing Date:

10 June 2024 (10.06.2024)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/507,331 09 June 2023 (09.06.2023) US

(71) Applicants: INSTITUTE FOR CANCER RESEARCH

d.b.a. THE RESEARCH INSTITUTE OF FOX CHASE CANCER CENTER [US/US]; 333 Cottman Avenue, Philadelphia, PA 19111 (US). THE UNIVERSITY OF CHICAGO [US/US]; 5801 S Ellis Avenue, Chicago, IL 60637 (US).

(72) Inventors: WANG, Y., Lynn; 1043 Elderberry Way, Chel-

tenham, PA 19012 (US). LU, Pin; 8 Giana Way, Glen Mills, PA 19342 (US). FRANZEN, Carrie; 7151 N Sioux Court, Chicago, IL 60646 (US).

(74) Agent: KANE, Richard, F. et al.; Howson & Howson LLP,

325 Sentry Parkway East, Five Sentry East, Suite 160, Blue Bell, PA 19422 (US).

(81) Designated States (unless otherwise indicated, for every

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

(84) Designated States (unless otherwise indicated, for every

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

(54) Title: COMPOSITIONS AND METHODS FOR THE TREATMENT OF CANCERS THROUGH INHIBITION OF CELL-IN-CELL PHENOMENON

(57) Abstract: Compositions and methods for the treatment of cancers through the inhibition of Cell-in-Cell Phenomenon are provided herein. Also provided are compositions and methods for modulating the CIC internalization process and identification of agents which modulate the same.



WO 2024/254584 A2

Compositions and Methods for the Treatment of Cancers Through Inhibition of Cell-in-Cell Phenomenon

By

Y. Lynn Wang

Pin Lu

Carrie Franzen

Cross Reference to Related Applications

This patent application claims the benefit of U.S. Provisional Patent Application No. 63/507,331, filed June 9, 2023. The entire contents of the foregoing application is incorporated herein by reference, including all text, tables, drawings, and sequences.

Field of the Invention

The present invention relates to the fields of cancer prognosis, risk, relapse, anti-cancer therapies, including chemotherapies, immunotherapies, chemo-immunotherapies and/or targeted therapies for the treatment of residual disease. More specifically, the invention provides combinations of agents which reduce Cell-In-Cell (CIC), also known as emperipolesis cellular processes, through inhibition of pathways, such as the CXCL12-CXCR4 axis, thereby increasing cancer cell death and minimizing the occurrence of residual disease.

Background of the Invention

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated by reference herein as though set forth in full.

Chronic lymphocytic leukemia (CLL) is the most common leukemia/lymphoma among the elderly population. CLL is a chronic lymphoid malignancy characterized by an accumulation of monoclonal mature B-cells in peripheral blood (PB), bone marrow (BM), and secondary lymphoid tissues. Historically, CLL has been considered a disease of defective apoptosis, since tumor cells isolated from the peripheral circulation are dormant and non-proliferating. More recently, however, cell proliferation has been recognized as playing an important role in CLL pathogenesis (Deaglio et al., Haematologica. 2009;94: 752-6; Messrner et al., The Journal of

clinical investigation. 2005; 115:755-64; Schmid et al., *Histopathology*. 1994; 24:445-51; Lampert et al., *Hum Pathol*. 1999; 30:648-54; Fabbri et al., *Nat Rev Cancer*. 2016; 16:145-62; and Gine et al., *Haematologica*. 2010; 95: 1526-33; herein incorporated by reference in their entireties).

5 CLL, overall, is a heterogeneous disease with different clinical presentations, IGHV mutational status, cytogenetic features, and genomic profiles. Patients typically are treated with chemotherapy, immunotherapy using monoclonal anti-CD20 antibodies, or chemo-immunotherapy regimens. A variety of targeted therapies, including ibrutinib (BTK inhibitor) and venetoclax (BCL2 inhibitor), have recently been developed and are generating high response
10 rates, revolutionizing CLL treatment. However, any given individual patient may be intolerant or refractory to a particular therapy, and patients who respond initially may relapse with dismal outcomes. Therapy selection for each patient currently is based on clinical factors such as age, comorbidities, and prior therapies, but the behavior of an individual patient's tumor cells is not taken into consideration. Individualized therapy is especially needed for CLL patients with high-
15 risk profiles, such as chromosomal 17p deletion or complex cytogenetics. These patients experience a higher rate of disease progression on the newer therapy ibrutinib (Maddocks et al., *JAMA oncology*. 2015; 1:80-7; and Kadri et al., *Blood advances*. 2017; 1 : 715-277; herein incorporated by reference in their entireties).

The ability of cancer cells to persist in response to anti-cancer therapy is the major reason
20 for treatment failure. Many cancers initially respond well to therapy but, after the initial response, patients can relapse due to the presence of drug resistant cancer cells. The molecular mechanism underlying drug resistant cancer cells is still poorly understood; however, recent studies have demonstrated a critical role for the Cell-in-Cell phenomenon in cancer disease persistence after therapy.

25 Cell-in-cell (CIC) describes a century old observation where an intact cell can be seen inside another whole cell. This phenomenon has been associated with the prognosis of cancers and promotion of tumor progression, especially in hematopoietic malignancies. These tumor cells evade drug treatment by hiding in the surrounding stromal cells, remain as residual disease after treatment and may become the origin for future disease relapse. However, in the absence of
30 systematic studies of case cohorts, understanding of the pathological significance of live CIC remains challenging.

Clearly, there is a major clinical need to understand the direct impact that the cancer CIC phenomenon has on cancer disease persistence after therapy. Such information would provide guidance for effective therapeutic strategies to eliminate the residual disease in certain cancers.

5

Summary of the Invention

In accordance with the present invention, methods for modulating tumor CIC internalization are provided. An exemplary method comprises incubating an ex vivo cell culture system, including a first cell culture of bone marrow fibroblasts (BMF) which express one or more cell signaling molecules and a second cell culture comprising leukemia or lymphoma cells isolated from a human; and optionally one or more exogenous soluble cell signaling molecules or growth factors, in the presence and absence of at least one anticancer agent. The number and quality of tumor CIC internalized into said first cell culture in the presence of said at least one anti-cancer agent relative to untreated cell cultures is then determined, thereby identifying agents which modulate the CIC tumor cell internalization process. In certain embodiments, the agent is a BTK inhibitor. In certain embodiments, the methods further comprise administering a CXCR4 antagonist. In another embodiment, the method further comprises detecting a CIC associated biomarker. In certain embodiments, the CIC internalization process is inhibited or increased. In certain embodiments, increased lymphoma or leukemia CIC internalization is indicative of increased risk for minimal residual disease (MRD).

20

In another aspect of the invention, combination regimens useful in cancer treatment comprising the co-administration to a subject in need thereof of effective amounts of a) at least one BTK inhibitor and b) at least one CXCR4 antagonist in at least one pharmaceutically acceptable carrier are provided. In certain embodiments the BTK inhibitor is selected from Ibrutinib, ONO/GS-4059 (tirabrutinib), AVL-292/CC-292/spebrutinib, BGB-3111 (Zanubrutinib), and ACP-196/acalabrutinib, M7583, MSC2364447C, BIIB068, ACO0058TA, DTRMWXHS-12, LOXO305 (pirtobrutinib). In certain embodiments, the CXCR4 antagonist is selected from Plerixafor, T140 analogs, BL-8040 (previously BKT140), TN14003, MSX-122, TG-0054, cyclic-pentapeptide-based antagonists, FC122 and FC131, tetrahydroquinolines-based antagonists, AMD070 AMD070 derivatives, indole-based antagonists, FC131, Para-xylyl-enediamine-based compounds, AMD3465, AMD3465 analogues WZ811, MSX122, guanidine-

based Antagonists, NB325, quinoline derivatives, NSC56612, KRH-3955, CTCE-9908, POL6326, motixafortide, and mavorixafor. In certain embodiments, the combination regimen further comprises at least one additional anti-cancer agent. In certain embodiments, the cancer is selected from lymphoma, leukemia, chronic lymphocytic leukemia (CLL), a non-Hodgkin's lymphoma (NHL), small lymphocytic lymphoma (SLL), follicular lymphoma (FL), marginal zone lymphoma (MZL), mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL) or a T-cell lymphoma selected from peripheral T-cell lymphoma and T-prolymphocytic lymphoma. In certain embodiments, the combination acts synergistically. In certain embodiments, the cancer has CIC tumor cells.

In certain embodiments, the at least one agent reduces undesirable side effects from administration of said one agent present in said combination of agents. In certain embodiments, the side effect is increased occurrence of CIC.

In another aspect of the invention, methods for treating CIC associated cancer in a subject in need thereof are provided. In one aspect, the method comprises administering an effective amount of at least one BTK inhibitor along with administration of an effective amount of at least one CXCR4 antagonist to reduce CIC occurrence. In certain embodiments the BTK inhibitor is selected from Ibrutinib, ONO/GS-4059 (tirabrutinib), AVL-292/CC-292/spebrutinib, BGB-3111 (Zanubrutinib), and ACP-196/acalabrutinib, M7583, MSC2364447C, BIIB068, ACO0058TA, DTRMWXHS-12, LOXO305 (pirtobrutinib). In certain embodiments, the CXCR4 antagonist is selected from Plerixafor, T140 analogs, BL-8040 (previously BKT140), TN14003, MSX-122, TG-0054, cyclic-pentapeptide-based antagonists, FC122 and FC131, tetrahydroquinolines-based antagonists, AMD070 AMD070 derivatives, indole-based antagonists, FC131, Para-xylylenediamine-based compounds, AMD3465, AMD3465 analogues WZ811, MSX122, guanidine-based Antagonists, NB325, quinoline derivatives, NSC56612, KRH-3955, CTCE-9908, POL6326, motixafortide, and mavorixafor. In certain embodiments the method further comprises administering at least one additional anti-cancer agent. In certain embodiments, the combination acts synergistically. In certain embodiments, the cancer comprises CIC tumor cells.

In certain embodiments, the at least one agent reduces undesirable side effects caused by administration of said one agent present in said combination of agents. In certain embodiments, the side effect is increased occurrence of CIC. In certain embodiments, the cancer is lymphoma,

leukemia, or chronic lymphocytic leukemia. In certain embodiments, the cancer comprises CIC tumor cells.

In another aspect, methods for treating BTK inhibitor resistant cancer in a subject in need thereof, comprising administering at least one CXCR4 antagonist are provided. Also provided are methods for increasing the efficacy of BTK inhibitor in the treatment of cancer, the method comprising co-administering at least one CXCR4 antagonist with said BTK inhibitor. Also provided are methods for preventing relapse of CLL cancer after treatment with at least one BTK inhibitor, the method comprising co-administering at least one CXCR4 antagonist with said BTK inhibitor

The present invention also provides compositions and methods which modulate the cell in cell (CIC) internalization process in order to reduce risk of minimal residual malignant disease. The inventive methods inhibit such cells from evading anti-cancer treatments including without limitation, chemotherapeutic drugs, immunotherapies, targeted agents or any combination thereby minimizing undesirable cancer cell survival and disease persistence. Resulting MRD arising from the presence of these evasive cancer cells, which often mutate and proliferate results in relapse of cancer after months or years of being cancer free. With additional mutations, the relapsed cancers often become refractory to additional therapies, resulting in very poor survival outcomes for the patients.

Still other aspects and advantages of these compositions and methods are readily apparent and described further in the following detailed description of the invention.

Brief Description of the Drawings

5 **Figure 1:** Summary of the experimental results. (*) BTK inhibitor (BTKi), such as Ibrutinib, drives chronic lymphocytic leukemia (CLL) cells into bone marrow fibroblasts (cell-in-cell). (#), Chemokine CXCR4-CXCL12 axis mediates this process. (+), A CXCR4 antagonist blocks ibrutinib (ibr)-driven CIC.

10 **Figure 2A-2B. CIC are observed in archived human lymphoma samples. Arrows highlight the CIC. Fig. 2A)** Pleural fluid (Left) and lymph node biopsy (right) from a patient with lymphoplasmacytic lymphoma. Note the membrane of the outer cell in the LN biopsy (faint but discernable). **Fig. 2B)** Pleural fluid from a patient with CLL.

Figure. 3A-3B. TME system. Fig. 3A) Major steps in setting up TME model system: 1- 2) Place collected human tissues with pre-seeded bone marrow fibroblasts (BMF); 3) Add B-cell growth factors such as CpG/IL15 and drugs; and co-culture for 5-7 days or longer; and 4) Examine with microscopy or perform flow cytometry. **Fig. 3B)** Cells divide into daughter cells. % of cell in division can be calculated as $\% \text{ proliferation} = \text{daughter}/(\text{daughter} + \text{parental}) \times 100\%$. Top, BMF only. Bottom, BMF+TME stimuli (CpG/IL15).

Figures 4A-4F: Fixed confocal images of CIC structures. Images are acquired with Leica TCS SP8 FSU laser scanning confocal microscope using a 40x oil lens. **FIG. 4A)** Confocal images showing tumor cells in cytoplasm labeled in green, BMF cytoplasm in red, and the nuclei of BMF and CLL in blue. **FIG. 4B)** Images are reconstructed in 3D using Imaris v10.0. BMF cells are visualized as the red surfaces and CLL cells as the green surfaces. **FIG. 4C)** Red surfaces are transformed to a transparent state. Track cell 1, 2, or 3 through Fig. 4A, 4B, and 4C. **FIG. 4D-4F)** Show one CLL cell that was captured halfway in the entry process.

Figure 5A-5F: Time lapsed microscopy showing CLL cells are internalized, remain viable and move within the BMF. BMF are visualized as either the red or transparent pink, and CLL cells as the green surfaces (40x oil lens). Bar = 20 μm . Track cell 1, 2 and 3 through **Figs. 5A-5C**, and compare solid images (**Figs. 5A-5C**) with the corresponding transparent images (**Figs. 5D-5F**). Time-lapsed images are acquired with Leica TCS SP8 SMD confocal microscope. Images are reconstructed in 3D using Imaris v10.0.

Figures 6: CIC is associated with prior therapies. The number of internalized CLL cells is greater in treated than in un-treated patients in response to TME stimuli, +/-, IL-15/CpG and CD40L. 20 cases of untreated CLL. NS, not significant. 21 cases of treated CLL. $P=0.0045$, analyzed by a paired, non-parametric, one-tailed t-test.

Figure 7A-7C: CIC increases with ex vivo ibrutinib exposure. Fig. 7A) CIC increases in CLL (N=11) but not in normal B-cells (N=5). NBC, normal B-cells isolated from 5 healthy donors. Cells were treated with 0.4 μM ibr for 5 hrs. **Fig. 7B)** CIC in both ibr-sensitive (orange, N=11) and ibr-resistant BTK-mutant CLL cases (blue, N=6). Cells were treated with 0.4 μM ibr for 5 hrs. **Fig. 7C)** Time course of CIC in both BTK WT (orange, N=3) and mutant cells (blue, N=3).

Fig. 8A-8B: CIC occurs in follicular lymphoma cell line and primary tumor cells. Fig. 8A) CIC in FSCCL cell line. Each dot represents one experiment. Ten repeat experiments

are shown. **Fig. 8B)** CIC increased with ex vivo ibr exposure in FL primary samples (N=9). Cells were treated with 0.4 μ M for 5 hrs. Each dot represents a case. Data was analyzed with paired, parametric, two-tailed t-test.

Fig. 9A-9B: CIC responses to newer BTK inhibitors. Fig. 9A) CIC increased upon exposure to the covalent BTKi zanubrutinib in CLL (N=5). NBC, normal B-cells (N=5). **Fig. 9B)** CIC increased upon exposure to the non-covalent BTKi pirtobrutinib (pirt) in CLL (N=10).

Fig. 10: CIC are observed in the BM of BTKi-treated patients. Four panels represent the bone marrow aspirate smears from 4 patients.

Fig. 11A-11B: Different CIC responses to BTK inhibitors and venetoclax. Fig. 11A) CIC in venetoclax-treated vs ibrutinib-treated cells (N=10). Cells were treated for 5 hrs. **Fig. 11B)** Percentage of changes based on data in A. % change = (drug – DMSO) / DMSO * 100%. Data was analyzed with paired, parametric, two-tailed t-test.

Fig. 12A-12D: Molecular determinants of CIC in the TME system. Fig. 12A) Cell proliferation determined by the CFSE assay. Top, cells cultured with BMF only. Bottom, cells cultured with BMF and TME stimuli CpG+IL15. Percentage of cell in proliferation are calculated as % proliferation = daughter / (daughter+parental) x100%. **Fig 12B)** The correlation between CLL cell proliferation (as quantitated by CSFE) and cell internalization. R^2 and P for simple linear regression are indicated. Blue circles represent samples with proliferation < 50%; purple circles represent samples with proliferation > 50%. Solid line is best fit line, dotted lines are the 95% confidence intervals. **Fig. 12C)** Table listing known ligand-receptor interactions and/or soluble factors in the TME. The last column indicates the types of transduced BMF created with lentiviral transduction. **Fig. 12D)** Effects of conditioned media from transduced BMF on CIC over time. Each line with different symbols represents a different patient sample.

Figure 13: Plerixafor (Pleri) antagonizes chemokine-induced cell internalization. FSCCL cells were treated as indicated for 24hrs. CM, conditioned media. CXCL, CM from CXCL12/13 transduced BMF. Ctrl, CM from non-transduced BMF. Pleri was added at 1 μ M and CIC counting conducted at 24 hr after the addition of drug. Each of the three experiments was represented by one dot. Data was analyzed with ANOVA test.

Fig. 14A-14B: Pleri antagonizes ibr-driven cell internalization. Fig. 14A) FSCCL cells were treated as indicated in the absence of CM. Ibr was added at 0.4 μ M and Pleri at 1 μ M. CIC counting was conducted 24 hrs following drug administration. Each of the five experiments

was represented by one dot. **Fig. 14B)** CXCR4 in FSCCL cells were knocked out using gRNA#1 (KO#1) and gRNA#2 (KO#2). CIC counting conducted was conducted at 5 hrs post co-culture.

Fig. 15: CIC was blocked by Motixafortide (motix), another CXCR4 antagonist.

FSCCL cells were treated as indicated. Ibr was added at 0.4 μ M and Motix at 0.4 μ M . CIC

counting was conducted 5 hrs following drug administration. The experiments were repeated twice and error bars represent the average of five fields at 40x magnification.

Figure 16: Pleri antagonizes chemokine-induced cell internalization in primary CLL cells. Primary CLL cells (N=6) were treated similarly to the cell line. Each dot represents a sample. Data was analyzed with paired, parametric, two-tailed t-test.

Detailed Description of the Invention

The data presented herein describe a novel disease persistence mechanism and efficacious combination therapies for treating, preventing, and/or inhibiting the progression of recurrent cancers caused by such drug evasion mechanisms. Using ex vivo co-culture models that mimic the lymph node tumor microenvironment, we have demonstrated that tumor cells remain sheltered within other cells, such as bone marrow fibroblasts, thereby rendering them resistant to anti-cancer therapies, such as chemotherapeutic drugs or targeted agents. . Such cells persist as residual disease after treatment and often contribute to future disease relapse.

The TME models described in Fig 2 further facilitated the development of anti-cancer drugs and drug combinations which prevent or minimize CIC occurrence, thereby reducing residual disease. For example, CXCR4 antagonists reduce CIC in ibrutinib-treated tumor cells. Accordingly, this data indicates that treating cancers with CXCR4 inhibitors enhances the therapeutic effects of BTK inhibitors and promotes long-term remission or cure.

Definitions

Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have meanings that are commonly understood by those of ordinary skill in the art. In addition to definitions included in this sub-section, further definitions of terms are interspersed throughout the text.

In this invention, “a” or “an” means “at least one” or “one or more,” etc., unless clearly indicated otherwise by context. The term “or” means “and/or” unless stated otherwise. In the case of a multiple-dependent claim, however, use of the term “or” refers back to more than one preceding claim in the alternative only.

5 The terms “about” or “approximately” in the context of numerical values and ranges refers to values or ranges that approximate or are close to the recited values or ranges such that the embodiment may perform as intended, such as having a desired amount of nucleic acids or polypeptides in a reaction mixture, as is apparent to the skilled person from the teachings contained herein. In some embodiments, about means plus or minus 10% of a numerical amount.

10 Furthermore, a compound “selected from the group consisting of” refers to one or more of the compounds in the list that follows, including mixtures (i.e. combinations) of two or more of the compounds. According to the present invention, an isolated, or biologically pure molecule is a compound that has been removed from its natural milieu. As such, “isolated” and “biologically pure” do not necessarily reflect the extent to which the compound has been
15 purified. An isolated compound of the present invention can be obtained from its natural source, can be produced using laboratory synthetic techniques or can be produced by any such chemical synthetic route.

 The terms “agent” and “test compound” denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials
20 such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Biological macromolecules include siRNA, shRNA, antisense oligonucleotides, peptides, peptide/DNA complexes, and any nucleic acid-based molecule which encoded the proteins described herein. Molecularly targeted agents, such as BTK inhibitors, BCL2 inhibitors and CXCR4 inhibitors are also described herein.

25 It is also contemplated that the term “compound” or “compounds” refers to the compounds discussed herein and includes precursors and derivatives of the compounds, and pharmaceutically acceptable salts of the compounds, precursors, and derivatives.

 The phrase “consisting essentially of” when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID NO. For example, when used in
30 reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the functional and novel characteristics of the sequence.

The term "delivery" as used herein refers to the introduction of foreign molecules (i.e., miRNA encoding the polypeptide of interest) into cells. The term "administration" as used herein means the introduction of a foreign molecule into a cell. The term is intended to be synonymous with the term "delivery".

5

Combinations

Combinations comprising at least one anti-cancer therapy, chemo-immunotherapeutic therapy, and at least one CIC signaling pathway antagonist are provided. The combinations provided herein act to reduce undesirable side effects of certain potent anticancer agents while also effectively killing tumor cells, thereby treating cancer. In certain embodiments, the therapies comprise at least one BTK inhibitor. In certain embodiments, the CIC signaling pathway antagonist is at least one CXCR4 antagonist.

Also provided herein are therapeutic combinations comprising at least one BTK inhibitor and at least one CXCR4 antagonist. In preferred embodiments, the combinations provided herein act synergistically to kill tumor cells and treat cancer.

The term "synergy" or "synergistic" refers to the interaction or cooperation of two or more substances, or other agents to produce a combined effect greater than the sum of their separate effects. In certain embodiments, the components of the therapeutic combinations provided herein act synergistically.

Optionally, the combination may contain at least a third active component, which may be an anti-cancer therapy, such as a chemotherapeutic agent or an immunotherapy or another targeted agent. In one embodiment, one or more of these anti-cancer agents may be used in a therapeutic combination described herein. When separately formulated, the at least two active components can be administered simultaneously or sequentially, optionally via different routes. Optionally, the treatment regimens for each of the active components in the combination have different but overlapping delivery regimens, e.g., daily, twice daily, vs. a single administration, or weekly. The second active component may be delivered prior to, substantially simultaneously with, or after, the second active component.

Also provided herein are formulations containing a single or more multiple active components useful in a combination therapy, and therapeutic regimens for treatment of cancer.

The terms “combination therapy” or “combined treatment” or “in combination” as used herein encompass any form of concurrent or parallel treatment with at least two distinct therapeutic agents. Combinations of interest, include without limitation, a minimum of at least one BTK inhibitor and at least one CXCR4 antagonist. Another combination of interest includes at least one anti-cancer therapy and at least one antagonist of CIC control signaling pathways. Optionally, three or more components may be used in a combination regimen. Additionally, the combinations provided herein may be used in conjunction with other types of treatment. For example, other anti-cancer treatment may be selected from the group consisting of chemotherapy, immunotherapies, other targeted agents such as BCL2 inhibitors, surgery, radiotherapy (radiation), and/or hormone therapy, amongst other treatments, associated with the current standard of care for the patient.

The combinations provided herein utilize pharmaceutical compositions or medicaments comprising a compound and/or drug and at least one pharmaceutically acceptable carrier, diluent, excipient and/or adjuvant. The carrier(s) are “acceptable” in the sense of being compatible with the other ingredients of the formulation and, in the case of a pharmaceutically acceptable carrier, not deleterious to the recipient thereof in an amount used in the medicament.

Compounds

The term “drug response” as used herein, means any biological response in an organism that is the result of exposure to the drug. Drug responses can be favorable, such as when a patient's disease is eradicated by treatment with the drug, or unfavorable, such as when a patient relapses or enters a coma upon treatment with a drug.

The terms “inhibition” or “inhibit” refer to a decrease or cessation of any event (such as protein ligand binding) or to a decrease or cessation of any phenotypic characteristic or to the decrease or cessation in the incidence, degree, or likelihood of that characteristic. To “reduce” or “inhibit” is to decrease, reduce or arrest an activity, function, and/or amount as compared to a reference. It is not necessary that the inhibition or reduction be complete. For example, in certain embodiments, “reduce” or “inhibit” refers to the ability to cause an overall decrease of 20% or greater. In another embodiment, “reduce” or “inhibit” refers to the ability to cause an overall decrease of 50% or greater. In yet another embodiment, “reduce” or “inhibit” refers to the ability to cause an overall decrease of 75%, 85%, 90%, 95%, or greater. Inhibition can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33,

34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100%.

The term “modulate” as used herein refers to the ability of a compound to change an activity in some measurable way as compared to an appropriate control. As a result of the presence of compounds in the assays, activities can increase or decrease as compared to controls in the absence of these compounds. Preferably, an increase in activity is at least 25%, more preferably at least 50%, most preferably at least 100% compared to the level of activity in the absence of the compound. Similarly, a decrease in activity is preferably at least 25%, more preferably at least 50%, most preferably at least 100% compared to the level of activity in the absence of the compound. A compound that increases a known activity is an “agonist”. One that decreases, or prevents, a known activity is an “antagonist”.

The term “inhibitor” refers to a natural or synthetic compound that has a biological effect to inhibit, significantly reduce or down-regulate the expression of a gene and/or a protein or that has a biological effect to inhibit or significantly reduce the biological activity of a protein. In certain embodiments, the inhibitor is a small nucleic acid inhibitor. A “small nucleic acid inhibitor” refers to any sequence based nucleic acid molecule which, when introduced into a cell expressing the target nucleic acid, is capable of modulating expression of that target. Small nucleic acid inhibitors including, without limitation, siRNA, antisense oligonucleotides, miRNA, shRNA and the like, may be utilized in this invention.

An “siRNA” refers to a molecule involved in the RNA interference process for a sequence-specific post-transcriptional gene silencing or gene knockdown by providing small interfering RNAs (siRNAs) that has homology with the sequence of the targeted gene. Small interfering RNAs (siRNAs) can be synthesized in vitro or generated by ribonuclease III cleavage from longer dsRNA and are the mediators of sequence-specific mRNA degradation. Preferably, the siRNAs of the invention are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. The siRNA can be synthesized as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions. Specific siRNA constructs for inhibiting mRNA, may be between 15-35 nucleotides in length, and more typically about 21 nucleotides in length.

An “antisense” nucleic acid sequence (antisense oligonucleotide) can include a nucleotide sequence that is complementary to a “sense” nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to the gene of interest (e.g. BTK or CXCR4). Antisense nucleic acid sequences and delivery methods are well known in the art (Goodchild, *Curr. Opin. Mol. Ther.*, 6(2):120-128 (2004); Clawson, et al., *Gene Ther.*, 11(17):1331-1341 (2004)), which are incorporated herein by reference in their entirety. An antisense nucleic acid can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. The antisense nucleic acid also can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest).

The terms “miRNA” and “microRNA” refer to about 10-35 nt, preferably about 15-30 nt, and more preferably about 19-26 nt, non-coding RNAs derived from endogenous genes encoded in the genomes of plants and animals. They are processed from longer hairpin-like precursors termed pre-miRNAs that are often hundreds of nucleotides in length. MicroRNAs assemble in complexes termed miRNPs and recognize their targets by antisense complementarity. These highly conserved, endogenously expressed RNAs are believed to regulate the expression of genes by binding to the 3'-untranslated regions (3'-UTR) of specific mRNAs as well as other regions on targeted mRNAs. Without being bound by theory, a possible mechanism of action assumes that if the microRNAs match 100% their target, i.e. the complementarity is complete, the target mRNA is cleaved, and the miRNA acts like a siRNA. However, if the match is incomplete, i.e. the complementarity is partial, then the translation of the target mRNA is blocked. The manner by which a miRNA base-pairs with its mRNA target correlates with its function: if the complementarity between a mRNA and its target is extensive, the RNA target is cleaved; if the complementarity is partial, the stability of the target mRNA is not affected but its translation is repressed.

The term “shRNA” (also referred to as “short hairpin RNA” or “small hairpin RNA”) refers to an artificial RNA having a tight hairpin turn that can be used to silence target gene expression.

The phrase “anti-cancer therapy” refers to a therapy useful in treating cancer. Types of anti-cancer therapies include, but are not limited to, chemotherapy, immunotherapy, targeted therapies, surgery, and radiation therapies. Examples of anticancer therapeutic agents include, but are not limited to, e.g., chemotherapeutic agents, growth inhibitory agents, cytotoxic agents, agents used in radiation therapy, anti-angiogenesis agents, apoptotic agents, antitubulin agents, and other agents to treat cancer, anti-CD20 antibodies, platelet derived growth factor inhibitors (e.g., Gleevec™ (Imatinib Mesylate)), a COX-2 inhibitor (e.g., celecoxib), interferons, cytokines, antagonists (e.g., neutralizing antibodies) that bind to one or more of the following targets PDGFR-beta, BlyS, APRIL, BCMA receptor(s), TRAIL/Apo2, and other bioactive and organic chemical agents, etc. Combinations thereof are also included in the invention.

Chemotherapeutic agents are compounds that exhibit anticancer activity and/or are detrimental to a cell (e.g., a toxin). Suitable chemotherapeutic agents for use in the methods disclosed herein include, but are not limited to: toxins (e.g., saporin, ricin, abrin, ethidium bromide, diphtheria toxin, Pseudomonas exotoxin, and others listed above); alkylating agents (e.g., nitrogen mustards such as chlorambucil, cyclophosphamide, ifosfamide, mechlorethamine, melphalan, and uracil mustard; aziridines such as thiotepa; methanesulphonate esters such as busulfan; nitroso ureas such as carmustine, lomustine, and streptozocin; platinum complexes such as cisplatin and carboplatin; bioreductive alkylators such as mitomycin, procarbazine, dacarbazine and altretamine); DNA strand-breakage agents (e.g., bleomycin); topoisomerase II inhibitors (e.g., amsacrine, dactinomycin, daunorubicin, idarubicin, mitoxantrone, doxorubicin, etoposide, and teniposide); DNA minor groove binding agents (e.g., plicamycin); antimetabolites (e.g., folate antagonists such as methotrexate and trimetrexate; pyrimidine antagonists such as fluorouracil, fluorodeoxyuridine, CB3717, azacitidine, cytarabine, and floxuridine; purine antagonists such as mercaptopurine, 6-thioguanine, fludarabine, pentostatin; asparaginase; and ribonucleotide reductase inhibitors such as hydroxyurea); tubulin interactive agents (e.g., vincristine, vinblastine, and paclitaxel (Taxol)); hormonal agents (e.g., estrogens; conjugated estrogens; ethinyl estradiol; diethylstilbestrol; chlortrianisene; idenestrol; progestins such as hydroxyprogesterone caproate, medroxyprogesterone, and megestrol; and androgens such as

testosterone, testosterone propionate, fluoxymesterone, and methyltestosterone); adrenal corticosteroids (e.g., prednisone, dexamethasone, methylprednisolone, and prednisolone); leutinizing hormone releasing agents or gonadotropin-releasing hormone antagonists (e.g., leuprolide acetate and goserelin acetate); and antihormonal antigens (e.g., tamoxifen, antiandrogen agents such as flutamide; and antiadrenal agents such as mitotane and aminoglutethimide). In a particular embodiment, the chemotherapeutic agent is selected from the group consisting of: paclitaxel (Taxol®), cisplatin, docetaxol, carboplatin, vincristine, vinblastine, methotrexate, cyclophosphamide, CPT-11, 5-fluorouracil (5-FU), gemcitabine, estramustine, carmustine, adriamycin (doxorubicin), etoposide, arsenic trioxide, irinotecan, and epothilone derivatives.

The term “immunotherapy” refers to the treatment of cancer by activating or suppressing the immune system. Immunotherapies designed to elicit or amplify an immune response may be referred to as activation immunotherapies or immune activators, whereas immunotherapies that reduce or suppress such response may be referred to as suppression immunotherapies or immune suppressors. As used herein, the term “cancer immunotherapy” refers to an immunotherapy used for the treatment of a cancer, said immunotherapy modulating the immune response of a subject with the aim of inducing and/or stimulating the immune response of the subject towards cancer cells. Common antibodies used for the treatment of cancer include without limitation, trastuzumab (Herceptin), pertuzumab (Perjeta), bevacizumab (Avastin) rituximab (Mabthera) and obinutuzumab.

In some embodiments, cancer immunotherapy comprises, or consists of, the adoptive transfer of immune cells (ACT), in particular of T cells (such as alpha beta ($\alpha\beta$) T cells or gamma delta ($\gamma\delta$) T cells), NK cells or NK T cells.

The phrase “targeted therapy” or “targeted molecular therapy” refers to treatment using any molecule which aims at one or more particular target molecules (e.g., proteins) involved in tumor genesis, tumor progression, tumor metastasis, tumor cell proliferation, cell repair, and the like. Targeted therapy agents include, for example, monoclonal antibodies and small molecule drugs. Non-limiting examples of targeted therapy agents include signal transduction inhibitors, growth factor inhibitors, tyrosine kinase inhibitors, EGFR inhibitors, histone deacetylase (HDAC) inhibitors, proteasome inhibitors, cell-cycle inhibitors, angiogenesis inhibitors, matrix-metalloproteinase (MMP) inhibitors, hepatocyte growth factor inhibitors, TOR inhibitors, KDR

inhibitors, VEGF inhibitors, fibroblast growth factors (FGF) inhibitors, MEK inhibitors, ERK inhibitors, PI3K inhibitors, AKT inhibitors, MCL-1 inhibitors, BCL-2 inhibitors, SHP2 inhibitors, HER-2 inhibitors, BRAF-inhibitors, gene expression modulators, autophagy inhibitors, apoptosis inducers, antiproliferative agents, and glycolysis inhibitors.

5 The enzyme Bruton's tyrosine kinase (BTK) is a member of the Tec family non-receptor tyrosine kinases. BTK is an immunological target expressed in most hematopoietic cells, including B cells, and innate immune cells such as neutrophils, macrophages, and mast cells. The enzyme is also expressed in platelets which participate in inflammatory responses, supporting complement generation and promoting cytokine release, coagulation, and neutrophil NET
10 formation (Busygina 2018). BTK plays a role in the development and activation of B cells and regulates immune cell functions through a variety of signaling pathways, including signaling pathways involving B cell receptors, Fc receptors, integrins, Toll-like receptor, and chemokine receptors (Rip 2018). In addition, BTK plays an important role in degranulation, migration, and retention of neutrophils in injured tissues (Herter 2018) and in monocyte/macrophage activation
15 and differentiation processes (Rip 2018). BTK inhibition results in the modulation of various inflammatory immune cell activities such as proliferation, differentiation, and cytokine production without depleting immune cells (Rip 2018).

Consequently, a "Bruton's tyrosine kinase inhibitor" or "BTK inhibitor" refers to a compound that has a biological effect to inhibit or significantly reduce or down-regulate the
20 expression of the gene encoding for BTK and/or the expression of BTK and/or the biological activity of BTK. BTK plays a key role in the B-cell receptor signaling. Exemplary BTK inhibitors include, without limitation, Ibrutinib, zanubrutinib, tirabrutinib, olmutinib, orelabrutinib, poseltinib, branebrutinib, remibrutinib, evobrutinib, BI-BTK-1, fenebrutinib, GDC-0834, RN-486, G-744, BIB068, BMS-935177, BMS986143, BMS-986142, ONO/GS-
25 4059 (tirabrutinib) (Ono Pharmaceuticals/Gilead Sciences), AVL-292/CC-292/spebrutinib (Celgene Corporation), BGB-3111 (zanubrutinib) (BeiGene), and ACP-196/acalabrutinib (AstraZeneca), M7583 (EMD Serono/Merck KGaA), MSC2364447C(EMD Serono/Merck KGaA), BIIB068 (Biogen), ACO0058TA (ACEA Biosciences), DTRMWXHS-12 (Zhejiang DTRM Biopharma), and LOXO305 (pirtobrutinib)(Loxo@Lilly's). Other BTK inhibitors are
30 known by those skilled in the art. For example, those found in Zhang, D. et al. Recent Advances

in BTK Inhibitors for the Treatment of Inflammatory and Autoimmune Diseases. Molecules 2021, 26, 4907, the entirety of which is incorporated herein by reference.

For administration of a BTK inhibitor, the dosage will depend on the mode of administration and the age, weight, and general health of the individual being treated. Dosage amounts may also be selected depending upon the combination partner (i.e., the other active components). If the drug is formulated for, or delivered by a non-oral route, it may be desirable to decrease the unit or daily dose amounts delivered. In view of these factors, the skilled artisan would adjust the particular dose so as to obtain an effective dose for treating an individual. For example, a dosage contemplated herein can include a single volume of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, or 3.0 mL of a pharmaceutical composition having a concentration of a BTK inhibitor at about 0.001, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 10, 15, 20, 50, 100 μ M in a pharmaceutically acceptable carrier.

The phrase “CIC signaling pathway” or “Cell-in-Cell signaling pathway” refers to the series of chemical reactions in which a group of molecules function to regulate CIC internalization. Several signaling pathways are involved in regulating CIC occurrence, including, without limitation, the CXCR4- CXCL12 axis, the VLA integrin pathway, the CpG ODN pathway, the IL-15 pathway, the BAFF/APRIL pathway, and the CD40-CD40L pathway.

A “CIC signaling pathway antagonist” refers to a class of chemical or biological agents that inhibit or significantly reduce or down-regulate CIC internalization. Exemplary CIC signaling pathway antagonists include, any antagonist of the above CIC signaling pathways, such as CXCR4 antagonists, VLA4 integrin pathway inhibitors, TLR9 antagonists, NF- κ B inhibitors, immune suppressants, corticosteroids, calcineurin inhibitors, Jak/STAT pathway inhibitors, BAFF inhibitors, APRIL inhibitors, and antibodies/fusion proteins that specifically target a CIC signaling pathway.

CXCR4 (C-X-C chemokine receptor type-4), also known as fusin, cluster of differentiation 184 (CD184), FB22, LCR1, LESTR, or HM89, is an alpha-chemokine receptor specific for stromal-derived-factor-1. CXCR4 is a chemokine receptor which belongs to the superfamily of G protein-coupled receptors. It is actively involved in several biological processes, including hematopoiesis, and immune response. CXCR4 plays a role in various diseases, such as HIV, cancer, and WHIM syndrome. CXCR4 dysregulation is involved in

cancer metastasis. CXCR4 antagonists block the binding of C-X-C motif chemokine 12 (CXCL12 or stromal cell-derived factor 1) and the resultant downstream effects (e.g., cell migration).

The phrase "CXCR4 antagonist" refers to a class of chemical or biological agents that inhibit or significantly reduce or down-regulate the CXCR4 receptor and block its biological activity. Typically, CXCR4 antagonists reduce the CIC phenomenon, thereby preventing the sheltering of the tumor cells from an anti-cancer therapy. Exemplary CXCR4 antagonists include, without limitation, Plerixafor, LY2510924, PF-06747143, T140 analogs, BL-8040 (previously BKT140), TN14003, MSX-122, TG-0054, cyclic-pentapeptide-based antagonists including but not limited to FC122 and FC131, tetrahydroquinolines-based antagonists, including but not limited to AMD070 and AMD070 derivatives, indole-based antagonists including but not limited to FC131, Para-xylyl-enediamine-based compounds including but not limited to AMD3465 and AMD3465 analogues WZ811, MSX122, guanidine-based Antagonists including, but not limited to NB325, quinoline derivatives, including but not limited to NSC56612, KRH-3955, CTCE-9908, POL6326, motixafortide, mavorixafor and combinations thereof. Other CXCR4 antagonists are known by those skilled in the art. For example, those found in Cho, B. S. et al. Antileukemia activity of the novel peptidic CXCR4 antagonist LY2510924 as monotherapy and in combination with chemotherapy. *Blood* 126, 222-232, (2015) and Liu, S. H. et al. A novel CXCR4 antagonist IgG1 antibody (PF-06747143) for the treatment of hematologic malignancies. *Blood Adv* 1, 1088-1100, (2017), the entirety each being incorporated herein by reference.

Dosage amounts may also be selected depending upon the combination partner (i.e., the other active components). If the drug is formulated for or delivered by a non-oral route, it may be desirable to decrease the unit or daily dose amounts delivered. In view of these factors, the skilled artisan would adjust the particular dose so as to obtain an effective dose for treating an individual. For example, a dosage contemplated herein can include a single volume of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, or 3.0 mL of a pharmaceutical composition having a concentration of a CXCR4 antagonist at about 0.001, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 10, 15, 20, 50, 100 μ M in a pharmaceutically acceptable carrier.

VLA-4 is a member of the integrin family. VLA-4 can bind to vascular cellular adhesion molecule 1 (VCAM-1). Inhibitors of the VLA4 integrin pathway include, without limitation,

Natalizumab (Tysabri), Vedolizumab (Entyvio), Etrasimod, Firsatgrast (SB-683699), Ponesimod (Ponvory), BMS-986168, AJM300, PF-06881893, AJM300, and BTT-3033.

CpG oligodeoxynucleotides (ODNs) bind to and activate Toll-like receptor 9 (TLR9), initiating an innate immune response that supports the subsequent development of adaptive immunity. Inhibition of the CpG ODN (CpG oligodeoxynucleotide) pathway typically involves the use of reagents that can interfere with CpG ODN signaling or downstream immune responses. While CpG ODNs are commonly used as immunostimulatory agents, there may be instances where their activity needs to be inhibited. Reagents that can inhibit the CpG ODN pathway include, without limitation, ODN inhibitors, such as G-ODNs (ODNs with guanine substitutions that competitively inhibit CpG ODN binding), TLR9 antagonists, such as IRS954 and ODN-based TLR9 inhibitors, NF- κ B inhibitors, such as BAY 11-7082 and Celastrol, and immune suppressants.

Interleukin-15 (IL-15) is a pleiotropic cytokine with a broad range of biological functions in many diverse cell types. It plays a major role in the development of inflammatory and protective immune responses to microbial invaders and parasites by modulating immune cells of both the innate and adaptive immune systems. Inhibitors of IL-15 include soluble IL-15 receptor alpha, IL-15 neutralizing antibodies, IL-15 receptor antagonists, Jak/STAT pathway inhibitors, such as tofacitinib and baricitinib, and downstream signaling inhibitors that target signaling molecules and pathways activated by IL-15 (such as mTOR inhibitors).

The BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand) pathway are involved in regulating B-cell development and function. Inhibitors targeting this pathway include, without limitation, Belimumab, Tabalumab, Atacicept, Branebrutinib (BMS-986195), Ublituximab, and VAY736.

The CD40L pathway plays a critical role in immune responses and activation of immune cells. Inhibition of the CD40 ligand (CD40L) pathway is of interest in the treatment of various diseases. Exemplary CD40L pathway inhibitors include, without limitation, antiCD40L monoclonal antibodies, IDEC-131, BG9588 (Hu5C8), CD40-Fc fusion proteins, BI-655064, and small molecules inhibitors such as ASKP1240, TRAP-1, and soluble CD40.

The compounds described herein can be formulated for parenteral or systemic administration. For example, parenteral administration may include administration to a patient intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially,

intraarticularly, intraprostatically, intrapleurally, intratracheally, intravitreally, intratumorally, intramuscularly, subcutaneously, subconjunctivally, intravesicularly, intrapericardially, intraumbilically, by injection, and by infusion. The compounds can be combined with one or more pharmaceutically acceptable carriers and/or excipients that are considered safe and effective and may be administered to an individual without causing undesirable biological side effects or unwanted interactions. The carrier is all components present in the pharmaceutical formulation other than the active ingredient or ingredients. Typical carriers and conventional methods of preparing pharmaceutical compositions that can be used in conjunction with the preparation of formulations of the compounds are known by those skilled in the art. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like.

Parenteral formulations can be prepared as aqueous compositions using techniques known in the art. Typically, such compositions can be prepared as injectable formulations, for example, solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a reconstitution medium prior to injection; emulsions, such as water-in-oil (w/o) emulsions, oil-in-water (o/w) emulsions, and microemulsions thereof, liposomes, or emulsomes.

For intravenous administration, the compositions may be packaged in solutions of sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent. The components of the composition are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or concentrated solution in a hermetically sealed container such as an ampoule or sachet indicating the amount of active agent. If the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water or saline can be provided so that the ingredients may be mixed prior to injection.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, one or more polyols (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), oils, such as vegetable oils (e.g., peanut oil, corn oil, sesame oil, etc.), and combinations thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of

surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

Solutions and dispersions of the active compounds as the free acid or base or pharmacologically acceptable salts thereof can be prepared in water or another solvent or dispersing medium suitably mixed with one or more pharmaceutically acceptable excipients including, but not limited to, surfactants, dispersants, emulsifiers, pH modifying agents, viscosity modifying agents, and combination thereof.

Suitable surfactants may be anionic, cationic, amphoteric or nonionic surface-active agents. Suitable anionic surfactants include, but are not limited to, those containing carboxylate, sulfonate and sulfate ions.

The formulation can contain a preservative to prevent the growth of microorganisms. Suitable preservatives include, but are not limited to, parabens, chlorobutanol, phenol, sorbic acid, and thimerosal. The formulation may also contain an antioxidant to prevent degradation of the active agent(s).

The formulation is typically buffered to a pH of 3-8 for parenteral administration upon reconstitution. Suitable buffers include, but are not limited to, phosphate buffers, acetate buffers, and citrate buffers.

Sterile injectable solutions can be prepared by incorporating the active compounds in the required amount in the appropriate solvent or dispersion medium with one or more of the excipients listed above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those listed above.

The compounds described herein can be administered in an effective amount to a subject that is in need of alleviation or amelioration from one or more symptoms associated with cancer cell growth and proliferation.

With respect to the small nucleic acid inhibitors, the polynucleotides may be delivered through any known method, such as through one or more vectors (e.g., encoding siRNA, antisense oligonucleotides or other type of inhibitory nucleic acid), to a host cell. In some aspects, the invention further provides cells produced with said vectors, and organisms or cells comprising or produced from such cells. Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids in cells or target tissues. Such methods can be

used to administer nucleic acids encoding inhibitory compounds to cells in culture, or in a host organism. Non-viral vector delivery systems include DNA plasmids, RNA (e.g. a transcript of a vector described herein), naked nucleic acid, and nucleic acid complexed with a delivery vehicle, such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada et al., in *Current Topics in Microbiology and Immunology* Doerfler and Bihm (eds) (1995); and Yu et al., *Gene Therapy* 1:13-26 (1994).

Methods of non-viral delivery of nucleic acids include lipofection, nucleofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in e.g., U.S. Pat. Nos. 5,049,386, 4,946,787; and 4,897,355) and lipofection reagents are sold commercially (e.g., TransfectamTM and LipofectinTM). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Feigner, WO 91/17424; WO 91/16024. Delivery can be to cells (e.g. in vitro or ex vivo administration) or target tissues (e.g. in vivo administration).

The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (see, e.g., Crystal, *Science* 270:404-410 (1995); Blaese et al., *Cancer Gene Ther.* 2:291-297 (1995); Behr et al., *Bioconjugate Chem.* 5:382-389 (1994); Remy et al., *Bioconjugate Chem.* 5:647-654 (1994); Gao et al., *Gene Therapy* 2:710-722 (1995); Ahmad et al., *Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

The use of RNA or DNA viral based systems for the delivery of nucleic acids take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (in vivo) or they can be used to treat cells in vitro, and the modified cells may optionally be administered to patients (ex vivo). Conventional viral based systems could include retroviral,

lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue.

Retroviral vectors are comprised of cis-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum cis-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof (see, e.g., Buchscher et al., J. Virol. 66:2731-2739 (1992); Johann et al., J. Virol. 66:1635-1640 (1992); Sommerfelt et al., Virol. 176:58-59 (1990); Wilson et al., J. Virol. 63:2374-2378 (1989); Miller et al., J. Virol. 65:2220-2224 (1991); PCT/US94/05700). In applications where transient expression is preferred, adenoviral based systems may be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors may also be used to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and for in vivo and ex vivo gene therapy procedures (see, e.g., West et al., Virology 160:38-47 (1987); U.S. Pat. No. 4,797,368; WO 93/24641; Kotin, Human Gene Therapy 5:793-801 (1994); Muzyczka, J. Clin. Invest. 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin et al., Mol. Cell. Biol. 5:3251-3260 (1985); Tratschin, et al., Mol. Cell. Biol. 4:2072-2081 (1984); Hermonat & Muzyczka, PNAS 81:6466-6470 (1984); and Samulski et al., J. Virol. 63:03822-3828 (1989).

Packaging cells are typically used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ 2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by producing a cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the polynucleotide(s) to be expressed. The missing viral functions are typically supplied in trans by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely rep and cap, but lacking ITR sequences. The cell line may also be infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV.

By means of non-limiting examples, the compounds and any other active drugs of interest may be formulated as separate pharmaceutical preparations, as a single pharmaceutical preparation, or mixtures thereof. Any suitable form may be selected, e.g., in a form suitable for oral administration, for parenteral administration (such as by intravenous, intramuscular or subcutaneous injection or intravenous infusion), for topical administration (including ocular), for administration by inhalation, by a skin patch, by an implant, by a suppository, etc. Such suitable administration forms—which may be solid, semi-solid or liquid, depending on the manner of administration—as well as methods and carriers, diluents and excipients for use in the preparation thereof, will be clear to the skilled person; reference is made to the latest edition of Remington's Pharmaceutical Sciences.

Some preferred, but non-limiting examples of such preparations include tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols, ointments, cremes, lotions, soft and hard gelatin capsules, suppositories, drops, sterile injectable solutions and sterile packaged powders (which are usually reconstituted prior to use) for administration as a bolus and/or for continuous administration, which may be formulated with carriers, excipients, and diluents that are suitable per se for such formulations, such as lactose,

dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, polyethylene glycol, cellulose, (sterile) water, methylcellulose, methyl- and propylhydroxybenzoates, talc, magnesium stearate, edible oils, vegetable oils and mineral oils or suitable mixtures thereof. The formulations can optionally contain other substances that are commonly used in pharmaceutical formulations, such as lubricating agents, wetting agents, emulsifying and suspending agents, dispersing agents, disintegrants, bulking agents, fillers, preserving agents, sweetening agents, flavoring agents, flow regulators, release agents, etc. The compositions may also be formulated so as to provide rapid, sustained or delayed release of the active compound(s) contained therein.

The pharmaceutical preparations are preferably in a unit dosage form, and may be suitably packaged, for example in a box, blister, vial, bottle, sachet, ampoule or in any other suitable single-dose or multi-dose holder or container (which may be properly labeled); optionally in a kit which also comprises with one or more leaflets containing product information and/or instructions for use.

Depending on the condition to be prevented or treated and the route of administration, each of the different active compounds may be independently administered as a single daily dose, divided over one or more daily doses, or essentially continuously, e.g. using a drip infusion.

Solutions and dispersions of the active compounds as the free acid or base or pharmacologically acceptable salts thereof can be prepared in water or another solvent or dispersing medium suitably mixed with one or more pharmaceutically acceptable excipients including, but not limited to, surfactants, dispersants, emulsifiers, pH modifying agents, viscosity modifying agents, and combination thereof.

Suitable surfactants may be anionic, cationic, amphoteric or nonionic surface-active agents. Suitable anionic surfactants include, but are not limited to, those containing carboxylate, sulfonate and sulfate ions.

The formulation can contain a preservative to prevent the growth of microorganisms. Suitable preservatives include, but are not limited to, parabens, chlorobutanol, phenol, sorbic acid, and thimerosal. The formulation may also contain an antioxidant to prevent degradation of the active agent(s).

Sterile injectable solutions can be prepared by incorporating the active compounds in the required amount in the appropriate solvent or dispersion medium with one or more of the excipients listed above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which
5 contains the basic dispersion medium and the required other ingredients from those listed above.

The term “solvate” is used herein to describe a compound that contains stoichiometric or sub-stoichiometric amounts of one or more pharmaceutically acceptable solvent molecule such as ethanol. The term “hydrate” refers to when the said solvent is water.

The term “prodrug” as used herein means the pharmacologically acceptable derivatives
10 of the compounds, such as for example amides, whose in vivo biotransformation product generates the biologically active drug. Prodrugs are generally characterized by increased bio-availability and are readily metabolized into biologically active compounds in vivo.

The term “prodrug”, as used herein, means any compound that will be modified to form a drug species, wherein the modification may take place either inside or outside of the body, and
15 either before or after the prodrug reaches the area of the body where administration of the drug is indicated.

The phrase “minimal residual disease (MRD)” refers to small numbers of cells (particularly leukemia or lymphoma cells or other cancer cells from the bone marrow) that remain in the person during treatment, or after treatment when the patient is in remission and
20 exhibits no symptoms or signs of disease. It is the major cause of relapse in cancer and leukemia.

Methods of Treatment

Methods of treating cancer in a subject in need thereof, the method comprising administering at least one anti-cancer therapy and at least one CIC signaling pathway antagonist
25 are provided. In certain embodiments, the anti-cancer therapy is at least one BTK inhibitor. In certain embodiments, the CIC signaling pathway antagonist is at least one CXCR4 antagonist.

Also provided herein are methods for treating cancer in a subject in need thereof, the method comprising administering at least one BTK inhibitor and at least one CXCR4 antagonist.

Also provided are methods for increasing efficacy of BTK inhibitor treatment of cancer
30 in a subject in need thereof, the method comprising administering at least one CXCR4 antagonist.

Additionally, methods for decreasing tumor growth in a subject in need thereof, the method comprising administering at least one BTK inhibitor and at least one CXCR4 antagonist are provided herewith.

In certain embodiments, the at least one BTK inhibitor and at least one CXCR4 antagonist are administered together or sequentially. By sequential administration, the BTK inhibitor may be delivered to a subject before or after administration of the CXCR4 antagonist. In further embodiments, the BTK inhibitor may be delivered to a subject at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days, or 1 week, 2 weeks, 3 weeks, 4 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, or 12 months before or after administration of the CXCR4 antagonist. In still further embodiments, the BTK inhibitor may be delivered to a subject in any combination of months, days, hours, minutes, and seconds within these ranges. In further embodiments, the administration of CXCR4 antagonist and BTK inhibitors may be repeated multiple times.

Various cancers are known in the art. The cancer may be metastatic or non-metastatic. The cancer may be familial or sporadic. In some embodiments, the cancer is lymphoma, leukemia, or chronic lymphocytic leukemia (CLL), a non-Hodgkin's lymphoma (NHL) selected from small lymphocytic lymphoma (SLL), follicular lymphoma (FL), marginal zone lymphoma (MZL), mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL), or other types of B-cell lymphoma or a T-cell lymphoma selected from peripheral T-cell lymphoma and T-prolymphocytic lymphoma.

Additional cancers that can be treated using the methods provided herein include, for example, benign and malignant solid tumors and benign and malignant non-solid tumors. In one embodiment, the cancer is benign solid tumors. In one embodiment, the cancer is malignant solid tumors. In one embodiment, the cancer is benign non-solid tumors. In one embodiment, the cancer is malignant non-solid tumors.

As used herein, the terms "tumor", "tumor growth" or "tumor tissue" can be used interchangeably, and refer to an abnormal growth of tissue resulting from uncontrolled progressive multiplication of cells and serving no physiological function. A solid tumor can be malignant, e.g. tending to metastasize and being life threatening, or benign. Examples of solid tumors that can be treated or prevented according to a method of the present invention include sarcomas and carcinomas such as, but not limited to: fibrosarcoma, myxosarcoma, liposarcoma,

chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, colorectal cancer, gastric cancer, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, liver metastases, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, thyroid carcinoma such as anaplastic thyroid cancer, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma such as small cell lung carcinoma and non-small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, glioblastoma, and retinoblastoma.

Examples of solid tumors include, but are not limited to: biliary tract cancer, brain cancer (including glioblastomas and medulloblastomas), breast cancer, cervical cancer, choriocarcinoma, colon cancer, endometrial cancer, esophageal cancer, gastric cancer, intraepithelial neoplasms (including Bowen's disease and Paget's disease), liver cancer, lung cancer, neuroblastomas, oral cancer (including squamous cell carcinoma), ovarian cancer (including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells), pancreatic cancer, prostate cancer, rectal cancer, renal cancer (including adenocarcinoma and Wilms tumour), sarcomas (including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma and osteosarcoma), skin cancer (including melanoma, Kaposi's sarcoma, basocellular cancer and squamous cell cancer), testicular cancer including germinal tumors (seminomas, and non-seminomas such as teratomas and choriocarcinomas), stromal tumors, germ cell tumors, and thyroid cancer (including thyroid adenocarcinoma and medullary carcinoma).

In one embodiment, the cancer is brain cancer, including gliomas, glioblastomas and medulloblastomas. In one embodiment, the cancer is breast cancer. In one embodiment, the cancer is cervical cancer. In one embodiment, the cancer is choriocarcinoma. In one embodiment, the cancer is colon cancer. In one embodiment, the cancer is endometrial cancer. In one embodiment, the cancer is esophageal cancer. In one embodiment, the cancer is gastric

cancer. In one embodiment, the cancer is intraepithelial neoplasms, including Bowen's disease and Paget's disease. In one embodiment, the cancer is liver cancer. In one embodiment, the cancer is lung cancer. In one embodiment, the cancer is neuroblastomas. In one embodiment, the cancer is oral cancer, including squamous cell carcinoma. In one embodiment, the cancer is ovarian cancer, including those arising from epithelial cells, stromal cells, germ cells and mesenchymal cells. In one embodiment, the cancer is pancreatic cancer. In one embodiment, the cancer is prostate cancer. In one embodiment, the cancer is rectal cancer. In one embodiment, the cancer is renal cancer, including adenocarcinoma and Wilms tumour. In one embodiment, the cancer is sarcomas, including leiomyosarcoma, rhabdomyosarcoma, liposarcoma, fibrosarcoma and osteosarcoma. In one embodiment, the cancer is skin cancer, including melanoma, Kaposi's sarcoma, basocellular cancer and squamous cell cancer. In one embodiment, the cancer is testicular cancer including germinal tumors (seminomas, and non-seminomas such as teratomas and choriocarcinomas).

The phrase "Cell-in-cell", "CIC", or "emperipolesis" refers to the presence of one cell (known as the "engulfed cell" or inner cells) within another (known as the "host cell" or outer cells). The phrase "CIC tumor cells" or "CIC cancer cells" refers to CIC structure where the engulfed cell is a tumor cell. In certain embodiments, the host cell may also be a tumor cell. The engulfed cells are protected from anti-cancer agents by the host cell. In certain embodiments, the engulfed cells are protected when the patient is in remission. The engulfed cell can then leave the host cell, repopulate and ultimately cause a relapse.

The CIC phenomenon occurs in response to changes in the tumor microenvironment (TME). In certain embodiments, changes in the TME can cause an increase in the number of CIC tumor cells. In certain embodiments, the increase of CIC tumor cells may be caused by administration of an anti-cancer agent.

The term "subject" refers to a mammal, preferably a human. In one embodiment, a subject may be a "patient", i.e. a warm-blooded animal, more preferably a human, who/which is awaiting the receipt of, or is receiving medical care or was/is/will be the object of a medical procedure, or is monitored for the development of a disease.

The term "human" refers to a person of both genders and at any stage of development (i.e. neonate, infant, juvenile, adolescent, adult).

The term “in need of treatment” as used herein refers to a judgment made by a caregiver (c.g. physician, nurse, nurse practitioner, or individual in the case of humans; veterinarian in the case of animals, including non-human mammals) that a subject requires or will benefit from treatment. This judgment is made based on a variety of factors that are in the realm of a caregiver’s expertise, but that includes the knowledge that the subject is ill, or will be ill, as the result of a condition that is treatable by the disclosed compounds.

By “treatment” and “treating” is meant the medical management of a subject with the intent to cure, ameliorate, or stabilize, a pathological condition or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder. It is understood that treatment, while intended to cure, ameliorate, or stabilize, a disease, pathological condition, or disorder, need not actually result in the cure, ameliorization, or stabilization. The effects of treatment can be measured or assessed as described herein and as known in the art as is suitable for the disease, pathological condition, or disorder involved. Such measurements and assessments can be made in qualitative and/or quantitative terms. Thus, for example, characteristics or features of a disease, pathological condition, or disorder and/or symptoms of a disease, pathological condition, or disorder can be reduced to any effect or to any amount.

In certain embodiments, the method of treatment effectively suppresses symptoms associated with cancer. Symptoms of vary according to the location and type of cancer being treated. In certain embodiments, symptoms of cancer include, fatigue, weight loss, lumps, swelling, pain, coughing, wheezing, new or unusual growth, discoloration, and no symptoms at all. In certain embodiments, the treatment reduces the risk of relapse. In the context of a cancer, treatment or inhibition may be assessed by inhibition of disease progression, inhibition of tumor growth, reduction of primary tumor, relief of tumor-related symptoms, inhibition of tumor secreted factors, delayed appearance of primary or secondary tumors, slowed development of primary or secondary tumors, decreased occurrence of primary or secondary tumors, slowed or

decreased severity of secondary effects of disease, arrested tumor growth and regression of tumors, increased Time To Progression (TTP), increased Progression Free Survival (PFS), increased Overall Survival (OS), among others. OS as used herein means the time from treatment onset until death from any cause. TTP as used herein means the time from treatment onset until tumor progression; TTP does not include deaths. Time to Remission (TTR) as used herein means the time from treatment onset until remisison, for example, complete or partial remission. As used herein, PFS means the time from treatment onset until tumor progression or death. In one embodiment, PFS rates will be computed using the Kaplan-Meier estimates. Event-free survival (EFS) means the time from study entry until any treatment failure, including disease progression, treatment discontinuation for any reason, or death. Relapse-free survival (RFS) means the length of time after the treatment ends that the patient survives without any signs or symptoms of that cancer. Overall response rate (ORR) means the sum of the percentage of patients who achieve complete and partial responses. Complete remission rate (CRR) refers to the percentage of patients achieving complete remission (CR). Duration of response (DoR) is the time from achieving a response until relapse or disease progression. Duration of remission is the time from achieving remission, for example, complete or partial remission, until relapse. In the extreme, complete inhibition, is referred to herein as prevention or chemoprevention. In this context, the term “prevention” includes either preventing the onset of clinically evident cancer altogether or preventing the onset of a preclinically evident stage of a cancer. Also intended to be encompassed by this definition is the prevention of transformation into malignant cells or to arrest or reverse the progression of premalignant cells to malignant cells. This includes prophylactic treatment of those at risk of developing a cancer.

The term “administration”, or a variant thereof (e.g. “administering”), means providing the active agent or active ingredient, alone or as part of a pharmaceutically acceptable composition, to the subject in whom/which the condition, symptom, or disease is to be treated or prevented.

By “pharmaceutically acceptable” is meant that the ingredients of a pharmaceutical composition are compatible with each other and not deleterious to the subject to which it is administered.

Kits and Articles of Manufacture

Any of the aforementioned products can be incorporated into a kit which may contain at least one anti-cancer therapy, such as a BTK inhibitor, at least one CIC controlling signaling pathway antagonist, such as a CXCR4 antagonist, a pharmaceutically acceptable carrier, and optionally at least one other anti-cancer therapy. In certain embodiments, that additional anti-cancer therapy includes, without limitation, chemotherapy, immunotherapy or targeted therapy, instructions for use, a container, a vessel for administration, and/or any combination thereof.

A kit for performing the diagnostic or prognostic assay of the invention comprises a Bone marrow fibroblasts (BMF) cell line or other stromal cell lines, culture medium suitable for culture of the BMF cell line or other stromal cell lines, and labeled CLL cells, other lymphoma or other tumor cells and addition of one or more B cell growth factors or other growth factors.

Compositions for Detecting Minimal Residual Disease (MRD) and Subjects at greater risk for MRD

Also provided herein are compositions and methods for detecting MRD in a subject after receiving an anti-cancer treatment via detection the number and quality of internalized cells, and measurement of biomarkers which modulate the CIC internalization process. including without limitation, CXCR4 and CXCL12. Methods are also provided for identifying subjects at greater risk for MRD from CIC internalization. Finally, screening methods for identifying agents which modulate the CIC internalization process are also disclosed. Agents so identified should serve as effective anticancer agents and minimize risk for MRD, particularly agents which inhibit the CIC process.

“Control” or “Control subject” as used herein refers to both an individual or numerical or graphical averages of the CIC internalization levels and/or expression levels of the selected biomarkers obtained from large groups of patients with cancer before and/or after treatment. As described in the examples, the chemokine CXCR4-CXCL12 axis mediates CIC internalization process. Such controls are the types that are commonly used in similar diagnostic assays for other biomarkers. Selection of the particular class of controls depends upon the use to which the diagnostic methods and compositions are to be put by the physician. As used herein, the term “predetermined control” refers to a numerical level, average, mean or average range of the expression of a biomarker in a defined cell population, i.e., biomarkers such as CXCL12 and CXCR-4 where modulation of expression levels can promote or inhibit CIC internalization. The

predetermined control level is preferably provided by using the same assay technique as is used for measurement of the subject's biomarker levels, to avoid any error in standardization. For example, the control may comprise a single healthy mammalian subject or cells from such a subject. In another embodiment, the control comprises a population of multiple healthy
5 mammalian subjects or cells from such subjects. In another embodiment, the control comprises the same subject having treated cancer. In addition, a predetermined control may also be a negative predetermined control. In one embodiment, a negative predetermined control comprises one or multiple subjects who have CIC tumor cells. As noted, the control can refer to a numerical average, mean or average range of the expression of one or more biomarkers, in a defined cell
10 population, rather than a single subject.

"Sample" as used herein means any biological fluid or tissue that contains the biomarkers. The most suitable samples for use in the methods and with the compositions are blood samples, including serum, plasma, whole blood, and peripheral blood. Bone marrow samples or tissue samples as illustrated in Fig.2. It is also anticipated that other biological fluids,
15 such as saliva or urine, may be used similarly. Such samples may further be diluted with saline, buffer or a physiologically acceptable diluent. Alternatively, such samples are concentrated by conventional means.

By "change in internalization" is meant an alteration, either an increase or decrease, in the number of cells undergoing the CIC internalization process, particularly cancer cells
20 internalized into fibroblasts when incubated or exposed to a test agent, more particularly an anti-cancer therapeutic agent or an agent which modulates the CXCR4-CXCL12 axis in a therapeutically beneficial way.

By "change in expression" is meant an increased expression level of a selected biomarker, or upregulation of the genes or transcript encoding it in comparison to the reference
25 or control; a decreased expression level of a selected biomarker or a downregulation of the genes or transcript encoding it in comparison to the reference or control; or a combination of certain increased/upregulated and decreased/down regulated biomarkers. The degree of change in target expression can vary with each individual and is subject to variation with each population and days or weeks before or after anti-cancer treatment. For example, in one embodiment, a large
30 change, e.g., 2-3 fold increase or decrease in the biomarkers is statistically significant.

The term “microarray” refers to an ordered arrangement of hybridizable array elements, e.g., primers, probes, ligands, on a substrate.

The term “ligand” refers to a molecule that binds to a protein or peptide, and includes antibodies and fragments thereof.

5 The term “polynucleotide,” when used in singular or plural form, generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as defined herein include, without limitation, single- and double-stranded DNA, DNA including single- and double-stranded regions, single- and double-stranded RNA, and RNA including single- and double-stranded
10 regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or include single- and double-stranded regions. In addition, the term “polynucleotide” as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term “polynucleotide” specifically includes cDNAs. The term includes DNAs (including cDNAs) and RNAs that contain one or more modified bases. In general, the
15 term “polynucleotide” embraces all chemically, enzymatically and/or metabolically modified forms of unmodified polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells.

 The term “oligonucleotide” refers to a relatively short polynucleotide of less than 20 bases, including, without limitation, single-stranded deoxyribonucleotides, single- or double-
20 stranded ribonucleotides, RNA:DNA hybrids and double-stranded DNAs. Oligonucleotides, such as single-stranded DNA probe oligonucleotides, are often synthesized by chemical methods, for example using automated oligonucleotide synthesizers that are commercially available. However, oligonucleotides can be made by a variety of other methods, including in vitro recombinant DNA-mediated techniques and by expression of DNAs in cells and organisms.

25 As used herein, “labels” or “reporter molecules” are chemical or biochemical moieties useful for labeling a nucleic acid (including a single nucleotide), polynucleotide, oligonucleotide, or protein ligand, e.g., amino acid, peptide sequence, protein, or antibody. “Labels” and “reporter molecules” include fluorescent agents, chemiluminescent agents, chromogenic agents, quenching agents, radionucleotides, enzymes, substrates, cofactors, inhibitors, radioactive isotopes,
30 magnetic particles, and other moieties known in the art. “Labels” or “reporter molecules” are

capable of generating a measurable signal and may be covalently or noncovalently joined to an oligonucleotide or nucleotide (e.g., a non-natural nucleotide) or ligand.

The “targets” of the compositions and methods of these inventions include, in one aspect, agent which modulate either the CIC internalization process or genes, gene fragments, transcripts
5 and the expression products which modulate the CXCR4-CXCL12 axis. In certain embodiments, the methods described herein use at least a plurality of additional biomarkers.

Diagnostic Reagents

In one embodiment, diagnostic reagents for use in the methods of detecting CIC comprise
10 without limitation an ex vivo cell culture system, including a first cell culture of bone marrow stromal cells (BMSC) which express one or more exogenous cell signaling molecules and a second cell culture comprising labeled leukemia or lymphoma cells isolated from a human; and optionally one or more soluble cell signaling molecule, agents of interest that modulate the CIC tumor cell internalization process and one or more growth factors. For these reagents, the labels
15 may be selected from among many known diagnostic labels, including those described above. Similarly, the substrates for immobilization, e.g., of a biomarker or an agent having affinity for the biomarker may be any of the common substrates, glass, plastic, a microarray, a microfluidics card, a chip or a chamber.

In another embodiment, the diagnostic reagent is a ligand that binds to a biomarker
20 recited above or a unique peptide thereof. Such a ligand desirably binds to a protein biomarker or a unique peptide contained therein, and can be an antibody which specifically binds a single biomarker described above, or a unique peptide in that single biomarker. Various forms of antibody, e.g., polyclonal, monoclonal, recombinant, chimeric, as well as fragments and components (e.g., CDRs, single chain variable regions, etc.) may be used in place of antibodies.
25 The ligand itself may be labeled or immobilized.

In another embodiment, the diagnostic reagent is a polynucleotide or oligonucleotide sequence that hybridizes to gene, gene fragment, gene transcript or nucleotide sequence encoding a biomarker of any one or more of the biomarkers described above or encoding a unique peptide thereof. Such a polynucleotide/oligonucleotide can be a probe or primer, and may itself be
30 labeled or immobilized. In one embodiment, these polynucleotide or oligonucleotide reagent(s) are part of a primer-probe set, and the kit comprises both primer and probe. Said primer-probe

set amplifies a gene, gene fragment or gene expression product that encodes a CXCL12 biomarker, optionally including one or more additional biomarkers. PCR can also be used to identify other gene targets exhibiting altered expression levels or genes which modulate the CIC internalization process.

For use in the compositions the PCR primers and probes are preferably designed based upon intron sequences present in the biomarker gene(s) to be amplified selected from the gene expression profile. The design of the primer and probe sequences is within the skill of the art once the particular gene target is selected. The particular methods selected for the primer and probe design and the particular primer and probe sequences are not limiting features of these compositions. A ready explanation of primer and probe design techniques available to those of skill in the art is summarized in US Patent No. 7,081,340, with reference to publicly available tools such as DNA BLAST software, the Repeat Masker program (Baylor College of Medicine), Primer Express (Applied Biosystems); MGB assay-by-design (Applied Biosystems); Primer3 (Steve Rozen and Helen J. Skaletsky (2000) Primer3 on the WWW for general users and for biologist programmers and other publications.

In general, optimal PCR primers and probes used in the compositions described herein are generally 17-30 bases in length, and contain about 20-80%, such as, for example, about 50-60% G+C bases. Melting temperatures of between 50 and 80°C, e.g., about 50 to 70 °C are typically preferred.

The compositions based on the CXCL12 biomarker, optionally associated with detectable labels, can be presented in the format of a microfluidics card, a chip or chamber, or a kit adapted for use with the assays described in the Examples, ELISAs or PCR, RT-PCR or Q PCR techniques described herein.

The selection of the ligands, poly/oligonucleotide sequences, their length, suitable labels and substrates used in the composition are routine determinations made by one of skill in the art in view of the teachings of which biomarkers form signature suitable for the detection of residual disease.

Methods of Use

Methods for detecting biomarkers modulating the CIC process in a biological fluid sample of the subject are disclosed. Such methods entail determination of the expression level of a protein or peptide fragment thereof from at least one protein in the CXCR4- CXCL12 axis. The method further involves comparing the subject's expression level of the selected biomarker or biomarker fragment with the level of the same protein or peptide in the biological fluid of a reference or control subject. Changes in expression of the subject's biomarker protein or peptide fragment, e.g., CXCL12, from those of the reference or control can be indicative of an altered risk for MRD. In this diagnostic method, a change in expression level of one or more of the selected biomarker proteins or peptide fragment in comparison to the IUP control reference may be an increase or decrease in the expression levels of the individual biomarkers. This method may employ any of the suitable diagnostic reagents or kits or compositions described above.

The measurement of the CIC related biomarkers in the biological sample may employ any suitable ligand, e.g., antibody (or antibody to any second biomarker) to detect the biomarker protein. Such antibodies may be presently extant in the art or presently used commercially, such as those available as part of commercial antibody ELISA assay kits or that may be developed by techniques now common in the field of immunology. As used herein, the term "antibody" refers to an intact immunoglobulin having two light and two heavy chains or any fragments thereof. Thus, a single isolated antibody or fragment may be a polyclonal antibody, a high affinity polyclonal antibody, a monoclonal antibody, a synthetic antibody, a recombinant antibody, a chimeric antibody, a humanized antibody, or a human antibody. The term "antibody fragment" refers to less than an intact antibody structure, including, without limitation, an isolated single antibody chain, a single chain Fc construct, a Fab construct, a light chain variable or complementarity determining region (CDR) sequence, etc. A recombinant molecule bearing the binding portion of an CXCL12 biomarker antibody, e.g., carrying one or more variable chain CDR sequences, may also be used in a diagnostic assay. As used herein, the term "antibody" may also refer, where appropriate, to a mixture of different antibodies or antibody fragments that bind to the selected biomarker. Such different antibodies may bind to different biomarkers or different portions of the same CXCL12 biomarker protein than the other antibodies in the mixture. Such differences in antibodies used in the assay may be reflected in the CDR sequences of the variable regions of the antibodies. Such differences may also be generated by the antibody backbone, for example, if the antibody itself is a non-human antibody containing a human CDR

sequence, or a chimeric antibody or some other recombinant antibody fragment containing sequences from a non-human source. Antibodies or fragments useful in the method of this invention may be generated synthetically or recombinantly, using conventional techniques or may be isolated and purified from plasma or further manipulated to increase the binding affinity thereof. It should be understood that any antibody, antibody fragment, or mixture thereof that binds one of the biomarkers or a particular sequence of the selected biomarker may be employed in the methods of the present invention, regardless of how the antibody or mixture of antibodies was generated.

Similarly, the antibodies may be tagged or labeled with reagents capable of providing a detectable signal, depending upon the assay format employed. Such labels are capable, alone or in concert with other compositions or compounds, of providing a detectable signal. Where more than one antibody is employed in a diagnostic method, e.g., such as in a sandwich ELISA, the labels are desirably interactive to produce a detectable signal. Most desirably, the label is detectable visually, e.g., colorimetrically. A variety of enzyme systems operate to reveal a colorimetric signal in an assay, e.g., glucose oxidase (which uses glucose as a substrate) releases peroxide as a product that in the presence of peroxidase and a hydrogen donor such as tetramethyl benzidine (TMB) produces an oxidized TMB that is seen as a blue color. Other examples include horseradish peroxidase (HRP) or alkaline phosphatase (AP), and hexokinase in conjunction with glucose-6-phosphate dehydrogenase that reacts with ATP, glucose, and NAD⁺ to yield, among other products, NADH that is detected as increased absorbance at 340 nm wavelength.

Other label systems that may be utilized in the methods of this invention are detectable by other means, e.g., colored latex microparticles (Bangs Laboratories, Indiana) in which a dye is embedded may be used in place of enzymes to provide a visual signal indicative of the presence of the resulting selected biomarker-antibody complex in applicable assays. Still other labels include fluorescent compounds, radioactive compounds or elements. Preferably, an anti-biomarker antibody is associated with, or conjugated to a fluorescent detectable fluorochromes, e.g., fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), coriphosphine-O (CPO) or tandem dyes, PE-cyanin-5 (PC5), and PE-Texas Red (ECD). Commonly used fluorochromes include fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), and also include the tandem dyes, PE-cyanin-5 (PC5), PE-cyanin-7

(PC7), PE-cyanin-5.5, PE-Texas Red (ECD), rhodamine, PerCP, fluorescein isothiocyanate (FITC) and Alexa dyes. Combinations of such labels, such as Texas Red and rhodamine, FITC + PE, FITC + PECy5 and PE + PECy7, among others may be used depending upon assay method.

5 Detectable labels for attachment to antibodies useful in diagnostic assays of this invention may be easily selected from among numerous compositions known and readily available to one skilled in the art of diagnostic assays. The biomarker-antibodies or fragments useful in this invention are not limited by the particular detectable label or label system employed. Thus, selection and/or generation of suitable biomarker antibodies with optional labels for use in this
10 invention is within the skill of the art, provided with this specification, the documents incorporated herein, and the conventional teachings of immunology.

Similarly, the particular assay format used to measure the selected biomarker in a biological sample may be selected from among a wide range of immunoassays, such as enzyme-linked immunoassays, such as those described in the examples below, sandwich immunoassays,
15 homogeneous assays, immunohistochemistry formats, or other conventional assay formats. One of the skills in the art may readily select from any number of conventional immunoassay formats to perform this invention.

Employing ligand binding to the biomarker proteins or multiple biomarkers forming the signature enables more precise quantitative assays, as illustrated by the ELISA assays.

20 In another embodiment, a method for detecting increased CIC numbers or identifying subject at increased risk for MRD in a subject, includes measuring in a co-culture such as that described above, the level of labeled leukemia or lymphoma CIC internalization occurring in the presence or absence of a test agent, agents that reduce the number of CIC events being effective to reduce MRD or the risk thereof. The method can further involve comparing the CIC
25 internalization level observed in cancer cells from the subject with the level of CIC observed with labeled bone marrow cells of a reference or control subject.

Still other methods useful in performing the diagnostic steps described herein are known in the art. Such methods include methods based on hybridization analysis of polynucleotides, methods based on sequencing of polynucleotides, proteomics-based methods or
30 immunochemistry techniques. The most commonly used methods known in the art for the quantification of mRNA expression in a sample include northern blotting and in situ

hybridization; RNase protection assays; and PCR-based methods, such as reverse transcription polymerase chain reaction (RT-PCR) or qPCR. Alternatively, antibodies may be employed that can recognize specific DNA-protein duplexes. The methods described herein are not limited by the particular techniques selected to perform them. Exemplary commercial products for
5 generation of reagents or performance of assays include TRI-REAGENT, Qiagen RNeasy mini-columns, MASTERPURE Complete DNA and RNA Purification Kit (EPICENTRE®, Madison, Wis.), Paraffin Block RNA Isolation Kit (Ambion, Inc.) and RNA Stat-60 (Tel-Test), the MassARRAY-based method (Sequenom, Inc., San Diego, CA), differential display, amplified fragment length polymorphism (iAFLP), and BeadArray™ technology (Illumina, San Diego,
10 CA) using the commercially available Luminex100 LabMAP system and multiple color-coded microspheres (Luminex Corp., Austin, Tex.) and high coverage expression profiling (HiCEP) analysis.

Thus, in yet another embodiment, a method for detecting residual cancer in a subject involves measuring in a biological fluid sample of the subject the expression level of a gene,
15 gene fragment, gene transcript (e.g., mRNA) or expression product encoding one or more of the CXCL12 biomarkers. The method further includes comparing the subject's selected biomarker gene, gene fragment, gene transcript or expression product expression level with the level of the same gene, gene fragment, gene transcript or expression product in the biological fluid of a reference or control subject. Changes in expression of the subject's selected biomarker gene,
20 gene fragment, gene transcript or expression products from those of the reference or control correlates with detection of residual disease.

In yet another embodiment, the methods and compositions described herein may be used in conjunction with clinical risk factors to help physicians make more accurate decisions about how to manage patients that were treated for cancer. Another advantage of these methods and
25 compositions is that detection may occur early.

MATERIALS AND METHODS

The following materials and methods are provided to facilitate the practice of the present invention.

Cell lines and culture conditions

WSU-FSCCL, a B cell lymphoma cell line, was used in vitro experiments. Different stromal cell lines BMF, NKTert, or HS-5 cells, were also used for co-culture purposes. All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 mg/ml penicillin/streptomycin in a humidified 37°C, and 5% CO₂ incubator. The medium was changed every two days. Passages were performed when stromal cells reached 80-90% confluence, using 0.25% trypsin and 0.5 mM EDTA.

Stable expression of Td-Tomato (TdT), hCD40L, BAFF/APRIL, CXCL12/13, and IL4/15/21 in stromal cells

mCherry-NKTert, TdT-HS5, TdT-BMF, BAFF/APRIL-BMF, CXCL12/13-BMF, and IL4/15/21-BMF cells were made via lentiviral transduction. mCherry, TdT, BAFF/APRIL, CXCL12/13, or IL4/15/21-expressing lentiviral particles were transduced individually into stromal cells (BMF, NKTert or HS-5). For transduction, stromal cells were seeded overnight at 100,000 cells/well in a 12-well plate and transduced overnight at 37°C with 20 µL viral solution in 400 µL media containing 5 µg/mL polybrene. For selection, puromycin (2 µg/mL) was added 48 hr later. After selection (7-10 days), mCherry and TdT overexpression were assessed by fluorescence microscopy, and BAFF/APRIL, CXCL12/13, and IL4/15/21 expression were determined by flow cytometry.

TdT-hCD40L-BMF cells were made by two transductions. First, Human CD40L-expression lentiviral particles with a viral titer of $>10^8$ TU/mL were transduced into BMF using the single transduction protocol but selected with 500 µg/mL neomycin. Secondly, TdT-expressing lentiviral particles ($>10^9$ TU/mL,) were transduced into hCD40L-BMF subsequently using the same protocol, selected with puromycin (2 µg/mL) for 7 days, and transduction was assessed by fluorescence microscopy.

B cells/stromal cells Co-Culture

Ex vivo co-culture was performed as described previously Blood Cancer J. 2021 Feb 18;11(2):39. doi: 10.1038/s41408-021-00429-z). WSU-FSCCL cell line; CLL, FL, and NBC primary samples were co-culture with stromal cells in 24-well plate. Briefly, for co-culture, B cells were cultured with stromal cells in RPMI-1640 medium supplemented with 20% fetal

bovine serum and 100 µg/mL penicillin/streptomycin in a humidified 37°C incubator containing 5% CO₂. The ratio B cells/stromal cells was indicated for each specific experiment below.

Drug treatment and Cell stimulation

5 B cells were treated in co-culture with different drugs, such as Ibrutinib (400 nM), Pirtobrutinib (15 µM), or plerixafor (1 µM) at clinically achievable concentrations for 5hs, 16hs, 24hs, 48hs or 5 days. For the control well, an equal volume of DMSO was added. Cell stimulation experiments were conducted with CpG (2 µg/mL) and IL-15 (10 ng/mL).

10 Conditioned media

To study soluble factors, BAFF/APRIL-BMF, CXCL12/13-BMF, and IL4/15/21-BMF cells were used to obtain conditioned media (CM). Briefly, transfected stromal cells were cultured into T-75 flasks containing 15 mL of RPMI-1640 growth medium. After reaching 80-90% confluence the first batch of conditioned media (CM) was harvested, centrifuged at 1000 rpm, and stored at -20°C. Stromal cells were trypsin-treated, split, and resuspended in 15 mL of new media to make the second batch CM. The cells were kept for 3 days and then the harvest procedure was repeated. The first and second batches were mixed at 1:1 ratio, aliquoted, and stored at -20°C.

20 Confocal Microscopy

Fixed cell imaging: TdT-BMF, mCherry-NKTert, or TdT-HS5 cells were pre-seeded overnight on coverslips in 24-well plates (120,000 cells/well). CFSE-labelled B cells (2,000,000 cells/well) were co-cultured with stromal cells for 5hr, 16hr, 24hr, 48hr, or 5 days. During these times, the B cells were treated with drugs, stimulated, or incubated with soluble factors, to make “Drug treatment experiments”, “stimulation experiments” or “soluble factor experiments”, respectively. After this time, the cells were gently washed with PBS to remove non-adherent CLL cells and then fixed with 4% paraformaldehyde for 10 minutes. After fixation, the cells were stained with Hoechst (Thermo Fisher) for 5 minutes, and the coverslips were mounted onto slides using PermOUNT (Fisher Scientific).

30 Images were acquired with Leica TCS SP8 FSU laser scanning confocal microscope using a 40x NA 1.3 oil lens. Reconstruction images were made in 3D using Imaris v10.0 program.

Using the program, stromal cells were transformed into red surfaces and CLL cells into green surfaces.

3D imaging: Td-Tomato-hCD40L-BMF (2,500 cells) and CFSE-CLL cells (200,000 cells) were simultaneously added and mixed in a cell suspension which was then plated on a 5 mm coverslip. 3D Time-lapse imaging was started immediately using a Lattice Light sheet Bessel Beam Illumination microscope (3i) with a Nikon 25 x NA 1.1 water immersion objective. Images were captured using a Hamamatsu Flash4v2+ sCMOS high-sensitivity/low-noise camera.

Live cell time-lapse microscopy: TdT-BMF, mCherry- NKtert, or TdT-HS5 cells (2,500 cells/well) were seeded overnight on 4-well inserts on a Nunc glass-bottom imaging dish. B cells (12,500 cells/well), labeled with CFSE (Thermo Fisher), were then added to the stromal cell culture. Time-lapse microscopy was started immediately, using Leica TCS SP8 FSU confocal microscope with 40x NA 1.3 oil lens. The procedure was made using heating chamber with temperature, CO₂, and humidity control. The Images were reconstructed in 3D using Imaris v10.0 program.

CLINICAL SAMPLES AND CLL CELL ISOLATION

CLL patient PB samples were collected after informed consent according to the Declaration of Helsinki. CLL cells were isolated by negative selection using RosettesepTM human B cell enrichment cocktail following the manufacturers' instructions as previously described. After isolation, CLL cell purity was assessed using flow cytometry and was >95% CD19+/CD5+ in all cases. Cell viability was assessed with MUSE Count & Viability Kit and was ≥90% in all cases.

PRE-CULTURE OF CLL CELLS WITH BMF, T-CELL DEPLETION AND CFSE LABELING

Bone marrow fibroblasts (BMF) were trypsinized and seeded onto 12-well plates (2×10^5 cells/2 mL/well) to reach 70–90% confluence on the next day. CLL cells were then added to the BMF monolayer in RPMI-1640 media containing 20% FBS, 50 U/mL penicillin, 50 mg/mL streptomycin and 2 mmol/L of l-glutamine (Mediatech. VA). Plating ratio of CLL to stromal cells fall in the range of 2:1–20:1 depending on the cell number availability in the CLL samples. After 72 h of co-culture, CLL cells were collected by gently pipetting, washed and resuspended in 1 mL media. The residual T cells were removed by Dynabeads® CD3 kit according to

manufacturer's protocol. In brief, CD3 Dynabeads® were added at 50 µL per mL cell suspension, and rotated at 4 °C for 30 min. The bead-bound CD3+ T cells were separated from cell suspension by EasySep™ Magnet.

After CD3 depletion, CLL cells were labeled with violet-CFSE using CellTrace™Violet Cell Proliferation Kit according to manufacturer's protocol. Briefly, 10 µL of 5 µM CFSE in DMSO was added into 10 mL pre-warmed PBS for each labeling. CLL cells were washed twice with PBS and re-suspended in 10 mL CFSE-PBS solution. After a 20-min of incubation at 37 °C, cells were spun down, washed once and kept in RPMI-1640 media with 20% FBS for the next step.

CELL STIMULATION AND DRUG TREATMENT

Cell stimulation and drug testing were conducted in 24-well plates with BMF monolayer. For activation of CFSE-labeled CLL cells, CpG (2 µg/mL) and IL-15 (10 ng/mL) were added into each well except the unstimulated control. For drug treatment, ibrutinib and/or plerixafor, at clinically achievable concentrations, was added to the co-culture 24 h after CpG/IL-15 addition. For the control well, an equal volume of DMSO was added. At day 7 of the stimulation and drug treatment, 400 µL of cultured CLL cells were collected from each well for flow cytometric analysis.

CELL SURVIVAL AND PROLIFERATION ANALYSES

Cell survival and proliferation were determined by flow cytometry using LSR2 flow cytometer (BD Biosciences). Briefly, CLL cells collected at day 7 of drug treatment were stained with FITC-anti-CD5, PE-anti-CD19, APC-anti-CD3 antibodies, and PI solution was added after the antibody staining and before the flow cytometry analysis. Flow acquisition was conducted for the fixed time duration (60 s). Data were analyzed using FlowJo software (Version 10; TreeStar). Live CLL cell number was calculated by counting CD19+/CD5+ and PI- events in comparison to the DMSO control. For CLL proliferation, the percentage of cells distributed in the dividing phases of the CFSE profiles is automatically calculated by the FlowJo software. For Ki67 staining, CLL cells were first stained with FITC-anti-CD19 antibody, fixed and the permeabilized prior to Alexa Fluor 647 anti-human Ki67 antibody staining.

STATISTICAL ANALYSIS

Statistical analysis of the data was performed using Graphpad Prism 8 software (GraphPad, La Jolla, CA, USA). Group comparisons were conducted using non-parametric paired t-test selected by the Prism 8 software since data points are not normally distributed. One-way ANOVA analysis was applied for comparison among multiple groups and F-ratios are shown when they are indicated. P-values of 0.05 are considered statistically significant.

The following examples are provided to illustrate certain embodiments of the invention. They are not intended to limit the invention in any way.

Example I:

Figure 1 depicts a schematic diagram of the CIC process. CIC have been observed in archived human lymphoma samples. (FIG. 2). Primary CLL samples were assessed in an ex vivo co-culture model system which mimics the CLL tumor microenvironment (TME, Fig. 3). To recreate the lymph node (LN) microenvironment, we added and compared several known B-cell growth factors, including CpG, various cytokines, various chemokines, CD40L and BAFF/APRIL in 28 different combinations during co-culture. This work identified that BMF plus CpG/IL15 best simulates the LN microenvironment by providing the correct amount of stimulation. Under these conditions, CLL cells become larger, downregulated surface CXCR4 (endocytosis leading to activation of the pathway), and exhibited increased MYC and E2F gene expression signatures. In addition, tumor cells divide and proliferate (Fig. 3B), which recapitulates one of the salient features of CLL cells residing in the histologically defined “proliferation centers” in the lymph nodes.

Collectively, these features reproduced the phenotype of lymph node-resident CLL cells and the model system thus recapitulates the LN microenvironment. The cells were then observed and analyzed with confocal microscopy and 3D reconstruction.

The data reveal that CLL cells were actively internalized by BMF in at least 41 cases of the CLL samples studied. Moreover, the internalized CLL cells were alive and mobile within the BMF's cytoplasm. Surprisingly, the tumor cells were seen leaving their BMF host and re-entering into other BMFs (Fig. 4 and 5). More specifically, Fig. 4 shows high-resolution 3D confocal evidence of this process. The confocal microscopic examination of the co-cultured CLL cells revealed an interesting cellular behavior whereby CLL cells appeared to enter the bodies of

BMF in 2-5 hrs. Further analysis with 3D rendering of confocal images showed that the CLL cells were completely or partially embedded by the BMF, giving these cells a nutty chocolate appearance (Fig. 4B). Comparing Fig. 4B and 4C, it is evident that some cells are outside the BMF (such as cell 1, 4B vs 4C), some are halfway in (cell 2) and others are completely within the fibroblast (cell 3). In Fig. 4D-4F, the image is magnified on one CLL cell captured halfway in the process. This observation was made in the absence of any externally added drugs.

To determine if the internalized CLL cells were alive and able to move, we performed confocal time-lapse microscopy (Fig. 5). Time-lapse images acquired by confocal microscopy showed that BMF were actively taking in CLL cells and internalized CLL cells were moving inside the BMF independent of the movement of BMF themselves. Several snapshots are shown in Fig. 5 where CLL is labelled green and BMF is shown in either red (Fig.5A-C) or transparent pink surfaces (Fig.5D-F). It is apparent that “cell 1” moved into the body of BMF from time 7h50m to 8h02m (also compare solid to transparent images). “Cell 2” stayed in during this time while “cell 3” moved out of the capture area. This observation was made in the absence of any externally added drugs.

To decipher what CIC phenomenon means, we started by investigating under what clinical or pathological contexts does CIC occur. We first examined a cohort of 43 CLL blood samples using our model system, employing Imaris software to quantify live CIC instances in each case. Internalization was observed in all 41 samples (Fig. 6). No correlation was noted between CIC and established CLL prognostic indicators like IGHV mutation status, or cytogenetic abnormalities such as del(17p). Nonetheless, under the TME condition (BMF+CpG/IL15), samples from patients undergoing CLL treatment at the time of collection showed a more pronounced increase in CIC compared to untreated patients (Fig. 6). The finding, suggested that CIC may be promoted by drug therapy.

These association studies prompted a testable hypothesis, live CIC may represent a novel cellular mechanism for residual disease whereby live CLL cells “hide” inside BMF to evade drug attacks. To determine if drug exposure promotes CIC, we treated CLL cells with either DMSO or the BTK inhibitor ibrutinib (ibr) in the TME system at a clinically achievable concentration of 0.4 μ M. Fig.7A shows that normal B-cells (NBC, N=5) treated with DMSO also got internalized by the BMF, but at a significantly lower level than CLL cells treated with DMSO (N=11, compared grey dots to grey dots). Notably, when NBC were treated with ibr, CIC events did not

change compared to the vehicle control (blue dots). In contrast, when CLL cells were treated with ibr, a significantly greater number of cells got internalized by BMF (orange dots).

We next determined if CIC behavior is related to the clinical ibr-resistance status. Approximately 15-20% ibr-treated patients relapse within 18-36 months and most of these (~80%) acquire BTK and/or PLCG2 mutations. We studied CIC in samples collected from ibr-resistant patients (Samples herein are ibr-sensitive unless specified as ibr-resistant). No statistical differences in CIC occurrences were detected at early time points between the BTK mutant (N=6) and wild-type groups (N=11) (Fig. 7B, blue to orange). But interestingly, over a longer period, a higher number of mutant cells are found in BMF than wild-type cells suggesting the resistant cells have an increased capacity to persist (Fig. 7C, blue to orange).

Since little is known about CIC, one of the foremost questions is whether live CIC can be found in other B-cell lymphomas. We investigated follicular lymphoma (FL), another indolent lymphoma. Unlike CLL, FL has established cell lines. We observed that in BCL2-IGH positive WSU-FSCCL cells, CIC events also increased in response to ibr (Fig. 8A). In primary FL (N=9), CIC also occurred and further increased when cells were exposed to ibr (Fig. 8B, green dots).

Collectively, these data demonstrated CIC is promoted by ibr and ibr-resistant cells stay longer within BMF. Furthermore, the phenomenon is not confined to CLL and may represent a common cellular mechanism for lymphoma cells to escape and endure therapy.

Next, we determined if lymphoma drugs of different classes or sub-classes would similarly augment CIC. Following ibr, the first-in-class covalent BTKi, several new 'covalent' BTKi have been developed including acalabrutinib and zanubrutinib (zanu). Like ibr, these two BTKi bind to C481 through a covalent bond, but they provide higher specificity and improved tolerability. Resistance to all three covalent BTK inhibitors has emerged via mutation of the C481 residue^{5 12 41 13}. To overcome this problem, non-covalent BTKi, such as pirtobrutinib (pirt), has been developed, and the drug was recently approved in Dec 2023 to treat relapsed CLL. In addition to BTKi, another effective small-molecule drug is venetoclax (ven), a BCL2 inhibitor that specifically targets the antiapoptotic BCL2 protein, causing direct apoptotic death of tumor cells.

Since primary samples have finite cell numbers, we took precautions to test only concentrations that represent the average plasma concentrations determined by pharmacokinetic

studies under standard dosing, such as 0.4 μ M for ibr and 0.2 μ M for zanu and 15 μ M for pirto^{36,42 43}. Similar to ibr, CIC events increased in cells treated with either zanu or pirto compared to DMSO (Fig.9 A&B) demonstrating both covalent and non-covalent BTKi promoted CIC.

5 To prove CIC is relevant to BTKi treatment in real patients, we examined the bone marrow aspirates from patients who were on treatment with BTKi. Fig. 10 shows that CIC formation can be identified in such patients (Fig. 10, N=4).

We next tested, BCL2 inhibitor ven. a drug of a different class. In contrast to ibr, ven did not significantly increase CIC, with some variability among individuals (Fig. 11A, green vs grey, 10 not significant). The average increase of ibr-driven CIC was 134.5% (ranged 68-181%), while the average CIC increase in ven-treated cells was significantly lower at 38% (-26 to 104%) (Fig.11B). These findings reveal a class differentiation between the BCL2 and BTK inhibitors. Notably, these laboratory findings align with clinical observations where MRD positivity is much more common with ibr therapy compared to ven^{6 44} (See Discussion below).

15 Our next objective is to identify the mechanism by which BTKi drives CIC. The occurrence of CIC depends on the microenvironment, which provides tumor cells with signals for survival and proliferation. We first examined if cell internalization depends on tumor cell proliferation within the TME system. Proliferation by CFSE staining (Fig. 12A-B) revealed a strong positive correlation between tumor cell internalization and cell proliferation (N=11) (Fig. 20 12B, $R^2 = 0.78$ and $p = 0.0003$).

Various ligand-receptor interactions and/or soluble factors in the TME are known to promote tumor proliferation (listed in Fig. 12C). We hypothesized that stromal cells that help promote cell proliferation may mediate CIC. To mimic different cell types, such as T-cells and macrophages, we engineered BMF cells to express CD40L, cytokines (IL4-IL21-IL15), 25 BAFF/APRIL, and chemokines (CXCL12-CXCL13) using lentiviral transduction (Fig. 12C last column). We then explored the impact of conditioned media (CM) from each of these BMFs on the process of CIC. Fig. 12D shows that CM from CD40L-transduced BMF (CD40L-BMF) had little effect on CIC compared with the un-transduced BMF (Fig. 12D, upper left panel, purple vs grey). CM from IL4/15/21-BMF promoted CIC in 2/4 cases (Fig. 12D, upper right panel, blue vs 30 grey) while CM from BAFF/APRIL-BMF and CXCL12/13 BMF promoted CIC in all 4 cases (Fig. 12D lower panels).

We then further explored CXCR4 inhibition and depletion. We first tested if CXCL12-induced CIC can be prevented by the CXCR4 antagonist plerixafor (pleri). FSCCL cells were incubated with CM from either control BMF or CXCL12/13-BMF. The co-culture was then treated with DMSO or pleri at a clinically achievable concentration of 1 μM ⁵⁰. CM from CXCL12/13-BMF significantly increased the number of CIC compared to the control CM (Fig. 13, orange vs grey). Notably, pleri reduced this stimulation back to the control level (green vs orange vs grey). Pleri also decreased the baseline CIC in cells treated with CM from untransduced BMF (blue vs grey).

We then questioned if pleri treatment can block *ibr-driven* CIC besides chemokine-induced CIC. In FSCCL cells, ibr exposure caused an increase in CIC events (Fig. 14A, orange vs grey). Pleri, by itself, reduced the CIC counts compared to the control (blue vs grey). Importantly, addition of pleri to ibr completely abrogated ibr-driven CIC (green vs orange vs grey). We have validated these results with another CXCR4 antagonist, motixafortide (motix) (Fig.15).

To corroborate this finding, we knocked out CXCR4 in FSCCL cells via CRISPR-Cas9 approach. In cells depleted with two different guiding RNAs (KO#1 and #2), CIC events were significantly reduced (Fig. 14B) comparing to cells which received mock gRNA (WT), providing additional genetic evidence that CXCL12-CXCR4 axis plays a pivotal role in mediating cell-in-cell.

Lastly, we examined the ability of pleri to block CIC in primary CLL cells. Fig. 16A shows that exposure to ibr increased CIC (N=6, orange vs grey) while pleri alone did not significantly affect CIC compared to the control (blue vs grey). Consistent with the cell line study, the addition of pleri completely abolished ibr-induced cell internalization (green vs orange vs grey). Altogether, these findings underscore the essential role CXCL12-CXCR4 in mediating CIC and demonstrate experimental blockade of this signaling axis can prevent ibr-induced CIC in both cell line and primary tumors (Fig.1).

References

1. Lu, P., Wang, S., Franzen, C.A. et al. Ibrutinib and venetoclax target distinct subpopulations of CLL cells: implication for residual disease eradication. Blood Cancer J. 11, 39 (2021). <https://doi.org/10.1038/s41408-021-00429-z>.

2. Wang X et. al., “Cell-in-Cell Phenomenon and Its Relationship With Tumor Microenvironment and Tumor Progression: A Review.”, *Front. Cell Dev. Biol.* 7:311. <https://doi.org/10.3389/fcell.2019.00311>

3. Gutwillig *et al.*, “Transient cell-in-cell formation underlies tumor relapse and resistance to immunotherapy”, *eLife* 2022;11: e80315. <https://doi.org/10.7554/eLife.80315>

4. Teicher BA and Fricker SP, “Molecular Pathways CXCL12 (SDF-1)/CXCR4 Pathway in Cancer”, *Clin Cancer Res* (2010) 16 (11): 2927–2931. <https://doi.org/10.1158/1078-0432.CCR-09-2329>

5. Sánchez-Ortega, I., Querol, S., Encuentra, M. *et al.* Plerixafor in patients with lymphoma and multiple myeloma: effectiveness in cases with very low circulating CD34+ cell levels and preemptive intervention vs remobilization. *Bone Marrow Transplant* **50**, 34–39 (2015). <https://doi.org/10.1038/bmt.2014.196>

6. Hübel K, et al. Plerixafor in non-Hodgkin's lymphoma patients: a German analysis of time, effort and costs. *Bone Marrow Transplant.* 2019 Jan;54(1):123-129. doi: 10.1038/s41409-018-0228-z. Epub 2018 May 24. PMID: 29795422; PMCID: PMC6320344.

7. International Patent Publication No. WO 2022/144885 A1

8. International Patent Publication No. WO 2019/213144 A1

Example II

Test and Treat Method for Ameliorating CIC Which Leads to Disease Relapse

The information herein above can be applied clinically to patients for therapeutic intervention. . In certain embodiments this can occur after the patient received a cancer therapy. In certain embodiments, according to the clinical criteria, the patient was not responsive, or partially responsive, to the cancer therapy. In certain embodiments the diagnostic/prognostic methods are performed to identify those subjects with cancer exhibiting CIC. This can be done via direct assessment of the CIC or CIC-related biomarkers described above. The derived therapeutic doses of BTK inhibitor and CXCR4 antagonist for human could be determined by those skilled in the art based on clinical trials. BTK inhibitors, such as ibrutinib, and CXCR4 antagonists, such as plerixafor, have been shown to be well tolerated and the residual disease can be assessed through clinical assays.

While certain features of the invention have been described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the scope of the invention.

What is claimed is:

1. A method for modulating tumor CIC internalization, comprising,
 - a) incubating an ex vivo cell culture system, including a first cell culture of bone
5 marrow fibroblasts (BMF) which express one or more cell signaling molecules and a second cell culture comprising leukemia or lymphoma cells isolated from a human; and optionally one or more exogenous soluble cell signaling molecules or growth factors, in the presence and absence of at least one anticancer agent and
 - b) determining the number and quality of tumor CIC internalized into said first
10 cell culture in the presence of said at least one anti-cancer agent relative to untreated cell cultures, thereby identifying agents which modulate the CIC tumor cell internalization process.
2. The method of claim 1, wherein said agent is a BTK inhibitor.
- 15 3. The method of claim 1 or 2 further comprising a CXCR4 antagonist.
4. The method of any one of claims 1, 2, or 3 comprising detection of a CIC associated biomarker.
- 20 5. The method of claim 1, wherein said CIC internalization process is inhibited.
6. The method of claim 1, wherein CIC internalization is increased.
7. The method of claim 1, wherein increased lymphoma or leukemia CIC internalization is
25 indicative of increased risk for minimal residual disease (MRD).
8. A combination regimen useful in cancer treatment comprising the co-administration or sequential administration to a subject in need thereof of effective amounts of
 - a) at least one BTK inhibitor and
 - b) at least one CXCR4 antagonist in at least one pharmaceutically acceptable carrier.

9. The combination regimen of claim 8, wherein the BTK inhibitor is selected from Ibrutinib, ONO/GS-4059 (tirabrutinib), AVL-292/CC-292/spcbrutinib, BGB-3111 (Zanubrutinib), and ACP-196/acalabrutinib, M7583, MSC2364447C, BIIB068, ACO0058TA, DTRMWXHS-12, LOXO305 (pirtobrutinib).
10. The combination regimen of claim 8 or claim 9, wherein the BTK inhibitor is Ibrutinib.
11. The combination regimen of any one of claims 8 to 10, wherein the CXCR4 antagonist is selected from Plerixafor, T140 analogs, BL-8040 (previously BKT140), TN14003, MSX-122, TG-0054, cyclic-pentapeptide-based antagonists, FC122 and FC131, tetrahydroquinolines-based antagonists, AMD070 AMD070 derivatives, indole-based antagonists, FC131, Para-xylyl-enediamine-based compounds, AMD3465, AMD3465 analogues WZ811, MSX122, guanidine-based Antagonists, NB325, quinoline derivatives, NSC56612, KRH-3955, CTCE-9908, POL6326, motixafortide, and mavorixafor.
12. The combination regimen of any one of claims 8 to 11, wherein the CXCR4 antagonist is Plerixafor, motixafortide, or mavorixafor.
13. The combination regimen of any one of claims 8 to 12, further comprising administration of at least one additional anti-cancer agent.
14. The combination regimen of any one of the preceding claims, wherein the cancer is selected from any of B-cell non-Hodgkin lymphoma/leukemia, chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), follicular lymphoma (FL), marginal zone lymphoma (MZL), mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL) or a T-cell lymphoma such as peripheral T-cell lymphoma and T-prolymphocytic lymphoma.
15. The combination of one of the preceding claims, wherein at least one agent reduces undesirable side effects from administration of said one agent present in said combination of agents.
16. The method of claim 15, wherein said undesired side effect increased occurrence of CIC.

17. The method of claim 8, wherein said agents in the combination act synergistically.
18. The combination regimen of any one of claims 8 to 17, wherein the cancer has CIC tumor cells.
19. A method for treating CIC associated cancer in a subject in need thereof, comprising,
 - a) administering an effective amount of at least one BTK inhibitor and
 - b) administering an effective amount of at least one CXCR4 antagonist, thereby reducing CIC occurrence.
20. The method of claim 19, wherein the BTK inhibitor is selected from Ibrutinib, ONO/GS-4059 (tirabrutinib), AVL-292/CC-292/spebrutinib, BGB-3111 (Zanubrutinib), and ACP-196/acalabrutinib, M7583, MSC2364447C, BIIB068, ACO0058TA, DTRMWXHS-12, LOXO305 (pirtobrutinib).
21. The method of any one of claims 19 or 20, wherein the BTK inhibitor is Ibrutinib.
22. The method of any one of claims 1-21, wherein the CXCR4 antagonist is selected from Plerixafor, T140 analogs, BL-8040 (previously BKT140), TN14003, MSX-122, TG-0054, a cyclic-pentapeptide-based antagonist, FC122, FC131, tetrahydroquinolines-based antagonists, AMD070 AMD070 derivatives, indole-based antagonists, FC131, Para-xylyl-enediamine-based compounds, AMD3465, AMD3465 analogues WZ811, MSX122, guanidine-based antagonists, NB325, quinoline derivatives, NSC56612, KRH-3955, CTCE-9908, POL6326, motixafortide, and mavorixafor.
23. The method of any one of claims 19 to 22, wherein the CXCR4 antagonist is Plerixafor, motixafortide, or mavorixafor.
24. The method of any one of claims 19 to 23, further comprising administering of at least one additional anti-cancer agent.

25. The method of any one of claims 19-24, wherein at least one agent reduces undesirable side effects from administration of said one agent present in said combination of agents.
26. The method of claim 25, wherein said undesired side effect is increased occurrence of CIC.
27. The method of claim 19, wherein said agents in the combination act synergistically.
28. The method of any one of claims 19 to 27, wherein the cancer is lymphoma, leukemia, or chronic lymphocytic leukemia.
29. The method of any one of claims 19 to 28, wherein the cancer has CIC tumor cells.
30. A method for treating BTK inhibitor resistant cancer in a subject in need thereof, the method comprising administering at least one CXCR4 antagonist.
- 5 31. A method for increasing the efficacy of BTK inhibitor in the treatment of cancer, the method comprising co-administering at least one CXCR4 antagonist with said BTK inhibitor.
32. A method for preventing relapse of CLL cancer after treatment with at least one BTK inhibitor, the method comprising co-administering or sequentially administering at least one
10 CXCR4 antagonist with said BTK inhibitor.



FIG. 2A



FIG. 2B

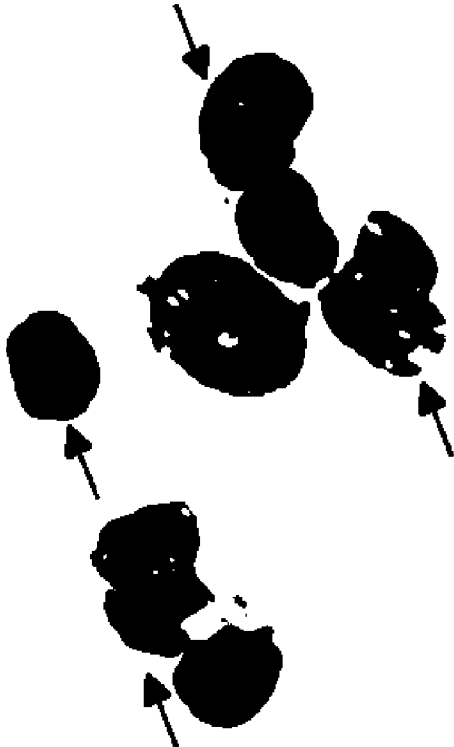
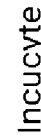
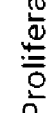
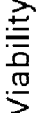
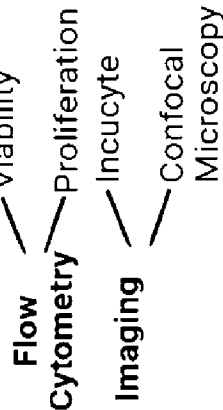
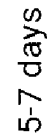
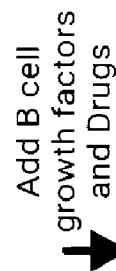
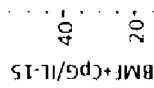
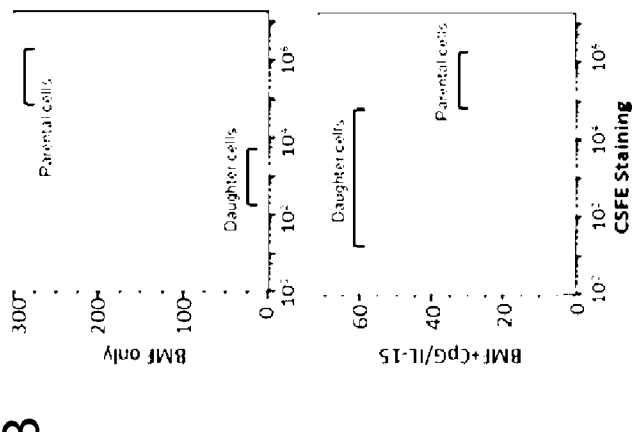
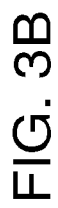
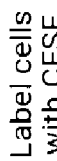
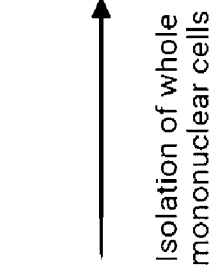
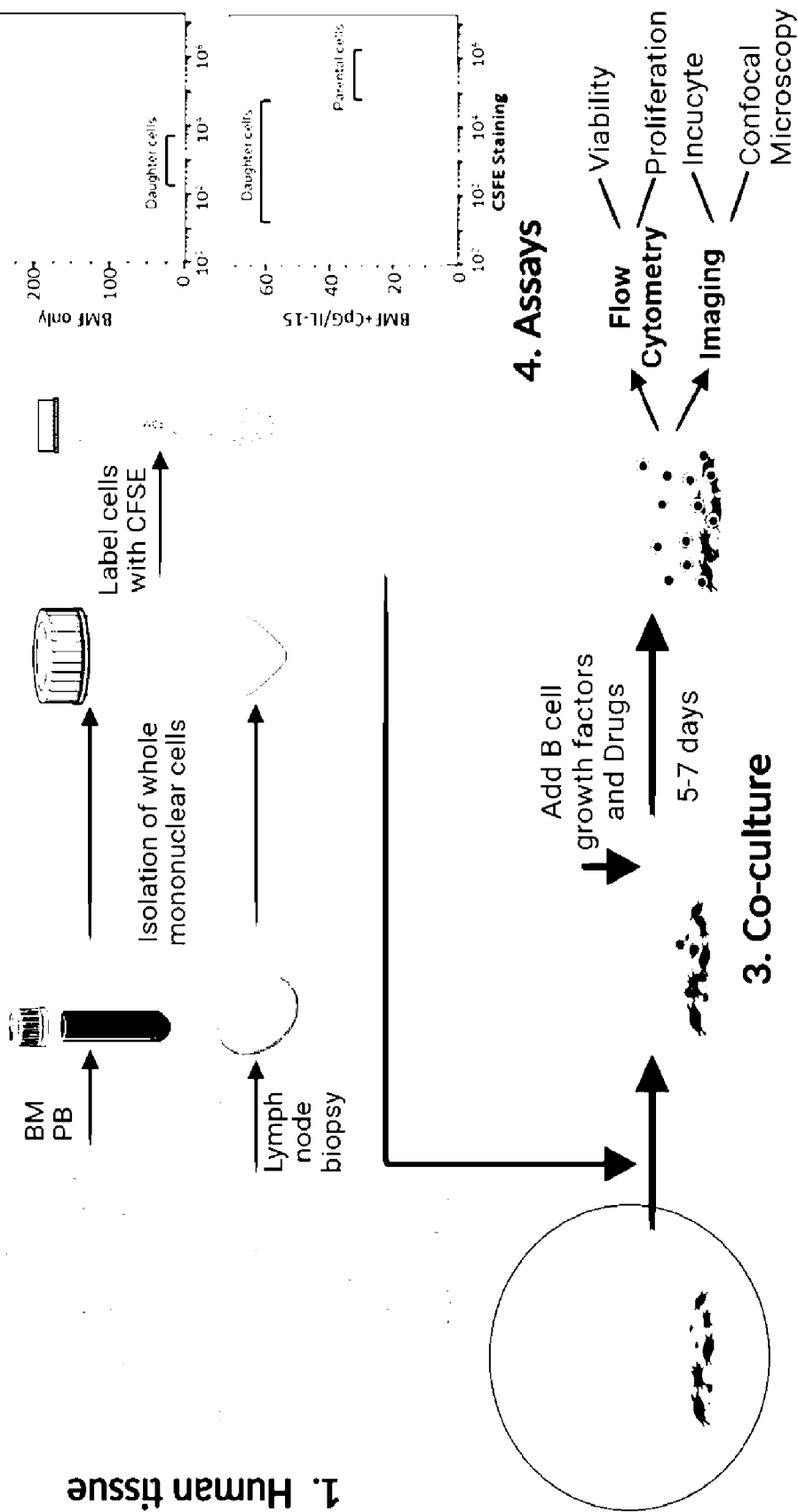


FIG. 3A



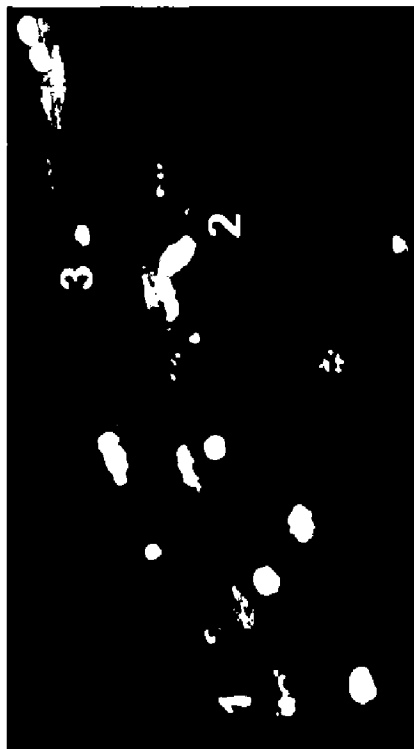


FIG. 4A



FIG. 4B



FIG. 4C

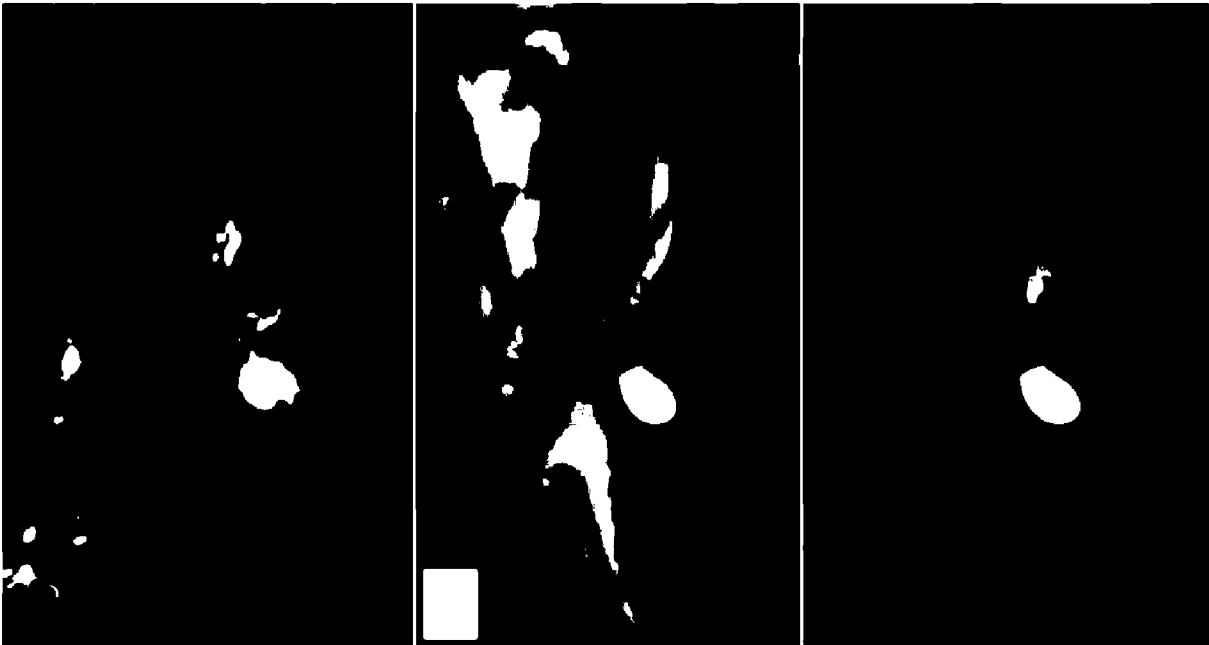


FIG. 4D

FIG. 4E

FIG. 4F



FIG. 5D



FIG. 5E



FIG. 5F



FIG. 5A



FIG. 5B



FIG. 5C

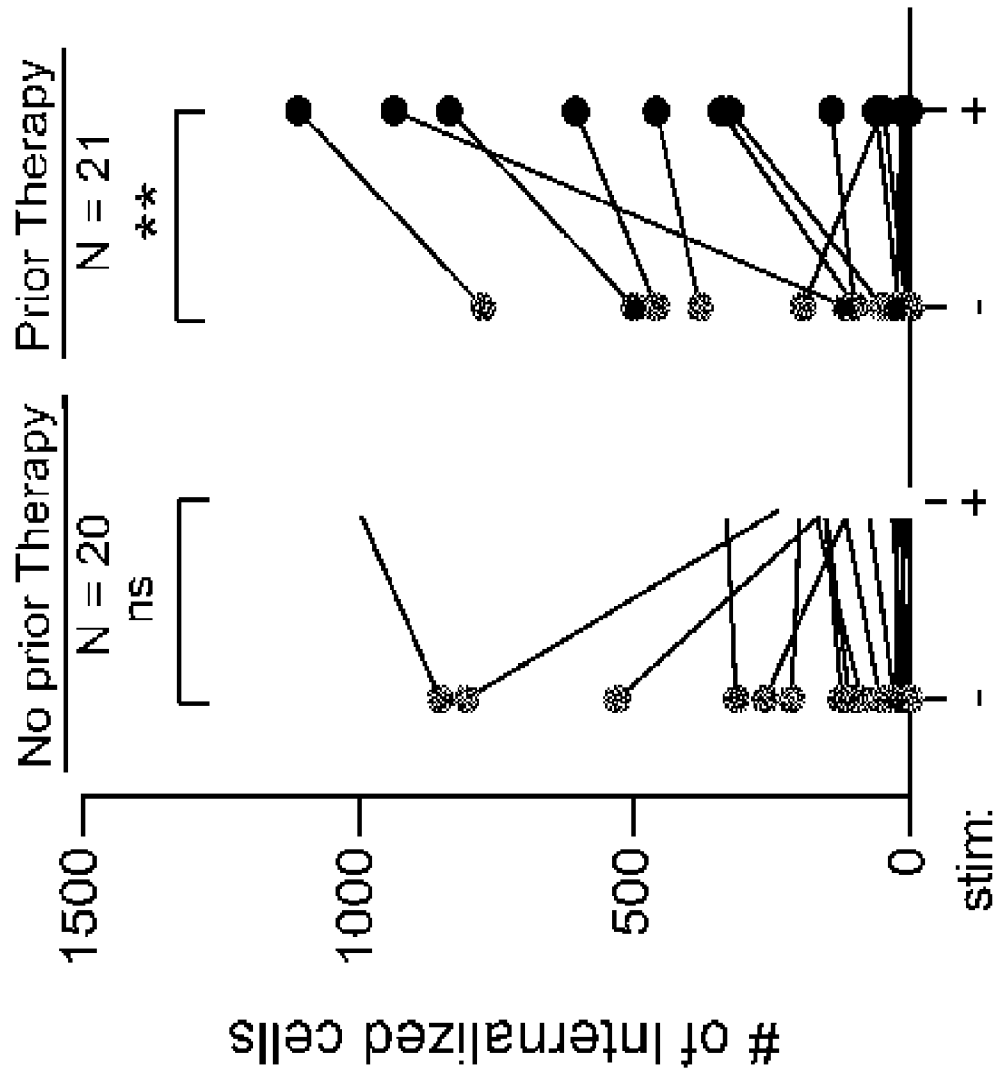


FIG. 6

FIG. 7A

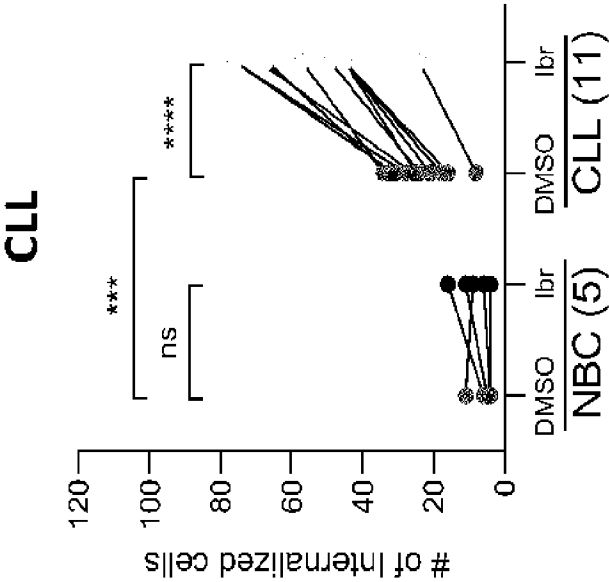


FIG. 7B

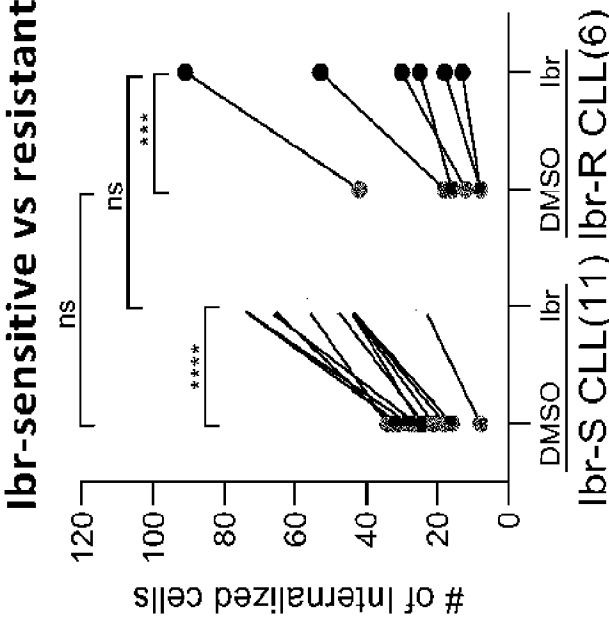


FIG. 7C

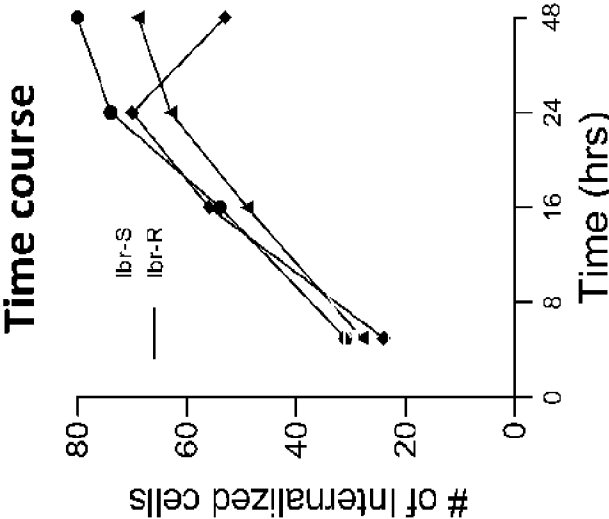


FIG. 8A FSCCL

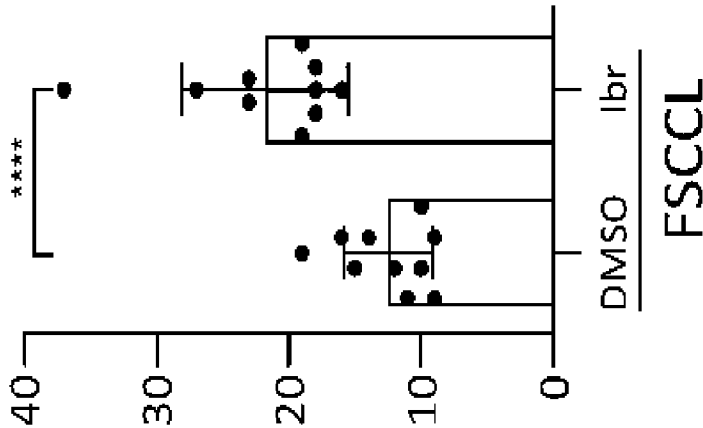


FIG. 8B

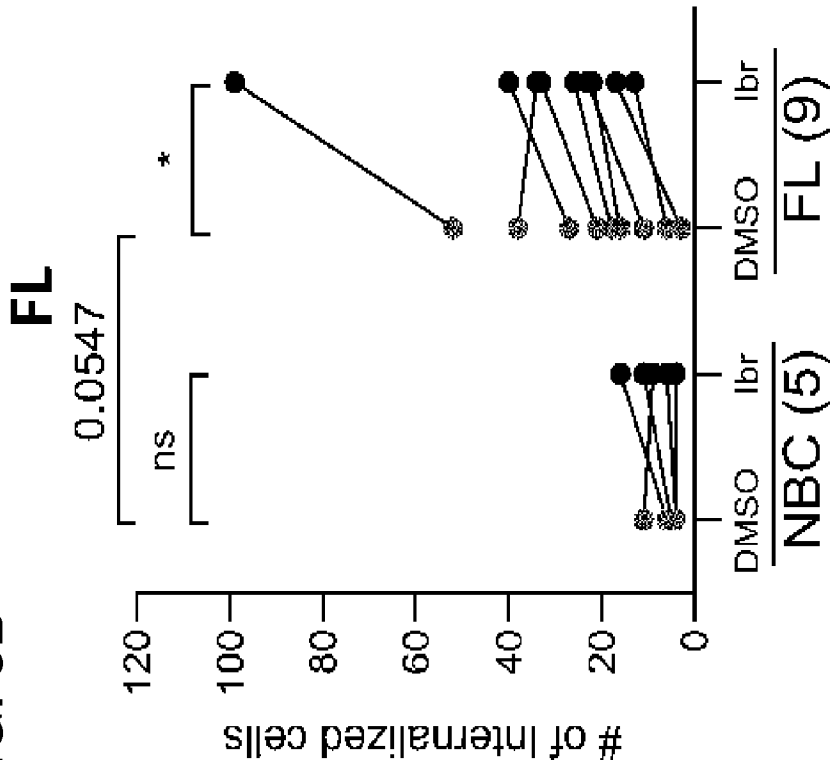


FIG. 9A

Zanubrutinib

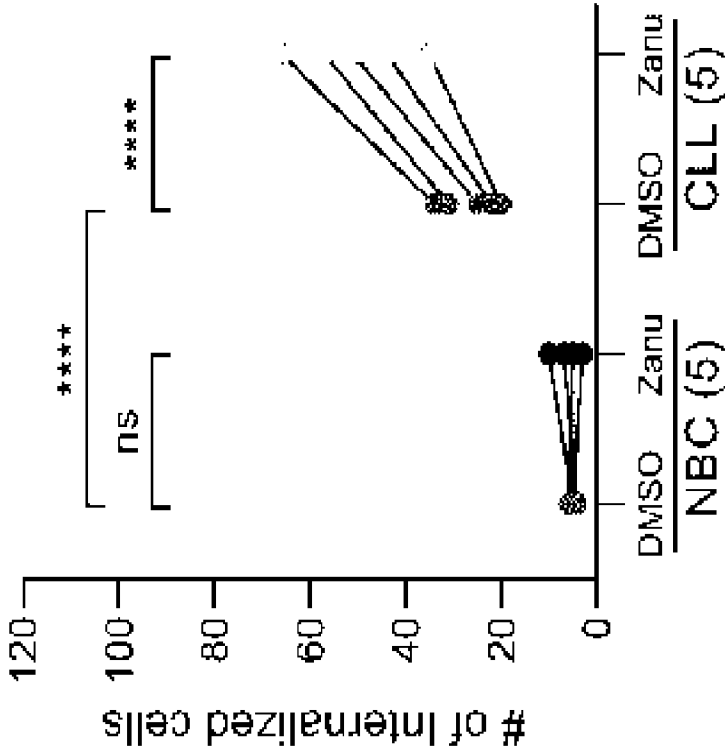
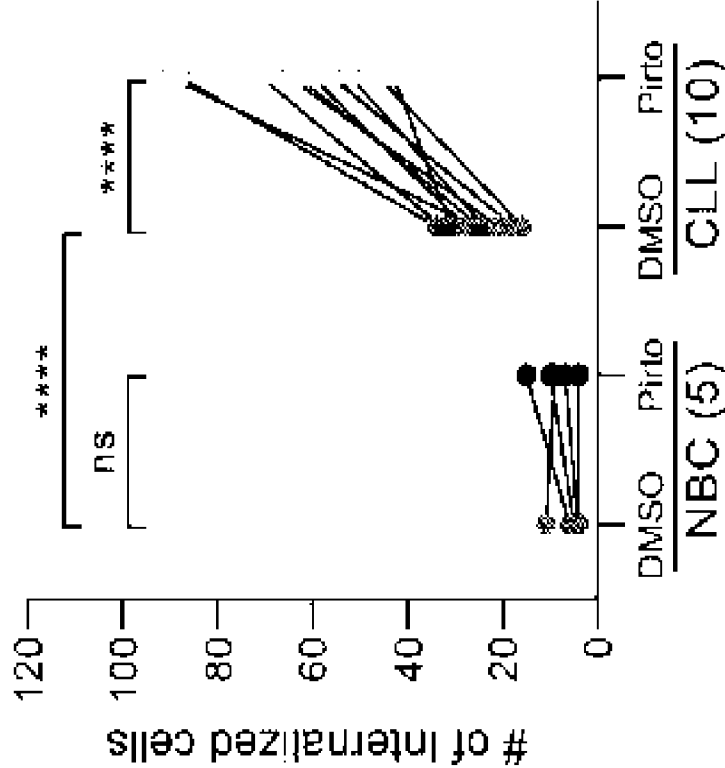


FIG. 9B

Pirtobrutinib



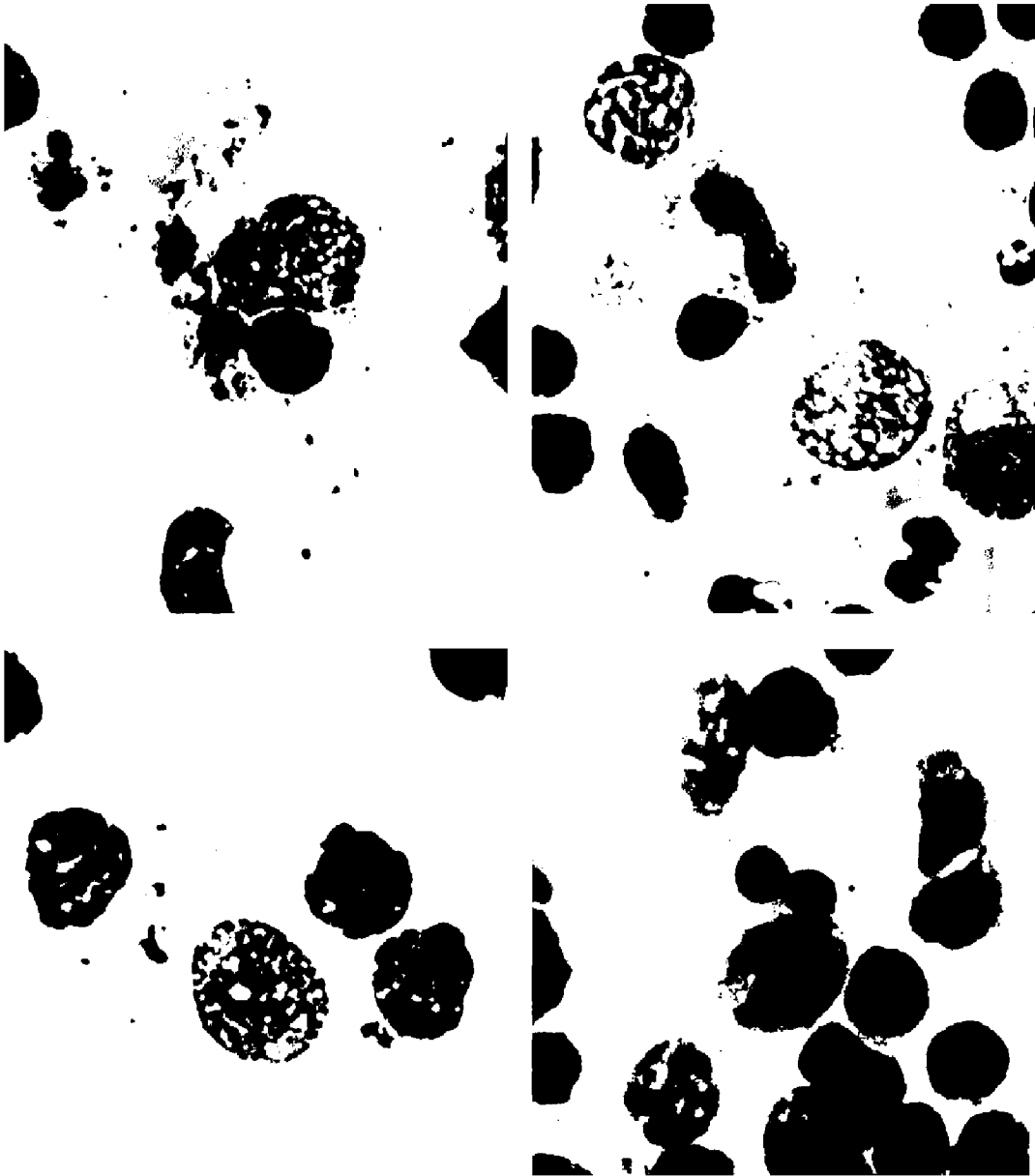


FIG. 10

FIG. 12A

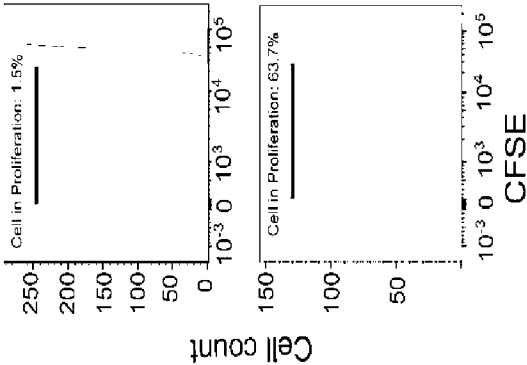


FIG. 12B

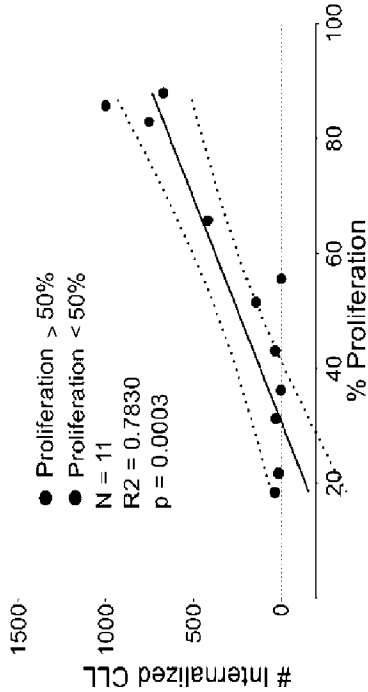


FIG. 12C

Growth Factors	Stroma cell types	Receptor on B-cells	Nature of interactions	Transduced BMF
CD40L	T-cells	CD40	Receptor-Ligand	CD40L-BMF
IL4	T-cells	IL4R	Cytokine	IL4/IL15/IL21-BMF
IL21	T-cells	IL21R	Cytokine	
IL15	Macrophage	IL15R	Cytokine	
BAFF/APRIL	Macrophage	BAFF-R	Receptor-Ligand	BAFF/APRIL-BMF
CXCL12	Macrophage Nurse-like cells	CXCR4	Chemokine	CXCL12/13-BMF
CXCL13	Macrophage Nurse-like cells	CXCR5	Chemokine	

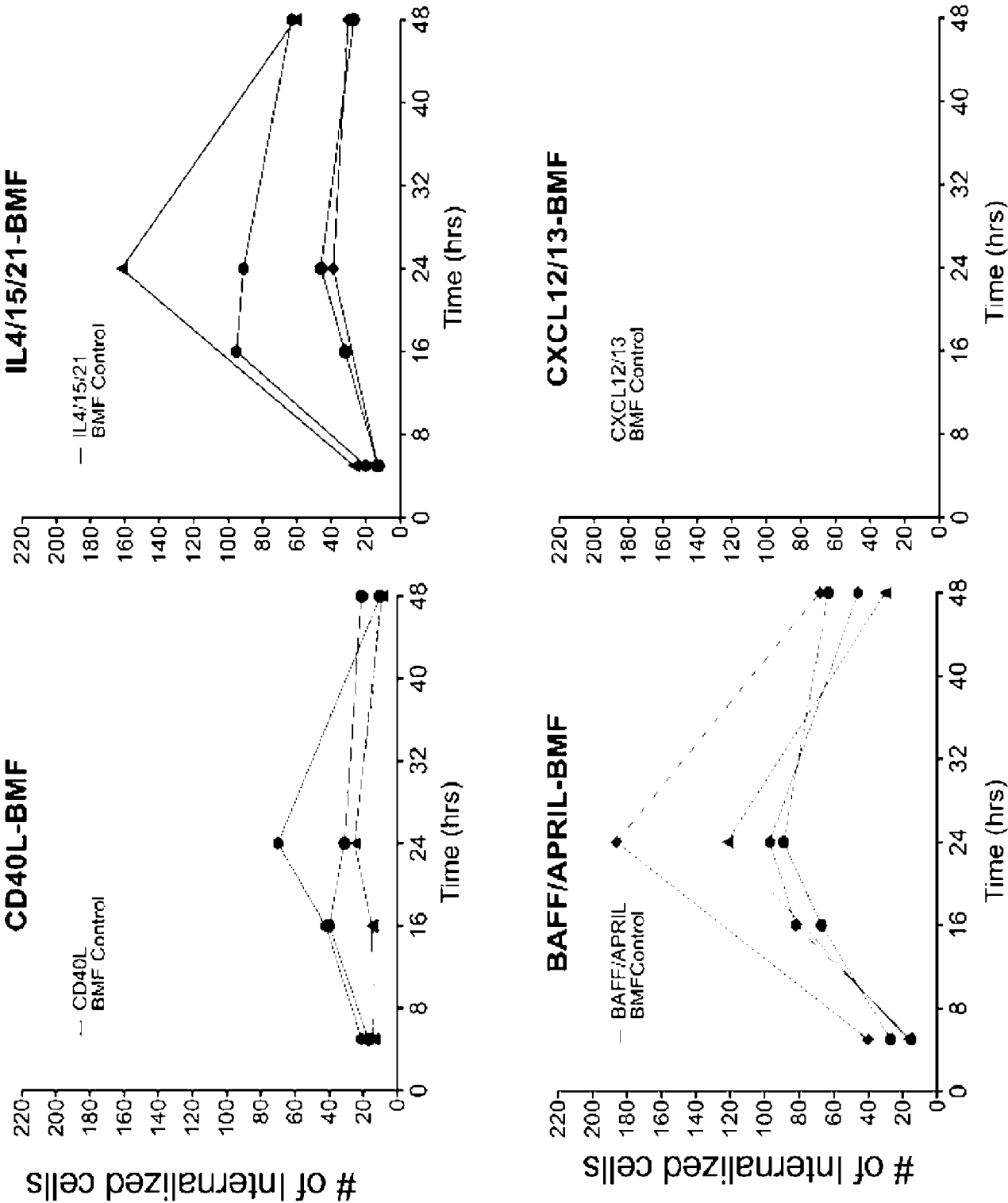


FIG. 12D

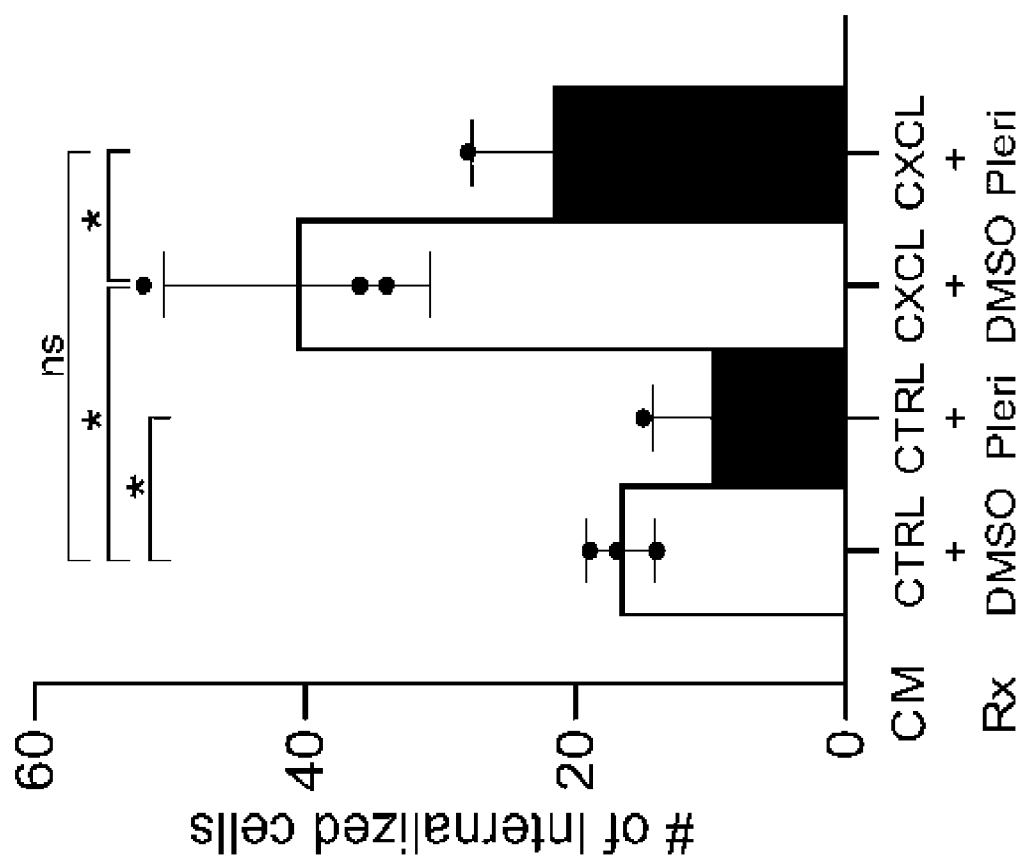


FIG. 13

FIG. 14B

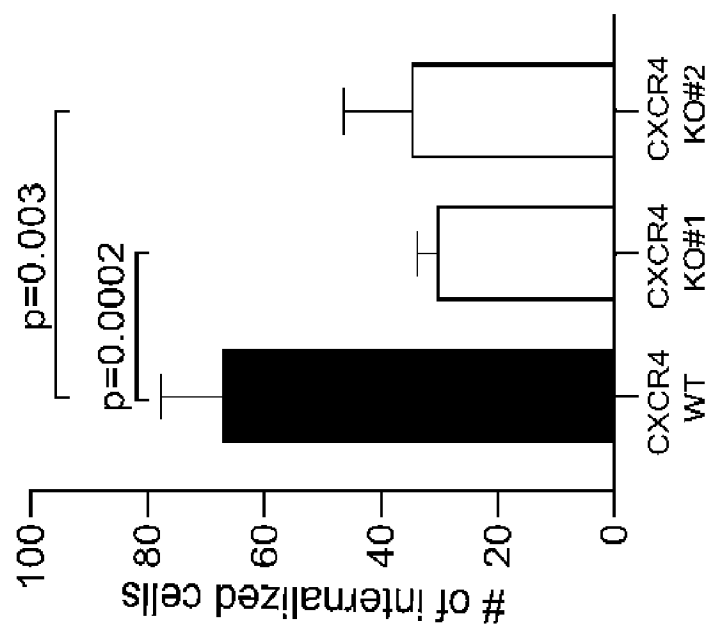
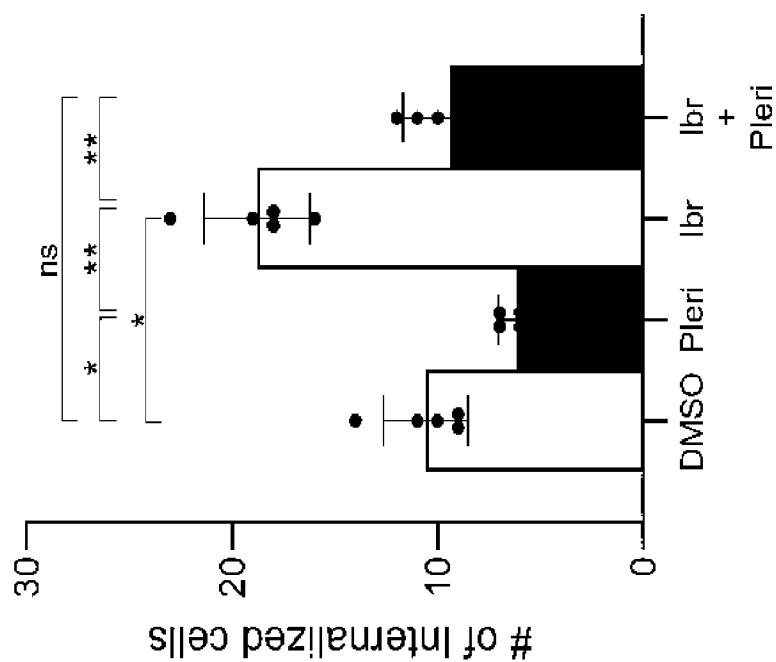


FIG. 14A



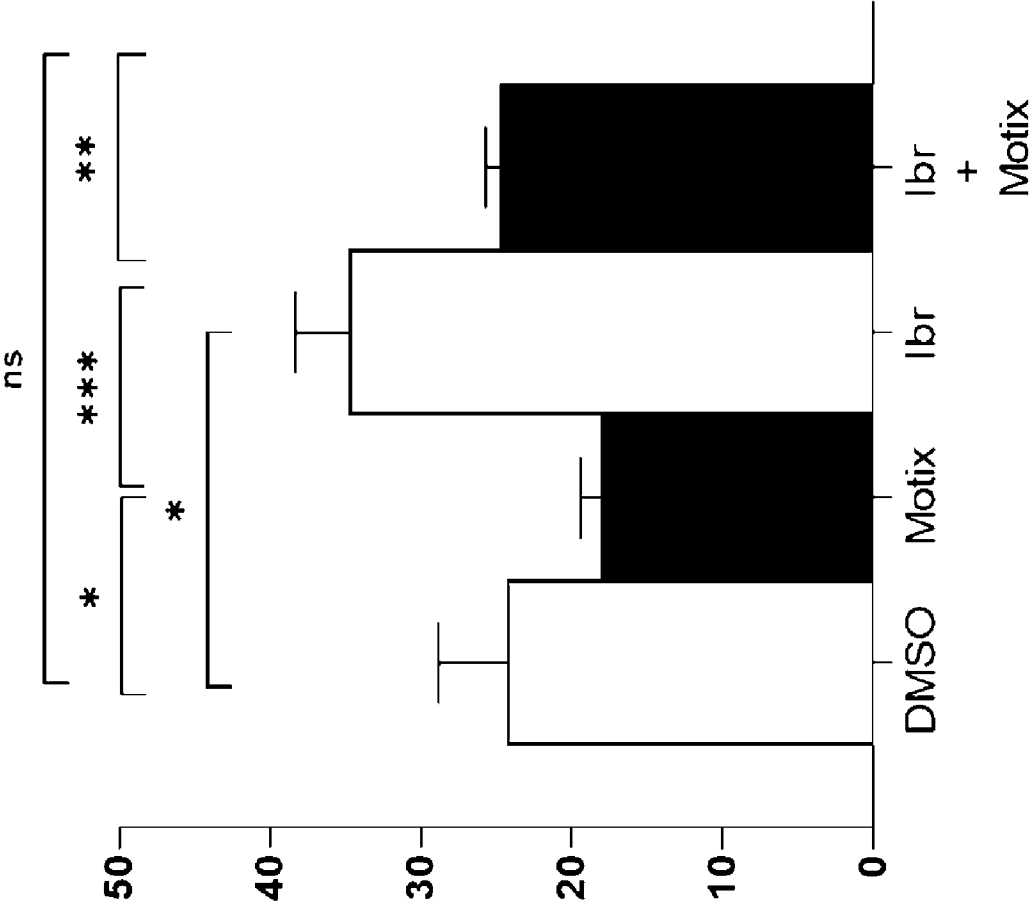


FIG. 15

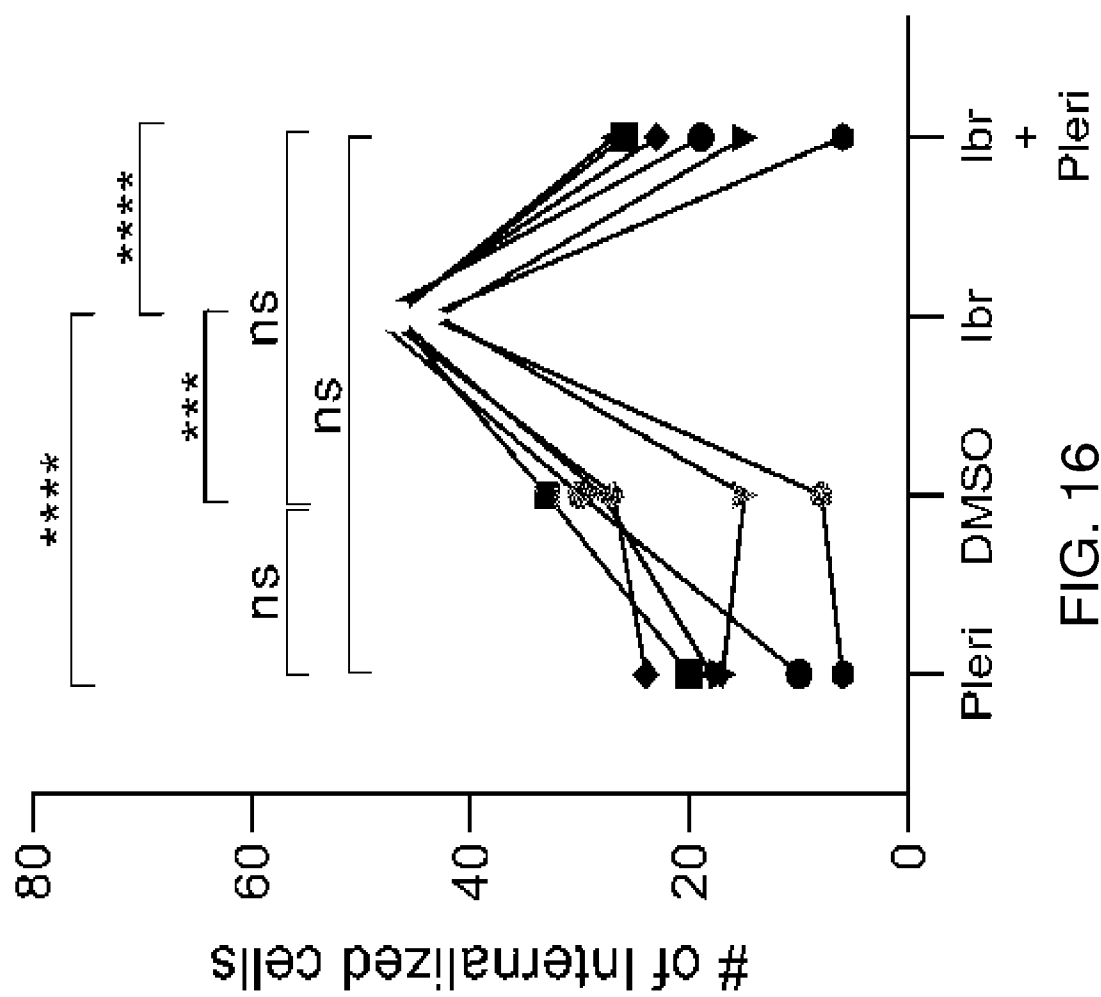


FIG. 16