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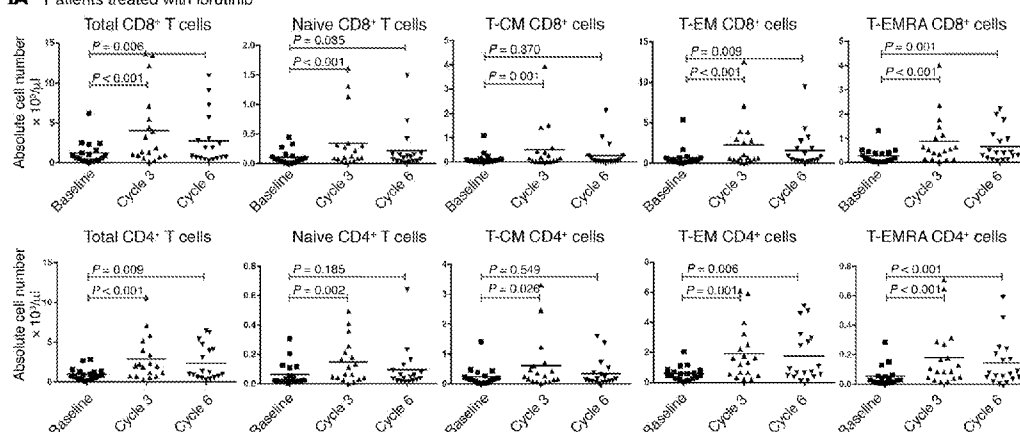
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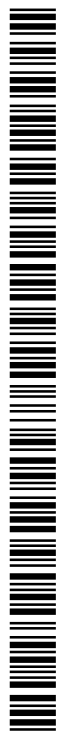
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(54) Title: EXPANSION OF IMMUNE CELLS WITH INTERLEUKIN-2 INDUCIBLE T CELL KINASE INHIBITING COMPOUNDS

FIG. 1A Patients treated with ibrutinib



(57) Abstract: Disclosed are methods for selectively expanding T cell and NK cell populations.



EXPANSION OF IMMUNE CELLS WITH INTERLEUKIN-2 INDUCIBLE T CELL KINASE INHIBITING COMPOUNDS

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5 awarded by the National Institutes of Health. The government has certain rights in the invention.

I. BACKGROUND

1. Chronic lymphocytic leukemia (CLL) patients are characterized by a profound
immune suppression and perturbation of both innate as well as adaptive immunity, highlighted
10 by increased susceptibility to infections, autoimmune conditions and a higher incidence of
secondary malignancies. In addition to global immunosuppression, CLL cells also evade
immune destruction due to tumor-specific immune suppression via multiple mechanisms
including expression of immunosuppressive molecules including CD200, HLA-G and PD-L1.
Malignant CLL B cells also phenotypically and functionally recapitulate the “B10” and
15 ”B10pro” regulatory B (Breg) cell types that produce IL-10, a major immunosuppressive
cytokine. In addition to direct tumor immunosuppressive effects, CLL cells can also increase the
number of regulatory T (Treg) cells that diminish cellular immune responses. Moreover, CLL
cells can induce IDO^{hi}CD14⁺HLA-DR^{lo} myeloid-derived suppressor cells (MDSC), which
inhibit T-cell responses both directly and indirectly through promoting Treg induction and
20 expansion. Collectively, these observations highlight the importance of immune interactions in
CLL and make this disease an ideal model to study systemic tumor-mediated immune
suppression.

2. It has been well-established that the T cell compartment in CLL patients is
dysfunctional. CLL T cell subsets are skewed toward a terminally differentiated phenotype,
25 with significant reduction in naïve T cells and expansion of effector memory/effector T cells.
CLL patient T-cells also demonstrate features of pseudo-exhaustion, with significant up-
regulation of checkpoint molecules and exhaustion markers such as PD-1, CTLA-4, CD160,
CD244 and CD57. These phenotypic changes are closely associated with profound functional
defects including reduced cytotoxic capacity of CD8 T cells and defective immunologic synapse
30 formation. A shift in the balance between Th1, Th2 and Th17 immune subsets in CLL patients
can also be an important factor in driving disease progression, and the T cell response in CLL
patients is skewed toward the Th2 polarization. Studies in CLL patients have also shown that a
decrease in IL-17-producing T cells is associated with Treg expansion and disease progression,

while increased Th17 cell numbers correlate with improved overall survival. To date, success in therapeutically enhancing cell-mediated immunity in CLL has been limited. Lenalidomide has favorable immune modulating properties on T-cells in CLL patients via down-regulation of IKZF1 through a cereblon-dependent mechanism, and can promote durable, sustained complete remissions in 50% or more of patients receiving this treatment. However, lenalidomide in this setting also can produce significant morbidity and sometimes fatal outcome from early-onset tumor flare.

3. New to the field of B-cell cancer therapy are agents that irreversibly target Bruton's tyrosine kinase (BTK) such as ibrutinib, which yields high response rates and durable remissions in patients with CLL. Although data in humans is lacking, multiple reports document the T cell effects of ibrutinib in mouse models. Ibrutinib irreversibly inhibits interleukin-2 inducible T cell kinase (ITK), leading to enhanced Th1 response *in-vitro* and *in-vivo*. In a CLL mouse model of bacterial infection, ibrutinib treatment led to a more than two-fold increase in expansion of Th1 pathogen-specific T cells and corresponding suppression of Th2 immunity. Ibrutinib treatment also leads to a shift in macrophages toward a Th1-supportive phenotype and increases CD8 T cell tumor infiltration in a mouse model of pancreatic cancer.

II. SUMMARY

4. Disclosed are methods and compositions related to expanding T cell and NK cell populations by contacting said cells with an interleukin-2 inducible T cell inhibitor.

5. In one aspect, disclosed herein are methods of selectively expanding CD8 and CD4 T cells but not CD25+Foxp3+ T cells in a subject comprising administering to a subject an agent that inhibits interleukin-2 inducible T cell kinase (ITK). Also disclosed are methods wherein the agent further inhibits Bruton's tyrosine kinase (BTK) (such as, for example ibrutinib).

6. In one aspect, disclosed herein are methods of any preceding aspect, wherein the expanded T cells are effector memory (CD45RA-CCR7-) T cells or CD45RA+CCR7- T cells.

7. Also disclosed herein are methods of inhibiting activation induced cell death of T cells and/or NK cells in a subject with chronic lymphocytic leukemia comprising administering to the subject an inhibitor of interleukin-2 inducible T cell kinase (ITK).

8. In one aspect, disclosed herein are methods of expanding NK cells comprising contacting NK cells with a stimulatory molecule and ibrutinib; wherein the stimulatory molecule is selected from the group consisting of IL-15, IL-21, IL-2, 41BBL, IL-12, IL-18, MICA, 2B4, LFA-1, and BCM1/SLAMF2.

9. Also disclosed herein are methods of treating cancer in a subject comprising administering to a subject NK cell therapy, wherein the NK cell therapy comprises a) expanding NK cells by stimulating NK cells with a stimulatory molecule ibrutinib; wherein the stimulatory molecule is selected from the group consisting of IL-15, IL-21, IL-2, 41BBL, IL-12, IL-18, MICA, 2B4, LFA-1, and BCM1/SLAMF2; and b) administering to the subject the expanded NK cell population.

10. In one aspect, disclosed herein are methods of treating cancer of any preceding aspect, further comprising administering to the subject ibrutinib prior to or after administration of the expanded NK cells to the subject.

11. Also disclosed herein are methods for expanding an immune cell (such as, for example, a chimeric antigen receptor (CAR) T cell, tumor infiltrating lymphocyte (TIL), or marrow-infiltrating lymphocyte (MIL), natural killer (NK) cell, NK-T cell, a cytokine-induced memory NK cell, or a cytokine-induced killer (CIK) cell) isolated from a subject for use in immune therapy, comprising contacting the isolated immune cell with an effective amount of interleukin-2 inducible T cell kinase (ITK) inhibitor (such as, for example, ibrutinib) to expand the immune cell in an amount effective for immunotherapy; and culturing the isolated immune cells in the presence of the ITK inhibitor (for example, culturing the cells in the presence of the ITK inhibitor).

12. In one aspect, disclosed herein are methods of increasing the cytotoxicity and survival of T cells to a tumor cell (such as, for example an autologous tumor) comprising contacting CD4 and/or CD8 T cells with an ITK inhibitor (such as, for example ibrutinib).

13. Also disclosed herein are methods of increasing the cytotoxicity and survival of T cells of any preceding aspect further comprising the administration of blinatumomab.

14. In one aspect, disclosed herein are methods of increasing the percentage of Th17 T cells comprising contacting a T cell population with an ITK inhibitor.

15. Also disclosed herein are methods of reducing checkpoint inhibition (such as, for example, checkpoint inhibition by immunosuppressive ligands such as PD-1/PD-L1, CTLA-4 and/or CD200) in a subject comprising administering to a subject an ITK inhibitor (such as, for example, ibrutinib).

III. BRIEF DESCRIPTION OF THE DRAWINGS

16. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments and together with the description illustrate the disclosed compositions and methods.

17. Figures 1A and 1B show that ibrutinib but not acalabrutinib treatment of CLL patients increases total T cell numbers: Figure 1A shows absolute numbers of CD8 (upper panel) and CD4 (lower panel) T cells before and during ibrutinib treatment (n=18). Figure 1B shows absolute numbers of CD8 (upper panel) and CD4 (lower panel) T cells before and during acalabrutinib treatment (n=12). Each cycle is 4 weeks. "Cycle 3" indicates samples obtained after two cycles (8 weeks into treatment), and "cycle 6" indicates samples obtained after 5 cycles (20 weeks into treatment). T cells were differentiated into subsets based on expression of CCR7 and CD45RA: naïve T cells (CCR7+CD45RA+), central memory T cells (CCR7+CD45RA-), effector memory T cells (CCR7-CD45RA-), and more differentiated effector memory T cells (T-EMRA; CCR7-CD45RA+).

18. Figures 2A and 2B show the effect of ibrutinib or acalabrutinib treatment on the frequency of different subsets of peripheral T cells. Figure 2A shows the percentage of different T cell subsets among total CD8 T cells(upper panel) and CD4 T cells (lower panel) before and after ibrutinib Treatment (n=18). Figure 2B shows the percentage of different T cell subsets among total CD8 T cells(upper panel) and CD4 T cells (lower panel) before and after acalabrutinib treatment (n=12). T cells are differentiated into subsets based on their expression of CCR7 and CD45RA: naïve T cells (CCR7+CD45RA+), central memory T cells (CCR7+CD45RA-), effector memory T cells (CCR7-CD45RA-), and most differentiated effector memory T cells (T-EMRA, CCR7-CD45RA+).

19. Figures 3A, 3B, 3C, 3D, and 3E show that ibrutinib treatment of human T cells or NK cells protects against activation induced cell death in a dose dependent manor. Figures 3A, 3B, and 3C show that T cells were isolated from healthy human donors blood samples, stimulated in vitro with CD3/CD28 for 3 days, rested in culture medium containing 50 IU IL-2 for 11 days, then were restimulated with plate bound CD3 for 6 hours (as in 3A. and 3B.) or 3 hours (as in 3C.) in the presence of IL2 to induce activation induced cell death in the presence of absence of ibrutinib. Each indicated condition. Figure 3A shows a representative FACS dot plots of Annexin V & PI staining. Figure 3B shows a Bar graphs that show the percentage of non-viable was done in triplicate. Data shown are representative of three independent experiments (apoptotic + necrotic, as defined by Annexin V positive and PI positive cells) cells after induction of AICD. Figure 3C shows a FAS-L mRNA upregulation in activated T cells upon induction of AICD was impaired by ibrutinib treatment. mRNA was isolated from the T cells after induction of AICD, cDNA was synthesized and qPCR for FAS-L and GAPDH was done. Figure A, B and C represent 3 independent experiments. Figures 3D and 3E show that Human CD56+/CD3-/14-/20- NK cells were isolated from peripheral blood from normal donors (N=3)

by negative enrichment, and were then sorted to greater than 99% purity by FACS Aria II sorter. Purified NK cells were plated at 5×10^4 cells/well and were cultured for three days. IL-15 (3D) and IL-2 (3E) were added as indicated for a final concentration of 10ng/mL. IL-12 (Miltenyi Biotec) was added where indicated at a concentration of 10ng/mL to induce activation induced cell death. Bar graphs that show the percentage of non-viable (apoptotic + necrotic, as defined by Annexin V positive and TO-PRO-3 positive cells) cells after induction of AICD. (N=3)

20. Figures 4A, 4B, and 4C show that ibrutinib treatment does not affect total numbers of stem memory T cells: Figure 4A shows the gating strategy for stem memory T cells. Naïve CD4 T cells (CCR7+CD45RA+) expressing CD62L were selected for further analysis. Of these, CD95+CD122hi cells were defined as stem memory T cells. Figure 4B left shows representative plots showing stem memory T cells in samples from a healthy donor and a CLL patient. Figure 4B right shows representative plots showing Tbet and Eomesodermin expression in naïve CD8 and CD4 T cells from a healthy donor and a CLL patient. Figure 4C shows stem memory T cells before and during ibrutinib treatment. Left: graphs showing absolute numbers (top) and percentages (bottom) of stem memory cells (n=15). Right: representative plots.

21. Figures 5A and 5B show that treatment with BTK inhibitors significantly reduces PD-1 expressing cells in CD8 T cell populations: Percentages of PD1+ cells among different subsets of CD8 T cells from CLL patients before and during treatment with (5A) ibrutinib (n=17) or (5B) acalabrutinib (n=10).

22. Figures 6A and 6B show that treatment with ibrutinib, as well as with acalabrutinib, leads to a significant reduction in the frequency of PD-1 positive cells in CD4 T cell populations. Figure 6A shows the percentage of PD1 positive cells among different subsets of CD4 T cells from CLL patients before and after ibrutinib treatment. (n=17) Figure 6B shows the percentage of PD1 positive cells among different subsets of CD4 T cells from CLL patients before and after acalabrutinib treatment. (n=10)

23. Figures 7A and 7B show that treatment with BTK inhibitors significantly reduces the frequency of T cells expressing intracellular CTLA4: Percentages of cells positive for intracellular CTLA4 expression among total CD4 T cells, CD45RA- CD4 T cells, and CD45RA+ CD4 T cells from CLL patients before and during treatment with (7A) ibrutinib (n=18) and (7B) acalabrutinib (n=9). Data for CD8 T cells are shown in Figure 8.

24. Figures 8A and 8B show that treatment with ibrutinib, as well as with acalabrutinib, leads to a significant reduction in the frequency of intracellular CTLA4 positive cells in CD8 T cell populations. Figure 8A shows the percentage of CTLA4 (intracellular) positive cells among total CD8 T cells, CD45RA- CD8 T cells and CD45RA+ CD8 T cells from CLL patients before

and after ibrutinib treatment.(n=18). Figure 8B shows the percentage of CTLA4 (intracellular) positive cells among total CD8 T cells, CD45RA- CD8 T cells and CD45RA+ CD8 T cells from CLL patients before and after acalabrutinib treatment.(n=9).

25. Figures 9A and 9B show that ibrutinib treatment does not affect T cell production of Th1 or Th2 cytokines but moderately increases frequency of Th17 cells: PBMC from CLL patients before and during treatment with (9A) ibrutinib (n=15) or (9B) acalabrutinib (n=11) were stimulated with PMA/ionomycin for 5 hours. Production of IFN γ , TNF, IL2, IL4, and IL17 was detected by intracellular cytokine staining. Percentages of cytokine-producing cells are shown.

26. Figures 10A, 10B, and 10C show that Ibrutinib treatment of CLL patients leads to a reduced frequency, but not reduced absolute number, of CD4+CD25+ Foxp3+ Treg cells: Fgiure 10A shows representative plots showing CD25 and intranuclear Foxp3 staining in CD4+CD3+ (upper panel) and CD8+CD3+ (lower panel) T cells. Figures 10B and 10C show the percentages (left) and absolute numbers (right) of CD25+ Foxp3+ regulatory T cells among total CD4 T cells before and during treatment with (10B) ibrutinib (n=18) or (10C) acalabrutinib (n=11).

27. Figures 11A and 11B show that ibrutinib and acalabrutinib treatment of CLL patients reduces CD200 and BTLA expression in CLL cells: Left panels: CD200 expression level as measured in mean fluorescence intensity (MFI) on CLL cells (CD19+CD5+) before and during treatment with (11A) ibrutinib (n=18) or (11B) acalabrutinib (n=12). Right panels: BTLA expression level as measured in mean fluorescence intensity (MFI) on CLL cells before and during treatment with (11A) ibrutinib (n=16) or (11B) acalabrutinib (n=12).

28. Figures 12A, 12B, and 12C show the ability of CLL cells to produce IL-10 is impaired with BTK inhibitor treatment: PBMC from CLL patients were collected before and during treatment with ibrutinib (12A, 12B, 12C). Cells were stimulated *in-vitro* with CpG and PMA/ionomycin for 5 hours (B10 conditions) or with CpG/CD40L for 48 hours, with PMA/ionomycin added for the last 5 hours (B10Pro conditions). IL-10 production was detected by intracellular cytokine staining. All events were gated on CLL cells (CD19+CD5+CD3-). Figure 12A shows representative flow cytometry plots of IL-10 expression in CLL cells under B10 conditions (top) and B10Pro conditions (bottom). Figure 12B shows CLL samples were collected at baseline and at the beginning of cycles 3 and 6 (8 and 20 weeks, respectively) after starting ibrutinib treatment. Graphs show the percentages of IL-10 producing CLL cells after *in-vitro* incubation under B10 conditions (left; n=13) and B10Pro conditions (right; n=18). Figure 12C shows that PBMC were collected from CLL patients at baseline and at the beginning of cycles 3 and 6 (8 and 20 weeks, respectively) after starting acalabrutinib treatment. Graphs show

the percentages of IL-10 producing CLL cells after *in-vitro* incubation under B10 conditions (left; n=10) and B10Pro conditions (right; n=12).

29. Figure 13 shows that ibrutinib treatment increases the number of activated leukemia specific T cells. Mice were engrafted with AML cell line (C1498) expressing OVA (a model antigen). OT-1 transgenic T cells (recognize OVA) were then adoptively transferred into AML engrafted mice. The mice were treated with Ibrutinib versus vehicle. Mice were sacrificed at day 6, spleens were harvested, the frequency and number of leukemia specific OT-1 T cells were counted and plotted. N=7 for each group.

30. Figure 14 shows that PD-1 expression is increased in all of the T cell subsets in CLL patients comparing to healthy donors. The increase is most prominent in naïve and central memory T cell compartment. Frequencies of PD-1 positive cells among different CD8 T cell subsets were shown. n=11 for healthy donor, n=15 for CLL patients.

31. Figure 15 shows that ibrutinib treatment in CLL patients improves their T cells' capability to mediated cytotoxicity against autologous CLL cells in the presence of blinatumammab. CLL patient's T cells (collected pre-ibrutinib vs. post-ibrutinib treatment) were mixed with autologous CLL cells (pre-ibrutinib) at 4:1 ratio and cultured overnight with blinatumomab. Percentage of live CLL cells at the end of the assay were shown in the left lower corner of each plot. Data shown are representative of three independent experiments.

32. Figure 16 shows that ibrutinib rescues CLL patient's T cells from AICD triggered by blinatumomab. CLL patient's T cells (collected pre-ibrutinib vs. post-ibrutinib treatment) were mixed with autologous CLL cells (pre-ibrutinib) at 4:1 ratio and cultured overnight with blinatumomab. Upper panel shows the number of viable CLL cells at the end of the assay. Lower panel shows the number of the viable T cells at the end of the assay.

IV. DETAILED DESCRIPTION

33. Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that they are not limited to specific synthetic methods or specific recombinant biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

A. Definitions

34. As used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for

example, reference to “a pharmaceutical carrier” includes mixtures of two or more such carriers, and the like.

35. Ranges can be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as “about” that particular value in addition to the value itself. For example, if the value “10” is disclosed, then “about 10” is also disclosed. It is also understood that when a value is disclosed that “less than or equal to” the value, “greater than or equal to the value” and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value “10” is disclosed the “less than or equal to 10” as well as “greater than or equal to 10” is also disclosed. It is also understood that throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point “10” and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

36. In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

37. “Optional” or “optionally” means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

38. The term “subject” refers to any individual who is the target of administration or treatment. The subject can be a vertebrate, for example, a mammal. In one aspect, the subject can be human, non-human primate, bovine, equine, porcine, canine, or feline. The subject can also be a guinea pig, rat, hamster, rabbit, mouse, or mole. Thus, the subject can be a human or veterinary patient. The term “patient” refers to a subject under the treatment of a clinician, e.g., physician.

39. The term “therapeutically effective” refers to the amount of the composition used is of sufficient quantity to ameliorate one or more causes or symptoms of a disease or disorder. Such amelioration only requires a reduction or alteration, not necessarily elimination.

40. The term “treatment” refers to the medical management of a patient with the intent to cure, ameliorate, stabilize, or prevent a disease, 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; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated 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.

41. A “decrease” can refer to any change that results in a smaller amount of a symptom, disease, composition, condition, or activity. A substance is also understood to decrease the genetic output of a gene when the genetic output of the gene product with the substance is less relative to the output of the gene product without the substance. Also for example, a decrease can be a change in the symptoms of a disorder such that the symptoms are less than previously observed. A decrease can be any individual, median, or average decrease in a condition, symptom, activity, composition in a statistically significant amount. Thus, the decrease can be a 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100% decrease so long as the decrease is statistically significant.

42. “Inhibit,” “inhibiting,” and “inhibition” mean to decrease an activity, response, condition, disease, or other biological parameter. This can include but is not limited to the complete ablation of the activity, response, condition, or disease. This may also include, for example, a 10% reduction in the activity, response, condition, or disease as compared to the native or control level. Thus, the reduction can be a 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, or any amount of reduction in between as compared to native or control levels.

43. An “increase” can refer to any change that results in a greater amount of a symptom, disease, composition, condition or activity. An increase can be any individual, median, or average increase in a condition, symptom, activity, composition in a statistically significant amount. Thus, the increase can be a 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100% increase so long as the increase is statistically significant.

44. Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this pertains. The references disclosed are also individually and specifically incorporated by reference herein for the material contained in them that is discussed in the sentence in which the reference is relied upon.

45. Immune cell therapy (such as NK cell therapy or T cell therapies including chimeric antigen receptor (CAR) T cell therapy and transfer of tumor infiltrating lymphocytes (TIL), or marrow-infiltrating lymphocyte (MIL)) has significant potential as a cancer therapy because immune cells can expand in large numbers to eradicate high volume disease, can traffic throughout disparate areas of the body to eradicate residual tumor sites, and can endow patients with long-lived tumor immunity. However, major disadvantages that limit the utility of adoptive immune cell therapy include the MHC restriction of antigen presentation to T cell receptors (TCR), MHC downregulation as a mechanism of immune escape, and the lengthy production time required to create a sufficient number of immune cells (including NK cells and tumor-specific T cells).

46. T-cells isolated from CLL patients prior to starting ibrutinib expand *ex vivo* chimeric antigen receptor (CAR) T-cells poorly, whereas those derived during treatment expand significantly better. Moreover, addition of ibrutinib to anti-CD19 CAR T Cells improves responses against mantle cell lymphoma. Furthermore, it has been reported that in mouse lymphoma models, ibrutinib enhances T cell-dependent antitumor immune responses and further potentiates the efficacy of immune checkpoint blockade. Immune modulatory effects have been preliminarily reported with the BTK inhibitor acalabrutinib (ACP-196), which also demonstrates promising clinical activity in CLL. However, unlike ibrutinib, acalabrutinib lacks inhibitory activity against the BTK-related kinase ITK. Clinically, this raises the question of whether more selective BTK inhibition will promote effective immune modulation and avoid the off-target effects observed with ibrutinib.

47. In one aspect, disclosed are methods and compositions related to expanding T cell and NK cell populations by contacting said cells with an interleukin-2 inducible T cell inhibitor. Here, the effects of ibrutinib on T-cells were comprehensively studied *in-vivo* and the ability of this agent to modulate the immune suppressive capacity of CLL cells, and compare the results to those achieved with acalabrutinib. The results indicate that while both agents diminish tumor-mediated immune suppressive molecules, ibrutinib has unique immune modulating capability in promoting expansion of chronically activated T-cells by diminishing activation-induced cell death. However, this expansion was not extended to Treg (CD25+Foxp3+) cells. Thus, in one

aspect, disclosed herein are methods of selectively expanding CD8 and CD4 T cells (such as, for example, effector memory (CD45RA-CCR7-) T cells or CD45RA+CCR7- T cells), but not CD25+Foxp3+ T cells in a subject comprising administering to a subject an agent that inhibits interleukin-2 inducible T cell kinase (ITK) (such as, for example ibrutinib).

48. It is understood and herein contemplated that the immune cells can be expanded in the presence of the ITK inhibitor for any amount of time sufficient to generate a therapeutically effective amount of immune cells for the adoptive immune cell therapy. Thus, in one aspect, disclosed herein are methods of expanding immune cells (such as, for example CD4 T cells, CD8 T cells, and/or NK cells) wherein the immune cells are incubated with the ITK inhibitor for at least 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 28, 30, 32, 34, 36, 48 hours, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days.

49. In some aspect, it is understood that the expansion of immune cells can occur ex vivo, in situ, or in vitro. However, the effect of the ITK inhibitor on immune cells can also occur in vivo. Accordingly, disclosed herein are methods of expanding immune cells (such as, for example CD4 T cells, CD8 T cells, and/or NK cells) wherein the ITK inhibitor (such as, for example, ibrutinib) is administered to the subject. This method can not only expand endogenous CD4, CD8, and/or NK cells, but will also work on endogenous TILs, MILs, or adoptively transferred TILs, MILs, and/or CAR T cells. Thus, in one aspect, disclosed herein are methods of increasing the number of tumor infiltrating lymphocytes in a subject with a tumor comprising administering to the subject an agent that inhibits interleukin-2 inducible T cell kinase (ITK).

50. Due to the effect ITK inhibitors (including, but not limited to broadly reactive BTK inhibitors that are also ITK inhibitors) have on expanding T cells and NK cells, the ITK inhibitor (such as, for example, ibrutinib) can be used to maximize cells being used for immune cell therapy for a cancer. Accordingly, disclosed herein are methods for expanding an immune cell (such as, for example, a chimeric antigen receptor (CAR) T cell, tumor infiltrating lymphocyte (TIL), or marrow-infiltrating lymphocyte (MIL), natural killer (NK) cell, NK-T cell, a cytokine-induced memory NK cell, or a cytokine-induced killer (CIK) cell) isolated from a subject for use in immune therapy, comprising contacting the isolated immune cell with an effective amount of interleukin-2 inducible T cell kinase (ITK) inhibitor (such as, for example, ibrutinib) to expand the immune cell in an amount effective for immunotherapy; and culturing the isolated immune cells in the presence of the ITK inhibitor (for example, culturing the cells in the presence of the ITK inhibitor).

51. As disclosed herein, ibrutinib is an ITK inhibitor and an inhibitor of Bruton's tyrosine kinase (BTK). Thus, in one aspect, also disclosed are methods wherein the agent

further inhibits Bruton's tyrosine kinase (BTK) (such as, for example ibrutinib). However, it is understood and herein contemplated that while ibrutinib inhibits both BTK and ITK, mere inhibition of BTK alone is not sufficient to perform the methods disclosed herein. For example, acalabrutinib (a BTK inhibitor) does not expand T cells.

5 52. While all T cells populations (naïve, effector, central memory, effector memory, and CD45RA⁺ effector memory cells) expanded herein upon contact with an ITK (such as, for example ibrutinib), expansion of effector memory T cells (T-EM) and CD45RA⁺ effector memory T cell (T-EMRA) populations saw greater expansion. Thus, in one aspect, disclosed herein are methods of any preceding aspect, wherein the method of claim 1, wherein the
10 expanded T cells are effector memory (CD45RA-CCR7⁻) T cells (T-EM) or CD45RA⁺CCR7⁻ T cells (T-EMRA).

 53. Beyond the expansion of T cell populations, it is shown herein that ITK inhibitors can also have an effect on the cytotoxicity and survival of T cells. Thus, in one aspect, disclosed herein are methods of increasing the cytotoxicity and survival of T cells to a tumor cell (such as,
15 for example an autologous tumor) comprising contacting CD4 and/or CD8 T cells with an ITK inhibitor (such as, for example ibrutinib). As the effect of the ITK inhibitor is not limited on the environment, the contact of the ITK inhibitor with the target T cell can occur in vitro or ex vivo (such as to effect T cells that would be adoptively transferred to subject for a cancer treatment) and/or in vivo (to increase the cytotoxicity and/or survival of endogenous T cells or TILs, MILs,
20 or CAR T cells that have previously been transferred to a subject or are being transferred concurrent with or following administration of the ITK inhibitor). Such methods can further comprise the administration of blinatumomab.

 54. In addition to the effect an ITK inhibitor can have on the cytotoxicity and survival of T cells, the ITK inhibitor (such as, for example, ibrutinib) can also polarize the T cell response
25 to a Th17 response. Studies have shown that increased Th17 cell numbers correlate with improved overall survival. Accordingly, in one aspect, disclosed herein are method of increasing the percentage of Th17 T cells comprising contacting a T cell population with an ITK inhibitor.

 55. As shown herein, the relative expansion of T cells following administration of
30 ibrutinib was more a consequence of decreased susceptibility to activation induced cell death (AICD) rather than in increased rate of expanding cells. This reduced susceptibility was also observed in cytokine activated NK cells which are prone to AICD in ex vivo expansion. Accordingly, in one aspect, disclosed herein are methods of inhibiting activation induced cell

death of T cells and/or NK cells in a subject with chronic lymphocytic leukemia comprising administering to the subject an inhibitor of interleukin-2 inducible T cell kinase (ITK).

56. As noted herein, the ability to reduce AICD in NK cells has important implications to NK cell therapy for cancer. In such treatment methods NK cells are typically expanded with cytokine activation, but the expanded NK cells are susceptible to AICD during expansion and after transfer to a subject to be treated. Administration of an ITK (such as, for example ibrutinib) can reduce AICD of the expanded NK cells making more available for treatment. Thus, in one aspect, disclosed herein are methods of expanding NK cells comprising contacting NK cells with a stimulatory molecule and ibrutinib; wherein the stimulatory molecule is a cytokine (such as, for example IL-15, IL-21, IL-2, 41BBL, IL-12, IL-18, MICA, 2B4, LFA-1, and BCM1/SLAMF2).

57. Because the reduced AICD on expanded NK cells has a role in NK cell therapy of cancer. Also disclosed herein are methods of treating cancer in a subject comprising administering to a subject NK cell therapy, wherein the NK cell therapy comprises expanding NK cells (including, but not limited to natural killer (NK) cell, NK-T cell, a cytokine-induced memory NK cell, or a cytokine-induced killer (CIK) cell) by stimulating NK cells with a stimulatory molecule ibrutinib; wherein the stimulatory molecule is a cytokine (such as, for example IL-15, IL-21, IL-2, 41BBL, IL-12, IL-18, MICA, 2B4, LFA-1, and BCM1/SLAMF2); and administering to the subject the expanded NK cell population.

58. One problem affecting most immune cell therapies to cancer is the cancer cells own defense in the way of upregulating the expression of molecules that turn off the host immune response. Programmed death-1 (PD-1) is an immune inhibitory co-receptor expressed on a variety of immune cells such as T cells, B cells and natural killer cells. When bound by its ligands, PD-L1/PD-L2, PD-1 functions by inhibiting an activated T cell response. Tumor cells up-regulate PD-L1 in response to inflammation thereby suppressing an anti-tumor immune response. Similar effects occur via CTLA-4 and CD200. Thus, in a normal situation immunosuppressive ligands such as PD-1, CD200, and CTLA-4 serve as checkpoint inhibitors to reduce the immune response. When turned on by a cancer cell, the immune response to the cancer can be thwarted. As shown herein, ibrutinib treatment can reduce the percentage of PD-1 positive CD4 and CD8 T cells as well as CTLA-4 CD4 and CD8 T cells. Thus, in one aspect, disclosed herein are methods of reducing checkpoint inhibition in a subject comprising administering to a subject an ITK inhibitor (such as, for example, ibrutinib). It is understood and herein contemplated that the disclosed methods of reducing checkpoint inhibition (by reducing the number of PD-1 positive, CD200 positive, and/or CTLA-4 positive CD4 and/or CD8 T cells)

can be used to augment any cancer immune cell therapy, including, but not limited to direct application to the subject for in vivo applications or used in conjunction with MIL, TIL, or CAR T cell therapy and thus, can also be applied to T cells ex vivo or in vitro. It is understood that the disclosed methods of reducing checkpoint blockade can further comprise the administration of any known immune checkpoint inhibitor, such as for example, a PD-1 inhibitor, a PD-L1 inhibitor, or CTLA-4 inhibitor (such as, for example, nivolumab, pembrolizumab, pidilizumab, BMS-936559, Atezolizumab, Durvalumab, or Avelumab).

59. Because the adverse effects of AICD on NK cell therapy and/or T cell therapy can also occur in vivo after transfer of expanded cells to the subject with a cancer, and because the effect of ITK (such as ibrutinib) can equally be applicable in vivo as ex vivo, disclosed herein are methods of treating cancer of any preceding aspect, further comprising administering to the subject ibrutinib prior to, concurrent with, or after administration of the expanded NK cells or T cells to the subject.

60. It is understood and herein contemplated that the disclosed methods can be used to treat any disease where uncontrolled cellular proliferation occurs such as cancers. A representative but non-limiting list of cancers that the disclosed compositions can be used to treat is the following: leukemia (including, but not limited to, acute myeloid leukemia (AML), chronic myeloid leukemia, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), lymphoma; B cell lymphoma,; T cell lymphoma, mantle cell lymphoma, mycosis fungoides; Hodgkin's Disease; leukemias, including but not limited to myeloid leukemia; plasmacytomas; histiocytomas; bladder cancer; brain cancer, nervous system cancer, head and neck cancer, squamous cell carcinoma of head and neck, urothelial cancer, kidney cancer, lung cancers such as small cell lung cancer and non-small cell lung cancer, neuroblastoma, glioblastoma, ovarian cancer, pancreatic cancer, prostate cancer, skin cancer, liver cancer, melanoma, squamous cell carcinomas of the mouth, throat, larynx, and lung; colon cancer; cervical cancer; cervical carcinoma; breast cancer; epithelial cancer; renal cancer, genitourinary cancer; pulmonary cancer; esophageal carcinoma; head and neck carcinoma; large bowel cancer; hematopoietic cancers; testicular cancer; colon and rectal cancers; prostatic cancer; AIDS-related lymphomas or sarcomas, metastatic cancers, or cancers in general; or pancreatic cancer.

61. It is further understood and herein contemplated that the disclosed methods of expanding NK and/or T cells can be used as one aspect of treatment of a cancer (such as, for example, CLL) and can be used in conjunction with additional anti-cancer agents. Anti-cancer agents that can be used in the disclosed methods can comprise any anti-cancer agent known in the art, the including, but not limited to Abemaciclib, Abiraterone Acetate, Abitrexate

(Methotrexate), Abraxane (Paclitaxel Albumin-stabilized Nanoparticle Formulation), ABVD, ABVE, ABVE-PC, AC, AC-T, Adcetris (Brentuximab Vedotin), ADE, Ado-Trastuzumab Emtansine, Adriamycin (Doxorubicin Hydrochloride), Afatinib Dimaleate, Afinitor (Everolimus), Akynzeo (Netupitant and Palonosetron Hydrochloride), Aldara (Imiquimod),

5 Aldesleukin, Alecensa (Alectinib), Alectinib, Alemtuzumab, Alimta (Pemetrexed Disodium), Aliqopa (Copanlisib Hydrochloride), Alkeran for Injection (Melphalan Hydrochloride), Alkeran Tablets (Melphalan), Aloxi (Palonosetron Hydrochloride), Alunbrig (Brigatinib), Ambochlorin (Chlorambucil), Amboclorin Chlorambucil), Amifostine, Aminolevulinic Acid, Anastrozole, Aprepitant, Aredia (Pamidronate Disodium), Arimidex (Anastrozole), Aromasin

10 (Exemestane), Arranon (Nelarabine), Arsenic Trioxide, Arzerra (Ofatumumab), Asparaginase Erwinia chrysanthemi, Atezolizumab, Avastin (Bevacizumab), Avelumab, Axitinib, Azacitidine, Bavencio (Avelumab), BEACOPP, Becenum (Carmustine), Beleodaq (Belinostat), Belinostat, Bendamustine Hydrochloride, BEP, Besponsa (Inotuzumab Ozogamicin), Bevacizumab, Bexarotene, Bexxar (Tositumomab and Iodine I 131 Tositumomab), Bicalutamide, BiCNU

15 (Carmustine), Bleomycin, Blinatumomab, Blinicyto (Blinatumomab), Bortezomib, Bosulif (Bosutinib), Bosutinib, Brentuximab Vedotin, Brigatinib, BuMel, Busulfan, Busulfex (Busulfan), Cabazitaxel, Cabometyx (Cabozantinib-S-Malate), Cabozantinib-S-Malate, CAF, Campath (Alemtuzumab), Camptosar, (Irinotecan Hydrochloride), Capecitabine, CAPOX, Carac (Fluorouracil--Topical), Carboplatin, CARBOPLATIN-TAXOL, Carfilzomib, Carmubris

20 (Carmustine), Carmustine, Carmustine Implant, Casodex (Bicalutamide), CEM, Ceritinib, Cerubidine (Daunorubicin Hydrochloride), Cervarix (Recombinant HPV Bivalent Vaccine), Cetuximab, CEV, Chlorambucil, CHLORAMBUCIL-PREDNISONE, CHOP, Cisplatin, Cladribine, Clafen (Cyclophosphamide), Clofarabine, Clofarex (Clofarabine), Clolar (Clofarabine), CMF, Cobimetinib, Cometriq (Cabozantinib-S-Malate), Copanlisib

25 Hydrochloride, COPDAC, COPP, COPP-ABV, Cosmegen (Dactinomycin), Cotellic (Cobimetinib), Crizotinib, CVP, Cyclophosphamide, Cyfos (Ifosfamide), Cyramza (Ramucirumab), Cytarabine, Cytarabine Liposome, Cytosar-U (Cytarabine), Cytosan (Cyclophosphamide), Dabrafenib, Dacarbazine, Dacogen (Decitabine), Dactinomycin, Daratumumab, Darzalex (Daratumumab), Dasatinib, Daunorubicin Hydrochloride,

30 Daunorubicin Hydrochloride and Cytarabine Liposome, Decitabine, Defibrotide Sodium, Defitelio (Defibrotide Sodium), Degarelix, Denileukin Diftitox, Denosumab, DepoCyt (Cytarabine Liposome), Dexamethasone, Dexrazoxane Hydrochloride, Dinutuximab, Docetaxel, Doxil (Doxorubicin Hydrochloride Liposome), Doxorubicin Hydrochloride, Doxorubicin Hydrochloride Liposome, Dox-SL (Doxorubicin Hydrochloride Liposome), DTIC-Dome

(Dacarbazine), Durvalumab, Efudex (Fluorouracil--Topical), Elitek (Rasburicase), Ellence (Epirubicin Hydrochloride), Elotuzumab, Eloxatin (Oxaliplatin), Eltrombopag Olamine, Emend (Aprepitant), Empliciti (Elotuzumab), Enasidenib Mesylate, Enzalutamide, Epirubicin Hydrochloride, EPOCH, Erbitux (Cetuximab), Eribulin Mesylate, Erivedge (Vismodegib),

5 Erlotinib Hydrochloride, Erwinaze (Asparaginase *Erwinia chrysanthemi*), Ethyol (Amifostine), Etopophos (Etoposide Phosphate), Etoposide, Etoposide Phosphate, Evacet (Doxorubicin Hydrochloride Liposome), Everolimus, Evista, (Raloxifene Hydrochloride), Evomela (Melphalan Hydrochloride), Exemestane, 5-FU (Fluorouracil Injection), 5-FU (Fluorouracil--Topical), Fareston (Toremifene), Farydak (Panobinostat), Faslodex (Fulvestrant), FEC, Femara

10 (Letrozole), Filgrastim, Fludara (Fludarabine Phosphate), Fludarabine Phosphate, Fluoroplex (Fluorouracil--Topical), Fluorouracil Injection, Fluorouracil--Topical, Flutamide, Folex (Methotrexate), Folex PFS (Methotrexate), FOLFIRI, FOLFIRI-BEVACIZUMAB, FOLFIRI-CETUXIMAB, FOLFIRINOX, FOLFOX, Folutyn (Pralatrexate), FU-LV, Fulvestrant, Gardasil (Recombinant HPV Quadrivalent Vaccine), Gardasil 9 (Recombinant HPV Nonavalent

15 Vaccine), Gazyva (Obinutuzumab), Gefitinib, Gemcitabine Hydrochloride, GEMCITABINE-CISPLATIN, GEMCITABINE-OXALIPLATIN, Gemtuzumab Ozogamicin, Gemzar (Gemcitabine Hydrochloride), Gilotrif (Afatinib Dimaleate), Gleevec (Imatinib Mesylate), Gliadel (Carmustine Implant), Gliadel wafer (Carmustine Implant), Glucarpidase, Goserelin Acetate, Halaven (Eribulin Mesylate), Hemangeol (Propranolol Hydrochloride), Herceptin

20 (Trastuzumab), HPV Bivalent Vaccine, Recombinant, HPV Nonavalent Vaccine, Recombinant, HPV Quadrivalent Vaccine, Recombinant, Hycamtin (Topotecan Hydrochloride), Hydrea (Hydroxyurea), Hydroxyurea, Hyper-CVAD, Ibrance (Palbociclib), Ibritumomab Tiuxetan, Ibrutinib, ICE, Iclusig (Ponatinib Hydrochloride), Idamycin (Idarubicin Hydrochloride), Idarubicin Hydrochloride, Idelalisib, Idhifa (Enasidenib Mesylate), Ifex (Ifosfamide),

25 Ifosfamide, Ifosfamidum (Ifosfamide), IL-2 (Aldesleukin), Imatinib Mesylate, Imbruvica (Ibrutinib), Imfinzi (Durvalumab), Imiquimod, Imlygic (Talimogene Laherparepvec), Inlyta (Axitinib), Inotuzumab Ozogamicin, Interferon Alfa-2b, Recombinant, Interleukin-2 (Aldesleukin), Intron A (Recombinant Interferon Alfa-2b), Iodine I 131 Tositumomab and Tositumomab, Ipilimumab, Iressa (Gefitinib), Irinotecan Hydrochloride, Irinotecan

30 Hydrochloride Liposome, Istodax (Romidepsin), Ixabepilone, Ixazomib Citrate, Ixempra (Ixabepilone), Jakafi (Ruxolitinib Phosphate), JEB, Jevtana (Cabazitaxel), Kadcylla (Ado-Trastuzumab Emtansine), Keoxifene (Raloxifene Hydrochloride), Kepivance (Palifermin), Keytruda (Pembrolizumab), Kisqali (Ribociclib), Kymriah (Tisagenlecleucel), Kyprolis (Carfilzomib), Lanreotide Acetate, Lapatinib Ditosylate, Lartruvo (Olaratumab), Lenalidomide,

Lenvatinib Mesylate, Lenvima (Lenvatinib Mesylate), Letrozole, Leucovorin Calcium, Leukeran (Chlorambucil), Leuprolide Acetate, Leustatin (Cladribine), Levulan (Aminolevulinic Acid), Linfolizin (Chlorambucil), LipoDox (Doxorubicin Hydrochloride Liposome), Lomustine, Lonsurf (Trifluridine and Tipiracil Hydrochloride), Lupron (Leuprolide Acetate), Lupron Depot (Leuprolide Acetate), Lupron Depot-Ped (Leuprolide Acetate), Lynparza (Olaparib), Marqibo (Vincristine Sulfate Liposome), Matulane (Procarbazine Hydrochloride), Mechlorethamine Hydrochloride, Megestrol Acetate, Mekinist (Trametinib), Melphalan, Melphalan Hydrochloride, Mercaptopurine, Mesna, Mesnex (Mesna), Methazolastone (Temozolomide), Methotrexate, Methotrexate LPF (Methotrexate), Methylnaltrexone Bromide, Mexate (Methotrexate), Mexate-AQ (Methotrexate), Midostaurin, Mitomycin C, Mitoxantrone Hydrochloride, Mitozytrex (Mitomycin C), MOPP, Mozobil (Plerixafor), Mustargen (Mechlorethamine Hydrochloride), Mutamycin (Mitomycin C), Myleran (Busulfan), Mylosar (Azacitidine), Mylotarg (Gemtuzumab Ozogamicin), Nanoparticle Paclitaxel (Paclitaxel Albumin-stabilized Nanoparticle Formulation), Navelbine (Vinorelbine Tartrate), Necitumumab, Nelarabine, Neosar (Cyclophosphamide), Neratinib Maleate, Nerlynx (Neratinib Maleate), Netupitant and Palonosetron Hydrochloride, Neulasta (Pegfilgrastim), Neupogen (Filgrastim), Nexavar (Sorafenib Tosylate), Nilandron (Nilutamide), Nilotinib, Nilutamide, Ninlaro (Ixazomib Citrate), Niraparib Tosylate Monohydrate, Nivolumab, Nolvadex (Tamoxifen Citrate), Nplate (Romiplostim), Obinutuzumab, Odomzo (Sonidegib), OEPA, Ofatumumab, OFF, Olaparib, Olaratumab, Omacetaxine Mepesuccinate, Oncaspar (Pegaspargase), Ondansetron Hydrochloride, Onivyde (Irinotecan Hydrochloride Liposome), Ontak (Denileukin Diftitox), Opdivo (Nivolumab), OPPA, Osimertinib, Oxaliplatin, Paclitaxel, Paclitaxel Albumin-stabilized Nanoparticle Formulation, PAD, Palbociclib, Palifermin, Palonosetron Hydrochloride, Palonosetron Hydrochloride and Netupitant, Pamidronate Disodium, Panitumumab, Panobinostat, Paraplat (Carboplatin), Paraplatin (Carboplatin), Pazopanib Hydrochloride, PCV, PEB, Pegaspargase, Pegfilgrastim, Peginterferon Alfa-2b, PEG-Intron (Peginterferon Alfa-2b), Pembrolizumab, Pemetrexed Disodium, Perjeta (Pertuzumab), Pertuzumab, Platinol (Cisplatin), Platinol-AQ (Cisplatin), Plerixafor, Pomalidomide, Pomalyst (Pomalidomide), Ponatinib Hydrochloride, Portrazza (Necitumumab), Pralatrexate, Prednisone, Procarbazine Hydrochloride, Proleukin (Aldesleukin), Prolia (Denosumab), Promacta (Eltrombopag Olamine), Propranolol Hydrochloride, Provenge (Sipuleucel-T), Purinethol (Mercaptopurine), Purixan (Mercaptopurine), Radium 223 Dichloride, Raloxifene Hydrochloride, Ramucirumab, Rasburicase, R-CHOP, R-CVP, Recombinant Human Papillomavirus (HPV) Bivalent Vaccine, Recombinant Human Papillomavirus (HPV) Nonavalent Vaccine, Recombinant Human

Papillomavirus (HPV) Quadrivalent Vaccine, Recombinant Interferon Alfa-2b, Regorafenib, Relistor (Methylnaltrexone Bromide), R-EPOCH, Revlimid (Lenalidomide), Rheumatrex (Methotrexate), Ribociclib, R-ICE, Rituxan (Rituximab), Rituxan Hycela (Rituximab and Hyaluronidase Human), Rituximab, Rituximab and , Hyaluronidase Human, , Rolapitant

5 Hydrochloride, Romidepsin, Romiplostim, Rubidomycin (Daunorubicin Hydrochloride), Rubraca (Rucaparib Camsylate), Rucaparib Camsylate, Ruxolitinib Phosphate, Rydapt (Midostaurin), Sclerosol Intrapleural Aerosol (Talc), Siltuximab, Sipuleucel-T, Somatuline Depot (Lanreotide Acetate), Sonidegib, Sorafenib Tosylate, Sprycel (Dasatinib), STANFORD V, Sterile Talc Powder (Talc), Steritalc (Talc), Stivarga (Regorafenib), Sunitinib Malate, Sutent

10 (Sunitinib Malate), Sylatron (Peginterferon Alfa-2b), Sylvant (Siltuximab), Synribo (Omacetaxine Mepesuccinate), Tabloid (Thioguanine), TAC, Tafenlar (Dabrafenib), Tagrisso (Osimertinib), Talc, Talimogene Laherparepvec, Tamoxifen Citrate, Tarabine PFS (Cytarabine), Tarceva (Erlotinib Hydrochloride), Targretin (Bexarotene), Tassigna (Nilotinib), Taxol (Paclitaxel), Taxotere (Docetaxel), Tecentriq , (Atezolizumab), Temodar (Temozolomide),

15 Temozolomide, Temsirolimus, Thalidomide, Thalomid (Thalidomide), Thioguanine, Thiotepe, Tisagenlecleucel, Tolak (Fluorouracil--Topical), Topotecan Hydrochloride, Toremifene, Torisel (Temsirrolimus), Tositumomab and Iodine I 131 Tositumomab, Totect (Dexrazoxane Hydrochloride), TPF, Trabectedin, Trametinib, Trastuzumab, Treanda (Bendamustine Hydrochloride), Trifluridine and Tipiracil Hydrochloride, Trisenox (Arsenic Trioxide), Tykerb

20 (Lapatinib Ditosylate), Unituxin (Dinutuximab), Uridine Triacetate, VAC, Vandetanib, VAMP, Varubi (Rolapitant Hydrochloride), Vectibix (Panitumumab), VeIP, Velban (Vinblastine Sulfate), Velcade (Bortezomib), Velsar (Vinblastine Sulfate), Vemurafenib, Venclexta (Venetoclax), Venetoclax, Verzenio (Abemaciclib), Viadur (Leuprolide Acetate), Vidaza (Azacitidine), Vinblastine Sulfate, Vincasar PFS (Vincristine Sulfate), Vincristine Sulfate,

25 Vincristine Sulfate Liposome, Vinorelbine Tartrate, VIP, Vismodegib, Vistogard (Uridine Triacetate), Voraxaze (Glucarpidase), Vorinostat, Votrient (Pazopanib Hydrochloride), Vyxeos (Daunorubicin Hydrochloride and Cytarabine Liposome), Wellcovorin (Leucovorin Calcium), Xalkori (Crizotinib), Xeloda (Capecitabine), XELIRI, XELOX, Xgeva (Denosumab), Xofigo (Radium 223 Dichloride), Xtandi (Enzalutamide), Yervoy (Ipilimumab), Yondelis

30 (Trabectedin), Zaltrap (Ziv-Aflibercept), Zarxio (Filgrastim), Zejula (Niraparib Tosylate Monohydrate), Zelboraf (Vemurafenib), Zevalin (Ibritumomab Tiuxetan), Zinecard (Dexrazoxane Hydrochloride), Ziv-Aflibercept, Zofran (Ondansetron Hydrochloride), Zoladex (Goserelin Acetate), Zoledronic Acid, Zolinza (Vorinostat), Zometa (Zoledronic Acid), Zydelig (Idelalisib), Zykadia (Ceritinib), and/or Zytiga (Abiraterone Acetate).

B. Examples

62. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

1. Example 1: Ibrutinib Treatment Improves T-cell Number and Function of CLL in patients

a) Results

(1) Ibrutinib treatment increases numbers of both CD4 and CD8 T cells via a BTK independent mechanism.

63. Serial samples were analyzed from 19 CLL patients who received ibrutinib treatment on a serial blood procurement study OSU-0025. Not all experiments were performed on each patient's serial sample, therefore the actual "N" for each experiment was less than 19. The absolute numbers of T-cells of various subsets were evaluated in CLL patients during the course of ibrutinib therapy. As shown in Figure 1A (N=18), a significant increase in total CD4 and CD8 T cell numbers was observed following ibrutinib treatment (approximately three-fold by 8 weeks, or the beginning of cycle 3); ($P < 0.01$ for both CD4 and CD8 T cells). Both CD4 and CD8 T cells were further categorized into naïve (CD45RA+CCR7+), central memory (T-CM; CD45RA-CCR7+), effector memory (T-EM; CD45RA-CCR7-) and CD45RA+ effector memory T cells (T-EMRA; CD45RA+CCR7-) (Figure 1A). The T-EMRA subset is considered to consist of more terminally differentiated effector memory/effector T cells. Among the different T cell subsets, the increase in total cell number was most prominent in the T-EMRA and T-EM compartments, whereas in the naïve and T-CM subsets, the increase was more modest and not significant at several time points examined. For example, CD4 T-EMRA cell numbers increased by more than three-fold (from 0.055 to $0.18 \times 10^3/\mu\text{l}$) 8 weeks into treatment (beginning of cycle 3), while naïve and central memory CD4 T cell numbers increased by about two fold at the same time point (from 0.065 to $0.147 \times 10^3/\mu\text{l}$ for naïve CD4 T cells). This same pattern coincides with the frequencies of different T-cell subsets, where the proportion of both CD4 and CD8 T-EM cells were increased modestly by cycle 6 of ibrutinib treatment (Figure 2A). For example, the mean proportion of CD4 T-EMRA cells increased from 5.1% to 7.3% ($P < 0.05$). In contrast,

the proportion of naïve and T-CM subsets were modestly but significantly reduced by cycle 6 of treatment in both CD4 and CD8 T cells ($P=0.006$ for CD8 T-CM cells and $P=0.001$ for CD4 T-CM cells; Figure 2A). The reduced frequencies of both CD4 and CD8 T-CM subsets after ibrutinib treatment appears to be a result of a dilution effect from preferential expansion of the T-EM and T-EMRA subsets as shown in Figure 1A, versus loss of these cells during treatment.

64. One concern is that the increase of the circulating T cells numbers after ibrutinib treatment may merely reflect the release of T-cells from the secondary lymphoid organs as opposed to true T-cell expansion. To address this, CLL engrafted mice were treated with ibrutinib and monitored peripheral blood T cell numbers before starting ibrutinib, 2 days and 4 days post starting ibrutinib. These time points correspond to the period when CLL cells numbers were transiently increased in peripheral blood post ibrutinib treatment. If ibrutinib causes translocation of T cells from secondary lymphoid organ to peripheral circulation like it does to CLL cells, an increase in T cell numbers was noticed in peripheral blood. However, no significant change was found in circulating T cell numbers before and after ibrutinib treatment. Furthermore, it was questioned if ibrutinib can enhance the expansion of activated antigen specific T cells using a mouse leukemia model. As shown in Figure 13, ibrutinib increased the number of tumor-antigen specific T cells in the secondary lymphoid organ (spleen) by approximately two fold.

65. To further examine if this effect on T-cells was dependent or independent of BTK inhibition, similar parameters were examined in CLL patients treated with acalabrutinib, a more selective BTK inhibitor that does not target other ibrutinib substrates such as ITK. Interestingly, similar increases in the cell numbers were not found after acalabrutinib treatment (Figure 1B, $N=12$). The frequency of CD4 and CD8 T cells trended up by cycle 6, which reflects the decrease of absolute lymphocyte count with acalabrutinib (Figure 2B). Given that activated ITK-deficient T-cells in murine systems have diminished activation-induced cell death (AICD), the ITK inhibitory activity of ibrutinib, but not acalabrutinib, can explain this observation. Therefore, the influence of these two agents was examined on T-cells *ex-vivo* with extended activation. As shown in Figure 3 A-C, activated T-cells treated with ibrutinib exhibited a dose-dependent decrease in AICD. Up-regulation of FAS ligand (FASL) by activated T cells and “suicide” by FAS-FASL interaction have been found to be an important mechanism for AICD. Moreover, ITK inhibition has been reported to impair AICD by reducing the upregulation of FASL. Upregulation of FASL expression in activated T cells after re-stimulation was indeed decreased by ibrutinib treatment (Figure 3C), indicating that ibrutinib ameliorates AICD by

decreasing TCR activation-induced FASL upregulation. In contrast, a similar protective effect against AICD was not observed with acalabrutinib at these same concentrations.

66. It has been reported that NK cells can also undergo apoptosis following activation via cytokine receptors. Ibrutinib targets such as ITK and BTK also play an important role in NK cell function and signaling processes. Therefore, it was investigated if ibrutinib can also ameliorate “AICD” of NK cells utilizing an established assay system in which NK cells are co-stimulated with IL-2/IL-12 or IL-15/IL-12. Here, a similar protection by ibrutinib, but not acalabrutinib was found (Figure 3).

(2) Ibrutinib treatment does not compromise stem memory T cells localized in the naïve T cell compartment.

67. Next stem memory T cells (TSCM), the long-lasting memory T cells that share many surface marker profiles with naïve T cells, were analyzed. TSCM were differentiated from naïve T cells by staining for CD122 and CD95, as shown in Figure 4A. Interestingly, CLL patients have more (>10%) TSCM within the naïve T cell gate (Figure 4B). This differs from healthy donors, who usually have less than ~1-2% of TSCM among the naïve T cell gate. Tbet and Eomesodermin are established Th1 differentiation markers. As expected, few naïve T cells from healthy donors express these markers (Figure 4B). However, a significant percentage of naïve T cells (more than 10% naïve CD4 cells) from CLL patients express Tbet or Eomesodermin. Therefore, a significant percentage of naïve (CCR7+/CD45RA+) T cells from CLL patients are not *bona fide* naïve T cells, but rather TSCM cells. In patients receiving ibrutinib, the absolute number of TSCM cells was increased by cycle 3 and was not significantly changed by cycle 6 (Figure 4C, N=15).

(3) Frequencies of PD-1 and CTLA-4 positive T cells are significantly reduced after ibrutinib treatment.

68. A key feature of T cells from CLL patients is the high percentage of cells expressing checkpoint inhibitor molecules such as PD-1 (Figure 14). As ibrutinib rescues AICD of T-cells, it is possible that these rescued cells can express a higher level of exhaustion markers that would indicate less immunologic potential toward target tumor cells. Therefore, these markers were examined in different T cell subsets before and after ibrutinib treatment. No significant changes were found in CD244, CD160 or CD57 after ibrutinib treatment. However, the percentages of PD-1 positive cells among total CD8 (Figure 5A, N=17) and total CD4 (Figure 6A, N=17) T cells were significantly reduced. The decrease in PD-1 was statistically significant by cycle 3 for CD8 T cells (P=0.001), and by cycle 6 for both CD4 (P=0.001) and CD8 (P<0.001) T cells. Although PD-1 positivity was reduced in all the T cell subsets among both CD4 and CD8 T

cells, the reduction was most dramatic in the T-CM compartment (Figures 5A and 6A). CD27 was down-regulated in the more differentiated effector and effector memory T cells, prompting the further differentiation of the T-EM and T-EMRA into CD27⁺ vs. CD27⁻ populations.

Significant differences in the distributions of CD27⁺ and CD27⁻ T cell subsets was not

observed. However, for CD4 T cells, the CD27⁺ T-EM and T-EMRA cells showed a statistically significant reduction in PD-1 after ibrutinib treatment ($p < 0.001$), while their CD27⁻ counterparts did not (Figure 6).

69. In the serial samples CTLA-4 expression was detected by intracellular staining (Figures 7A and 8A, N=18). A significant reduction in intracellular CTLA-4⁺ cells among both CD4 T cells (Figure 7A; $P < 0.001$ for both cycle 3 and cycle 6) and CD8 T cells (Figure 8A; $P < 0.01$ for both cycle 3 and cycle 6) was detected, with the reduction being more dramatic in CD4 T cells. Cells from patients treated with acalabrutinib showed similar patterns of change in PD-1 expression (Figures 5B/6B, N=10). Acalabrutinib treatment also reduced CTLA4 expression on T cells (Figures 7B/8B, N=9). Indicating that ibrutinib affects PD-1 and CTLA-4 expression in T-cells indirectly by inhibiting BTK.

(4) Ibrutinib treatment does not significantly change T cell polarization.

70. Ibrutinib enhances Th1 polarization *in-vitro* and *in-vivo* in murine models. To study T cell polarization in human CLL patients, PBMCs were re-stimulated from ibrutinib-treated patients with PMA/ionomycin and assessed their cytokine production profile. As shown in Figure 9A (N=15), ibrutinib treatment did not alter Th1 (IFN- γ), Th2 (IL-4) and Th0 (IL-2 and TNF) cytokine expression profile. However, there was a noticeable increase in the percentage of cells producing IL-17 in patients who received ibrutinib treatment ($P = 0.008$, Figure 9A). In contrast, acalabrutinib-treated patients did not have a post-treatment change in the frequency of IL-17 producing cells (Figure 9B, N=11). These results indicate that inhibition of non-BTK targets such as ITK play a key role in the expansion of Th17 cells. The average percentage of IFN- γ , IL-4 and TNF positive cells showed a trend of decrease post treatment with acalabrutinib (Figure 9B) compared to ibrutinib (Figure 9A), indicating a BTK but not ITK dependent mechanism.

(5) Ibrutinib decreases the T-reg:CD4 ratio but not absolute number of CD25⁺Foxp3⁺ Treg cells.

71. *In-vitro* experiments have shown enhanced differentiation toward Foxp3⁺ Treg cells by ITK-deficient conventional CD4 T cells. Therefore, it was investigated as to how ibrutinib treatment in human CLL patients affects Treg cells *in-vivo*. Treg cells were identified as

CD4+CD25+Foxp3+ cells (Figure 10A). An approximately two-fold reduction in the percentage of CD25+Foxp3+ Treg cells was found among CD4 T cells by cycle 3 ($p<0.001$) that persisted to cycle 6 ($P<0.001$) of ibrutinib treatment (Figure 10B, left panel, N=18). However, this reduction of the Treg:CD4 ratio was associated with an increase in the absolute number of CD4 T cells and the total number of Treg cells remained unchanged (Figure 10B, right panel). In acalabrutinib-treated patients, the number of CD4+CD25+Foxp3+ Treg cells and the Treg:CD4 ratio was not significantly changed (Figure 10C, left panel, N=11).

**(6) Ibrutinib down-regulates immunosuppressive molecules
CD200 and BTLA on CLL cells.**

72. CLL cells have been reported to express a variety of immunosuppressive ligands; the expression levels of these immunosuppressive molecules was evaluated in CLL cells before and after ibrutinib treatment. The surface expression of PD-L1, HLA-G and CD276 was low in general, and a significant change of their expression in CLL cells was not detected after ibrutinib treatment. However, CD200 expression was significantly reduced as early as cycle 3 of ibrutinib treatment, and this reduction persisted through cycle 6 ($p<0.001$ for both; Figure 11A, N=18). BTLA expression was not significantly changed in T cell subsets, while significant reduction in CLL cells was observed ($P<0.001$ for both, N=16). Similar reductions were observed in samples from patients treated with acalabrutinib (Figure 11B, N=12), indicating a BTK-dependent mechanism for these changes.

**(7) The capacity of CLL cells to make IL-10 after prolonged
B10Pro conditioning is impaired after ibrutinib treatment.**

73. It has been reported that CLL cells share phenotypic and functional features with regulatory-B cells and can produce IL-10 after *in-vitro* stimulation under “B10” (5 hour stimulation) or “B10Pro” (48 hour stimulation) conditions. Activation through the BCR, TLRs and CD40 is required for production of IL-10 by B cells or CLL cells. As BTK is involved in signal transduction of all these receptors, BTK inhibition can affect IL-10 production in CLL cells. Significant IL-10 production by CLL cells was detected after a brief *in-vitro* stimulation under B10 conditions in less than half of the CLL patient samples and significant changes in the frequency of these rare malignant B10 cells were not detected after ibrutinib treatment (Figure 12A and 12B, left panel, N=13). However, by stimulating cells under B10Pro conditions, IL-10 production was induced by CLL cells from most of the samples tested. Interestingly, after ibrutinib treatment, CLL cells showed a dramatically reduced capacity to secrete IL-10 after being stimulated under B10pro conditions ($P<0.001$). This reduction was observed as early as cycle 3 of ibrutinib treatment, when the majority of the patients studied still showed transient

lymphocytosis (Figure 12B, right panel, N=18). Samples from patients treated with acalabrutinib showed similar results (Figure 12C, N=10 for B10 conditions, N=12 for B10Pro condition), indicating that the reduced capacity of CLL cells to make IL-10 following ibrutinib treatment is from BTK inhibition.

(8) Ibrutinib treatment increases T cell cytotoxicity

74. Next, the effect of ibrutinib on T cell cytotoxicity in CLL patients was investigated. CLL patient T cells were collected pre-ibrutinib treatment and post-ibrutinib treatment and mixed with autologous CLL cells (pre-ibrutinib treatment). Cells were then either cultured with or without the addition of blinatumab and with or without Treg depletion. Cells were then stained for annexin V and propidium iodide (Figure 15). After treatment with ibrutinib, CLL patients' T cells demonstrated superior survival after being stimulated with blinatumomab plus autologous CLL cells. The underlying mechanism is likely rescuing activated T cells from activation induced cell death(AICD) by inhibition of ITK (Figure 16).

b) Discussion

75. Numerous studies in mice have reported the favorable immune modulating effect of ibrutinib that likely occurs through multiple mechanisms. Here, in the first comprehensive human study of ibrutinib's effects on T cells, significant increases were identified in both CD4 and CD8 T cell numbers following ibrutinib treatment in CLL patients. These T cells, while increased in number following ibrutinib treatment, lack the typical immunophenotypic features of CLL-exhausted T-cells, as evidenced by significantly lower PD-1 and intracellular CTLA-4 expression. Unlike other immune modulating agents such as IL-2 that expand Treg cells, ibrutinib in fact decreases the T-reg:CD4 T cell ratio by selectively expanding conventional T cells. Separate from the favorable effects on T-cells, ibrutinib also modulates the expression of several immune suppressive molecules on/in CLL cells including CD200, BTLA4 and IL-10. Acabrutinib is a second generation, selective BTK inhibitor. As shown table 1, while ibrutinib has comparable IC50 for BTK and ITK, acalabrutinib has virtually no affinity for ITK. The pharmacologic studies in patients clearly differentiate ibrutinib from the more selective BTK inhibitor acalabrutinib in its ability to inhibit AICD via ITK inhibition. Collectively, ibrutinib represents a novel T-cell immune modulating agent, and the data clearly differentiates it from other immunotherapeutics used in cancer.

Table 1: IC50 values for inhibition of enzymatic activity by ibrutinib versus acalabrutinib.

Kinase	IC50 (nM) of acalabrutinib	IC50 (nM) of ibrutinib
BTK	5.1 ± 1.0	1.5 ± 0.2
BMX	46 ± 12	0.8 ± 0.1

ITK	>1000	4.9 ± 1.2
TEC	93 ± 35	7 ± 2.5
TXK	368 ± 141	2.0 ± 0.3
EGFR	>1000	5.3 ± 1.3
ERBB2	~ 1000	6.4 ± 1.8
ERBB4	16 ± 5	3.4 ± 1.3
JAK3	>1000	32 ± 15
BLK	>1000	0.1 ± 0.0
FGR	>1000	3.3 ± 1.1
FYN	>1000	29 ± 0
HCK	>1000	29 ± 0
LCK	>1000	6.3 ± 1.3
LYN	>1000	20 ± 1
SRC	>1000	19 ± 1
YES1	>1000	4.1 ± 0.2
CSK	86	2.25
BRK	79	3.34
FLT3	100	72.9

76. The remarkable and new finding of the work is the notable increase of both CD4 and CD8 T-cells in patients receiving ibrutinib. This T-cell expansion is unlikely to be caused by BTK inhibition, as increased T cell numbers were not observed in patients treated with the more selective BTK inhibitor acalabrutinib that lacks ITK inhibitory activity. While discerning the mechanism of T-cell expansion *in-vivo* in CLL patients is not possible, was provided herein that ibrutinib treatment of activated T-cells diminishes AICD by targeting ITK, a finding also reported in murine models of ITK deficiency. ITK has been demonstrated to be involved in TCR-induced up-regulation of FASL and AICD of T cells. *In-vitro*, ITK-deficient T cells have been found to have impaired proliferation whereas *in-vivo*, activated ITK^{-/-} T cells survived to a much greater degree than normal T cells, leading to a greater accumulation. Targeting ITK with kinase inhibitors showed a similar pattern. *In-vitro*, ITK inhibitors inhibit IL-2 secretion and T-cell proliferation, whereas *in-vivo* ITK inhibitor was found to reduce AICD, leading to a 2-3 fold increase of activated T cell numbers.

77. In contrast to the findings herein, it was previously understood that ibrutinib treatment led to a decrease in circulating T cell numbers in CLL patients that paralleled the progressive decrease of CLL tumor burden and “normalization” of T-cell counts in most patients. This difference can best be explained by the impact of CLL tumor burden. It has been postulated that CLL cells promote chronic stimulation of T cells and lead to an “exhaustion” phenotype by inducing a CLL-specific immune response, or by modifying T cell response to chronic infections including cytomegalovirus (CMV). In either case, CLL cells cause chronic activation of T cells, and it is the activated T cells, but not resting T cells that are susceptible to AICD and can be

rescued by ibrutinib. Therefore, this effect on T cell numbers by ibrutinib is likely only to be seen in patients who still have significant tumor burden, since those patients who have achieved remission will no longer have CLL-induced aberrant activation of T cells. To more selectively study the direct impact of BTK inhibition on T cells, earlier time points (8 and 20 weeks into treatment) were selected and studied patients with persistent lymphocytosis (absolute lymphocyte counts at cycle 1 and cycle 6 are comparable; Table 2) to address this confounding factor. Consistent with this, in mouse models, ibrutinib strongly increased the number of activated T cells during listeria infection, while it has no significant impact on the resting T cell populations from healthy non-infected mice. Furthermore, in ibrutinib-treated patients, the increase in T cell numbers was most prominent in the effector and effector memory T cells compared to the resting naïve T cells and central memory T cells. As a side note, it is possible that some of the changes in T cell populations are due to redistribution rather than expansion, as has been observed in HIV patients after institution of antiretroviral therapy. However, the durable effects make it unlikely that all the effects are due to redistribution. Moreover, the animal experiments (Figure 13) provided evidence that ibrutinib treatment did lead to a bona fide increase of the numbers of activated antigen specific T cells in secondary lymphoid organs, while did not cause significant translocation of T cells from secondary lymphoid organ to peripheral circulation.

78. Ibrutinib treatment leads to preferential expansion of more differentiated T cells subsets (*e.g.* T-EM and EMRA), but it does not have a deleterious effect on the absolute number of naïve and central memory T cells. This is another feature that is desired for cancer immunotherapy. In contrast, although IL-2 is able to increase effector cell proliferation it also compromises the persistence of the less differentiated memory T cells, and therefore has deleterious effect on the long term persistence of antitumor immunity. Also, ibrutinib treatment does not compromise the total numbers of the stem memory T cells (TSCM), which represent the earliest and long-lasting memory T cells. The self-renewal capacity and long-term survival of these cells make them ideal vehicle for the cancer immunotherapy. Furthermore, while T cells from CLL patients demonstrate features of exhaustion similar to those exposed to chronic stimulation by viral infections and ibrutinib preferentially increases the number of these exhausted T cells from AICD, enrichment of such cells was not observed. Instead, diminished PD-1 surface and intracellular CTLA-4 expression was detected.

79. Here no significant changes in the percentage of T-cells expressing intracellular Th1 (IFN γ) or Th2 (IL4) cytokines was observed in CLL patients treated with ibrutinib. However, a moderate increase in the frequency of T cells capable of producing IL-17 (Th17 cells) was

found. Ibrutinib has also been recently found to enhance IL-17 response indirectly by modulating the function of antigen presenting cells such as dendritic cells. *In-vivo*, Th17 cells have been found to undergo FAS-mediated AICD, a process that can also be blocked by ITK inhibition. The findings indicate that the net effect of ibrutinib treatment in CLL patients is the increased percentage of Th17 cells. There is accumulating evidence that a Th17 response can play a role in CLL pathogenesis. Decreased frequency of Th17 cells has been found to be associated with regulatory T cell expansion and disease progression in CLL patients. In contrast, elevated Th17 cells in CLL patients is associated with improved survival.

80. The influence of ibrutinib in CLL patients also has direct positive influence on the immunosuppressive capacity of the primary tumor cells. CD200 and BTLA are significantly down-regulated on the surface of CLL cells as early as cycle 3 of ibrutinib treatment. While the function of BTLA on CLL cells is uncertain, CD200 regulates both innate and adaptive immunity and plays a key role in both tumor-specific and global immune suppression in CLL patients. Moreover, CD200 expression on tumor cells has been found to promote the expansion of Tregs, and CD200 blockade significantly decreases Treg cell numbers. Ibrutinib treatment of CLL patients dramatically reduced the frequency of malignant B10pro cells, which can express IL-10 after prolonged *in-vitro* stimulation, and similar findings with acalabrutinib indicate this is a BTK-dependent effect. To induce IL-10 expression in B10/B10Pro cells, stimulation via BCR, TLR4/9 and CD40L are required. BTK is involved in signaling transduction of all these receptors. Recently it was found that chemokine CXCL12 enhances IL-10 production in CLL cells via the CXCR4-STAT3 pathway, and BTK inhibition was reported to impair CXCR4 surface expression and signaling in CLL cells. In support of this, mice with BTK deficiency (XID mice) showed a more severe reduction in the numbers of B1a cells, which are also CD5+ B cells and are enriched with “B10” (~30%) and “B10pro”(30-40%) cells. Therefore, BTK inhibition can reduce the frequency of B10Pro-like CLL cells in two mutually non-exclusive mechanisms: by directly inhibiting the IL-10 production in CLL cells, and/or by selective depleting B10Pro-like CLL cells. IL-10 is a major immunosuppressive cytokine that can be produced by multiple cell types. Surprisingly, it has been found that B cells are actually a dominant source of IL-10 *in-vivo* in both naive and immune system-activated mice. Secretion of IL-10 by CLL cells can be triggered by Infections or host inflammatory responses in CLL. Given the significant elevated number of the malignant B cells in CLL patients, production of IL-10 by even a small fraction of the tumor cells can cause significant immune suppression. Endogenous B10/Breg cells were shown to inhibit CD20 mAb-induced lymphoma regression by secreting IL-10 and inhibiting mAb-mediated monocyte activation. CLL cell IL-10 production

was also found to significantly inhibit monocyte activation. Therefore, by reducing the IL-10 production by CLL cells, ibrutinib treatment can lead to relief of CLL-induced immune suppression. Moreover, as B10/B10pro cells share many phenotypic and functional features with CLL cells, it is possible that ibrutinib treatment also depletes these immunosuppressive cells or impairs their function.

81. The disclosure includes comparative data from CLL patient samples obtained at matched time points during treatments with either an irreversible ITK/BTK inhibitor or more selective BTK inhibitor. Although descriptive, these studies identify effects such as expansion of effector T-cells, increased proportion of Th17 producing cells, and distinct changes in CTLA-4 intracellular expression between CD4 and CD8 subsets that are likely attributable to alternative, non-BTK targets such as ITK that are inhibited by ibrutinib but not acalabrutinib. Also provided herein is in-vitro evidence that ibrutinib but not acalabrutinib prevents AICD of activated T-cells and NK cells. In contrast, decreased expression of surface PD-1 on CD4 and CD8 T cells and intracellular CTLA-4 on CD4 T-cells was observed with both agents, implicating BTK as an indirect factor in this change. This can be through modulation of immune suppressive molecules (CD200, IL-10, and others) on CLL tumor cells, which are impacted by both agents, and further studies to understand these effects are ongoing. Due to the smaller sample size for acalabrutinib treated patients available for analysis, some of negative findings on acalabrutinib were underpowered. For example, there is also a trend towards a decrease in the percentage of Treg cells after acalabrutinib treatment, and with increased sample size, this can be a statistically significant difference- albeit more modest comparing to the difference observed in ibrutinib treated samples. Nonetheless, the data presented here identify ibrutinib and acalabrutinib as distinctly different immune modulating agents. Despite potential added toxicity with ibrutinib due to alternative target inhibition, the data show improved immune modulation in-vivo with this agent compared to acalabrutinib and support its use in combination with other immune therapies.

82. In summary, by studying CLL patients treated with ibrutinib, its superior immunomodulatory effects was identified by virtue of being a less specific inhibitor of BTK. Ibrutinib induces significant increases in T cell numbers that are not achieved by a more selective BTK inhibitor. The underlying mechanism is likely to be ITK inhibition that leads to the rescue of chronically stimulated T cells from AICD. The data therefore provide support for ibrutinib therapy as an ideal cellular immune modulating agent for CLL and potentially other types of hematologic and solid cancers. For example, ibrutinib can be incorporated as part of cellular immune therapy. *In-vivo* persistence and expansion of antigen-specific T cells is the

most critical determining factor for the success of adoptive immunotherapy with TIL cells and CAR T cells. Expanding such cells with systemic administration of IL-2 is toxic and can have deleterious effect on the long term persistence of antitumor immunity. IL-2 also leads to preferentially expansion of Treg cells. Pre-conditioning with lymphocyte depletion enhances homeostatic proliferation and depletes host Tregs. However, it also carry along significant toxicities, and Tregs can out-proliferate conventional T cells in the lymphopenic environment. Low persistence of infused T cells can also be a result of T cell exhaustion, and ongoing clinical trials are investigating immune checkpoint blockade to boost the persistence of tumor-specific T cells. Of note, checkpoint blockade of CTLA-4 has been found to expand functional Treg cells. Whereas ibrutinib enhances persistence/expansion of activated T cells and shows the following desirable qualities: a) it has no deleterious effects on the central memory or naïve T cells; b) it does not cause collateral expansion of the Treg cells; and c) it partially reverses the exhausted T cell phenotype by reducing the expression of PD-1 and CTLA-4. Together, these findings provide further rationale for use of ibrutinib as an adjuvant for expansion of TIL or CAR-T cells.

c) Methods and procedures

(1) Flow cytometry(details) :

83. For surface staining, cells were stained with various markers at room temperature for 15 minutes, washed with PBS and then re-suspended in PBS containing 1:200 dilution of LIVE/DEAD® Fixable Near-IR stain. Cells were then incubate for 15 minutes under room temperature and were fixed with 1.5% formaldehyde for 20 minutes under room temperature, washed one time and resuspended in FACS buffer (PBS with 5% fetal calf serum) before analysis on flow cytometer.

84. For intracellular cytokine staining, PBMC were stimulated with PMA (25ng/ml, Sigma-Aldrich) and Ionomycin (500ng/ml, Sigma-Aldrich) in the presence of monensin (2mM; eBioscience) for 5 hours, or as described below under B10/B10 Pro conditions. Cells were then harvested and were stained with surface markers and then LIVE/DEAD® Fixable Near-IR stain (Thermo Fisher Scientific) as described above, with the exception that monensin was added to all the staining buffers. Cells were then fixed with 1.5% formaldehyde for 20 minutes under room temperature, and were then washed twice with permeablization buffer (FACS buffer containing 0.25% Saponin, from Sigma-Aldrich), stained with appropriate cytokine antibodies, washed again with permeablization buffer, and were then analyzed by flow cytometer.

85. For intracellular/intranuclear staining of Foxp3 and CTLA4, cells were first stained with surface maker and then labeled with LIVE/DEAD® Fixable Near-IR stain (Thermo Fisher Scientific) as described above. Cells were then fixed/permeablized using the Foxp3 /

Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's protocol and were stained with Foxp3 and Foxp3 antibodies.

86. All the samples were analyzed using Beckman Coulter Gallios™ Flow Cytometer, which can detect up to 10 different fluorochrome conjugated antibodies simultaneously.

(2) Analysis of IL-10 production by CLL cells.

87. PBMC cells were resuspended (2×10^6 cells/mL) in in Iscove's Modified Dulbecco's Media (IMDM) containing 10% fetal bovine serum (FBS), 200 µg/mL penicillin, 200 U/mL streptomycin, and 4mM L-glutamine (all from Gibco™ Thermo Fisher) and stimulated with CpG (ODN 2006, 10 µg/mL; Invivogen), CD40L (1 µg/mL; R&D Systems), PMA (50 ng/mL; Sigma-Aldrich), Ionomycin (1 µg/mL; Sigma-Aldrich), monensin (2mM; eBioscience), as indicated in 48-well flat-bottom plates before staining and flow cytometry analysis. For “B10” condition, cells were stimulated with CpG, PMA and Ionomycin in the presence of monensin for 5 hours. For “B10 Pro” condition, cells were stimulated with CpG/CD40L for 48 hours, with PMA/Ionomycin/ monensin added for the last 5 hours.

88. After stimulation, cells were stained for surface markers, including CD19, CD5, CD3, CD4, and CD8. PECE-594 labeled CD14, CD11b, CD16, CD56 and CD123 were added as a “dump channel” to gate out corresponding cell types. After surface staining, cells were labeled with LIVE/DEAD® Fixable Dead Cell Stains from ThermoFisher before being fixed with 1.5% Formaldehyde. Fixed cells were then permeablized with FACS buffer containing 0.25% Saponin and were stained with IL-10 antibody.

(3) Activation induced cell death in human T cells. :

89. T cells were isolated from healthy human donors using EasySep™ Human T Cell Isolation Kit. Isolated T cells were stimulated in vitro with plate bound CD3/CD28 for 3 days. Cells were then rested in complete medium containing 50IU/ml IL-2 for additional 7-11 days before they were treated with vehicle, Ibrutinib or acalabrutinib for 30 minutes. Cells were then plated on to 48 well plates coated with CD3; incubate for 6 hours (for flow cytometry based apoptosis assay) or 3 hours (to isolate mRNA for qPCR to quantify FAS-L expression.) in the presence of IL2 to induce AICD.

90. For AICD analysis, cells were stained with annexin-V fitc and Propidium Iodide (PI) using the BD biosciences 10X staining buffer according to the manufacturer's protocol before being analyzed on flow cytometer.

91. For FAS Ligand mRNA quantification, mRNA were extracted from T cells after 3 hours of re-stimulation using QIAGEN “RNeasy Mini”RNA Isolation Kit. mRNA was then reverse transcribed to cDNA using the M-MLV Reverse Transcriptase from Thermo Fisher.

Quantitative PCR for FAS-L were performed using the Taqman probe/primer mix (FAM labeled) from Thermo Fisher using GAPDH as internal control.

(4) Activation induced cell death in human NK cells

92. Human CD5696). NK cells were then sorted to greater than 99% purity with a FACS Aria II cell sorter (BD Biosciences). Purified NK cells were plated at 5×10^4 /CD3⁺/14⁻/20⁻ NK cells were isolated from peripheral blood leuko-Paks from normal donors (American Red Cross) by incubation with an NK cell RosetteSep negative enrichment cocktail (Stem Cell Technology), followed by Ficoll-Hypaque density gradient centrifugation (cells/well in a 96-well round bottom plate and cultured for three days at 37°C. Medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic/antimycotic (Life Technologies). The cytokines IL-2 (Peprotech) and IL-15 (National Cancer Institute) were supplemented as indicated for a final concentration of 10ng/mL. IL-12 (Miltenyi Biotec) was added where indicated at a concentration of 10ng/mL to induce activation induced cell death.

93. Cell viability and apoptosis were assessed after three days in culture by annexin V (BD Biosciences) apoptotic and TO-PRO-3 (Molecular Probes) viability flow cytometric analysis. NK cells were harvested and stained with annexin V per manufacturer's instructions (BD Biosciences). TO-PRO-3 was added immediately prior to acquisition, and all samples were analyzed with a LSRII cytometer (BD Biosciences) within one hour of annexin V staining. Analysis of dual staining of annexin V and TO-PRO-3 was analyzed using FlowJo (TreeStar).

(5) Patient samples

94. All patients provided written informed consent to provide material as part of an institution review board approved trial. Pre-treatment blood samples were collected pre-treatment, at completion of 2 months(cycle 3 day 1) and 5 months(cycle 6 day 1) of ibrutinib or acalabrutinib treatment derived from CLL patients enrolled on the blood collection study OSU-0025 that allows serial collection of samples at a monthly interval in leukemia patients. Samples described here are from NCT01589302 and NCT02029443 (clinicaltrials.gov). PBMC were isolated from blood samples by density gradient centrifugation using Ficoll. PBMC were then washed twice, resuspended in freezing medium containing 10% DMSO, and were stored at -170 degree. Patient characteristics are shown in table 2.

Table 2: Clinical characteristics of the studied patients

Characteristics	value
Age at diagnosis	
Mean (SD)	54 (11.5)
Median	53

Age at starting Ibrutinib

Mean (SD)	64 (8.6)
Median	64
Male sex	11

Rai stage at Ibrutinib treatment

0-2	1
3	1
4	17

Number of prior therapies

0	0
1	6
2	4
3-4	4
5 or more	5

Prior allogeneic transplant

2

Cytogenetics

Del(13q)	13
Del(11q)	9
Del(17q)	4
Trisomy 12	0
Complex karyotype	8

IgVH mutation status, unmutated, mutated, unknown

14, 5, 0

Bone marrow involvement at the time of Ibrutinib therapy: %(range)

88.1(70-99)

ALC (baseline before Ibrutinib)

Mean (SD)	46.5(40.1)
Median	29.9

ALC (cycle 3 day1)

Mean (SD)	122.6(66.9)
Median	99.1

ALC (cycle 6 day1)

Mean (SD)	60.9(52.9)
Median	47.6

(6) Mice, cell line and antibodies

95. All animal experiments were carried out under protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. TCL-1 transgenic mice (on C57BL/6 background) were used for these experiments. Breeding pairs were provided by Dr. Carlo M. Croce (the Ohio State University, Columbus, OH). OT-1 TCR transgenic mice were purchased from The Jackson Laboratory. C1498-OVA is a murine myeloid leukemia cell line (H-2b, C57BL/6 background) expressing the experimental surrogate antigen ovalbumin. It is kindly provided by Dr. Bruce R. Blazar (University of Minnesota, Minneapolis, MN).

(7) Statistical analysis:

96. Separate linear mixed effects models were used to assess differences (cycles 3 and 6 vs. baseline) in cell number and percentage as well as MFI among the different subsets for patients treated with ibrutinib and acalabrutinib. Absolute cell number and MFI data were first log-transformed to reduce skewness and stabilize variances. An initial analysis was performed based on 17 patients treated with ibrutinib and 9 patients treated with acalabrutinib. Later, additional 2 patients with ibrutinib and 4 patients with acalabrutinib were added to the original cohorts and the analysis was repeated. Results are shown for the updated data with a total of 19 ibrutinib patients and 13 acalabrutinib patients. Not all experiments were performed on each patient's serial sample, therefore the actual "N" for each experiment was less than 19 and 13 for ibrutinib and acalabrutinib treated patients, respectively. All clinical-sample analyses were performed using SAS/STAT software, Version 9.4 of the SAS System for Windows (SAS Institute Inc., Cary, NC). For the in-vitro/animal experiments described Figures 3 and 13, two tailed Student's t test were used. A p-value less than 0.05 was considered significant for all the experiments.

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V. CLAIMS

What is claimed is:

1. A method of selectively expanding CD8 and CD4 T cells but not CD25+Foxp3+ T cells
5 in a subject comprising administering to a subject an agent that inhibits interleukin-2 inducible T cell kinase (ITK).
2. The method of claim 1, wherein the agent further inhibits Bruton's tyrosine kinase (BTK).
3. The method of claims 1 and 2, wherein the agent is ibrutinib.
- 10 4. The method of claim 1, wherein the expanded T cells are effector memory (CD45RA-CCR7-) T cells or CD45RA+CCR7- T cells.
5. A method of inhibiting activation induced cell death of T cells in a subject with chronic lymphocytic leukemia comprising administering to the subject an inhibitor of interleukin-2 inducible T cell kinase (ITK).
- 15 6. A method of expanding NK cells comprising contacting NK cells with a stimulatory molecule and ibrutinib; wherein the stimulatory molecule is selected from the group consisting of IL-15, IL-21, IL-2, 41BBL, IL-12, IL-18, MICA, 2B4, LFA-1, and BCM1/SLAMF2.
7. A method of treating cancer in a subject comprising
20 a. expanding NK cells by stimulating NK cells with a stimulatory molecule and ibrutinib; wherein the stimulatory molecule is selected from the group consisting of IL-15, IL-21, IL-2, 41BBL, IL-12, IL-18, MICA, 2B4, LFA-1, and BCM1/SLAMF2
b. administering to the subject the expanded NK cell population.
- 25 8. The method of claim 7, further comprising administering to the subject ibrutinib prior to or after administration of the expanded NK cells to the subject.
9. A method of increasing the number of tumor infiltrating lymphocytes in a subject with a tumor comprising administering to the subject an agent that inhibits interleukin-2 inducible T cell kinase (ITK).

10. A method for expanding an immune cell isolated from a subject for use in immune therapy, comprising
- 5 a) contacting the isolated immune cell with an effective amount of interleukin-2 inducible T cell kinase (ITK) inhibitor to expand the immune cell in an amount effective for immunotherapy; and
- b) culturing the isolated immune cells in the presence of the ITK inhibitor.
11. The method of claim 10, wherein the immune cells are incubated with the ITK inhibitor for at least 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 28, 30, 32, 34, 36, 48 hours, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days.
- 10 12. The method of claim 10, wherein the immune cell comprises a chimeric antigen receptor (CAR) T cell, tumor infiltrating lymphocyte (TIL), or marrow-infiltrating lymphocyte (MIL).
13. The method of claim 10, wherein the immune cell comprises a natural killer (NK) cell, an NK-T cell, a cytokine-induced memory NK cell, a cytokine-induced killer (CIK) cell,
- 15 or a $\gamma\delta$ T cell.
14. A method of increasing the cytotoxicity and survival of T cells to a tumor cell comprising contacting CD4 and/or CD8 T cells with an ITK inhibitor.
15. The method of claim 14, further comprising the administration of blinatumomab.
16. A method of increasing the percentage of Th17 T cells comprising contacting a T cell
- 20 population with an ITK inhibitor.
17. A method of reducing checkpoint inhibition in a subject comprising administering to a subject an ITK inhibitor.

FIG. 1A Patients treated with ibrutinib

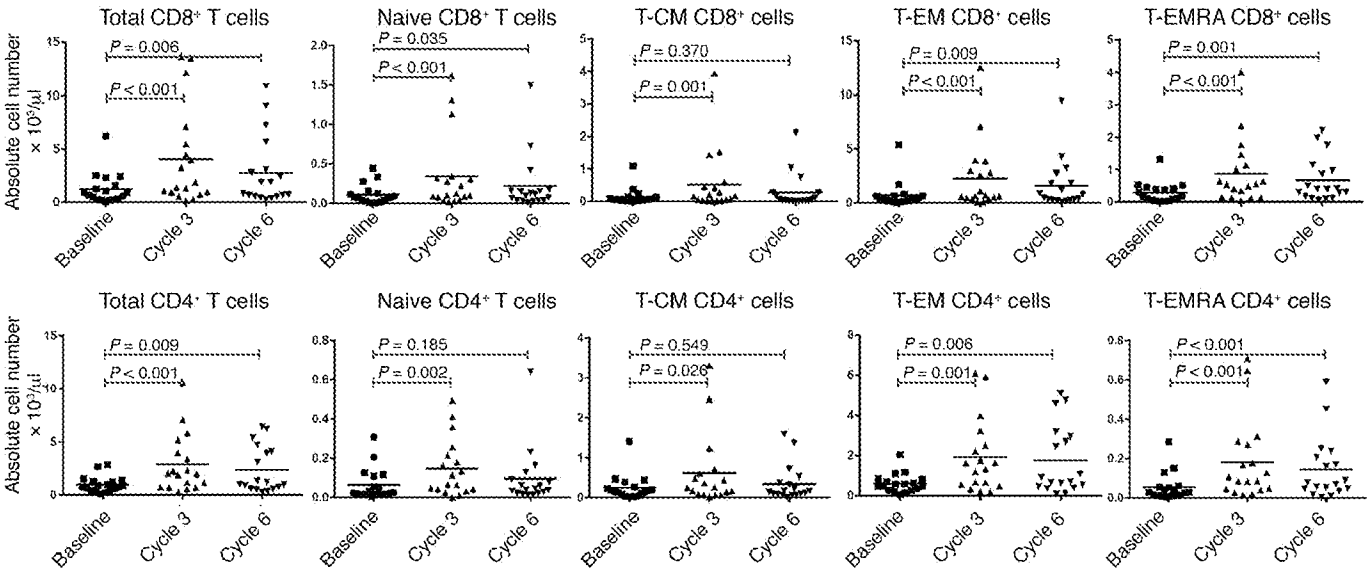


FIG. 1B Patients treated with acalabrutinib

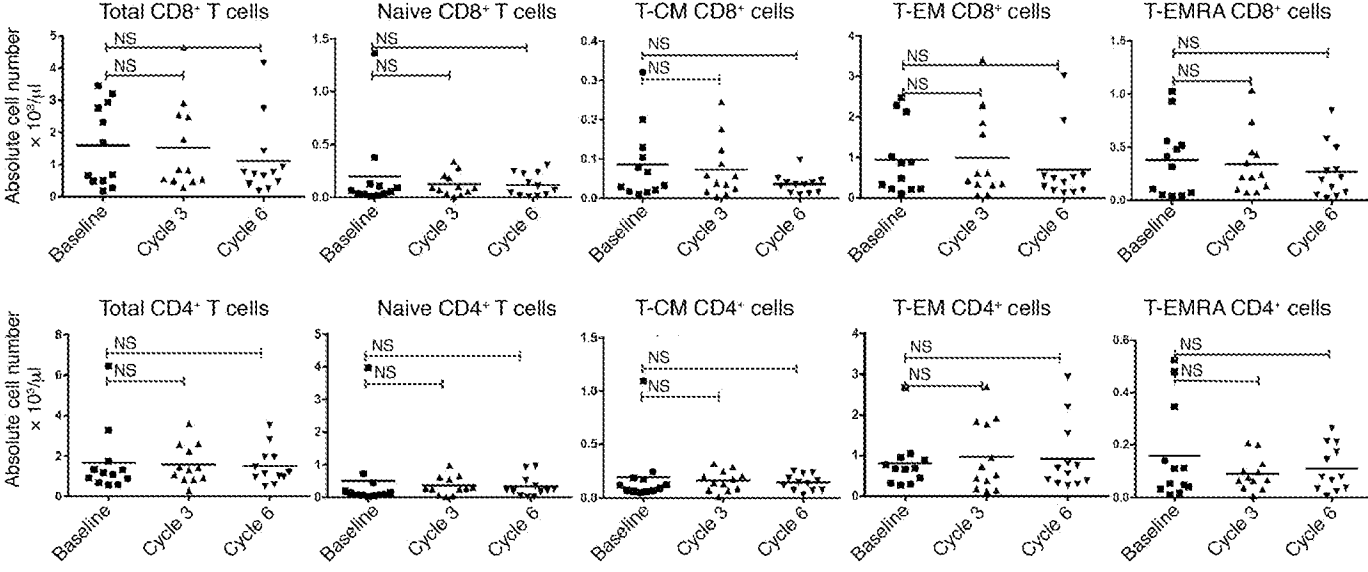


FIG. 1A and FIG. 1B

FIG. 2A and FIG. 2B

FIG. 2A. Patients treated with Ibrutinib

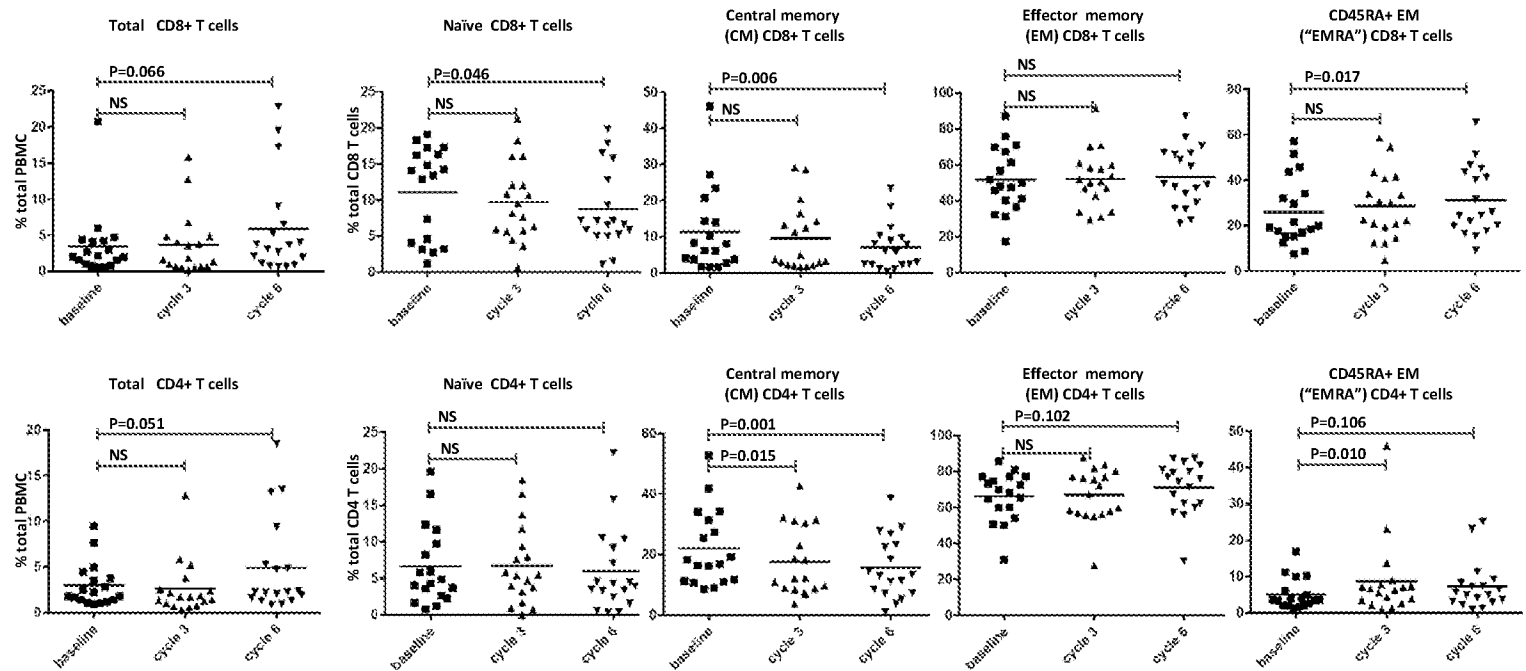
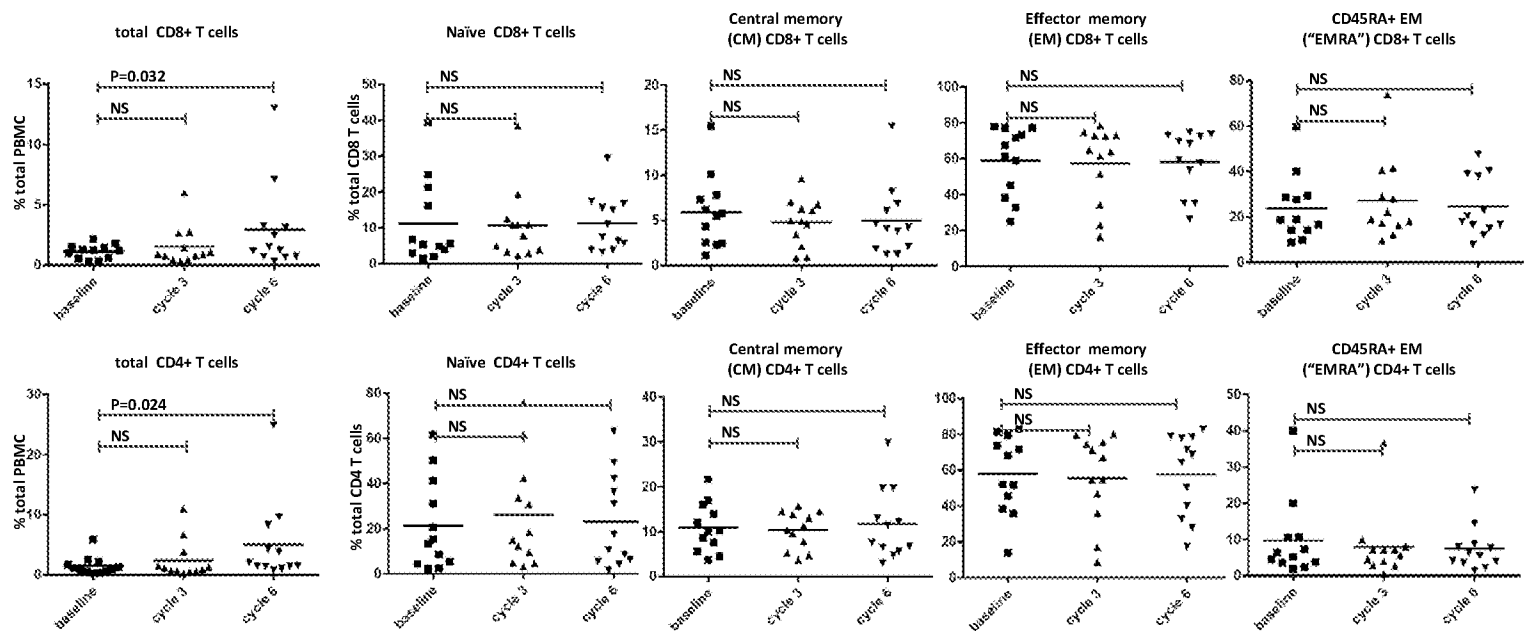


FIG. 2B. Patients treated with acalabrutinib



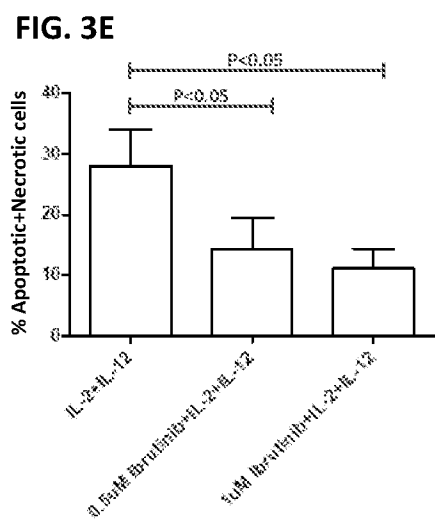
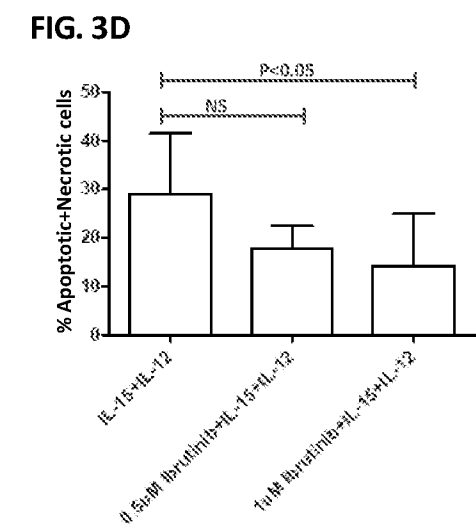
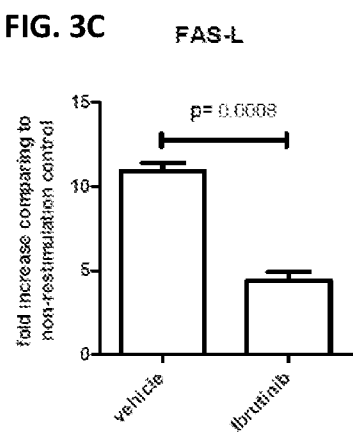
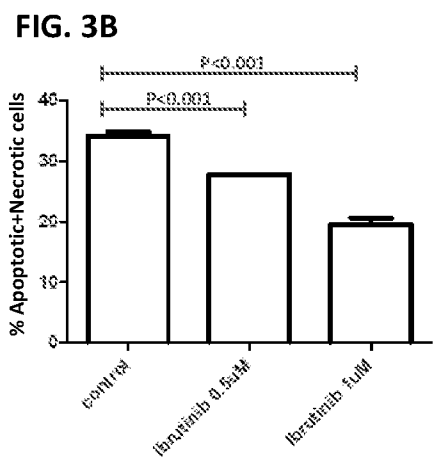
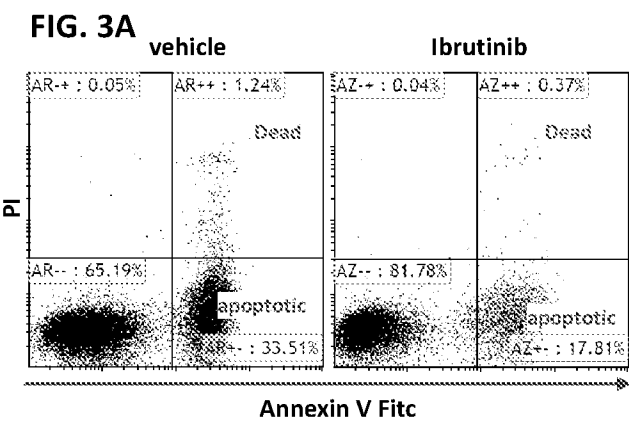


FIG. 3A, FIG. 3B, FIG. 3C, FIG. 3D, and FIG. 3E

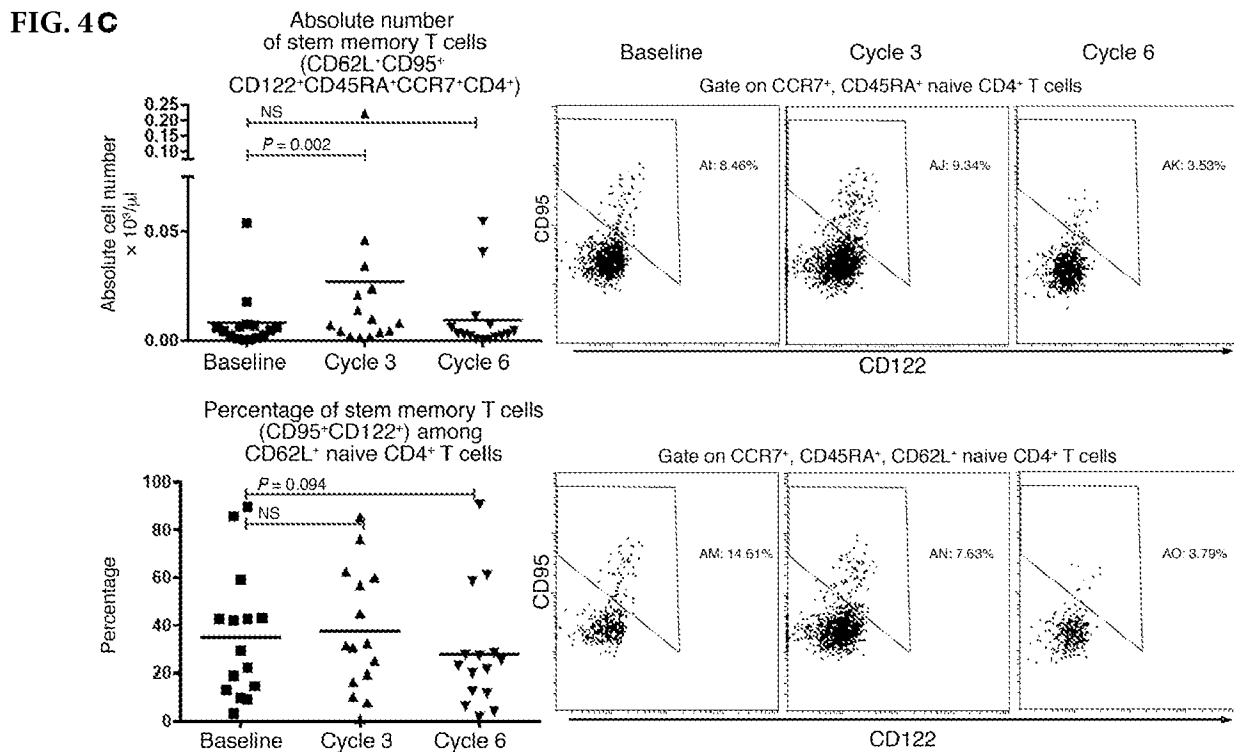
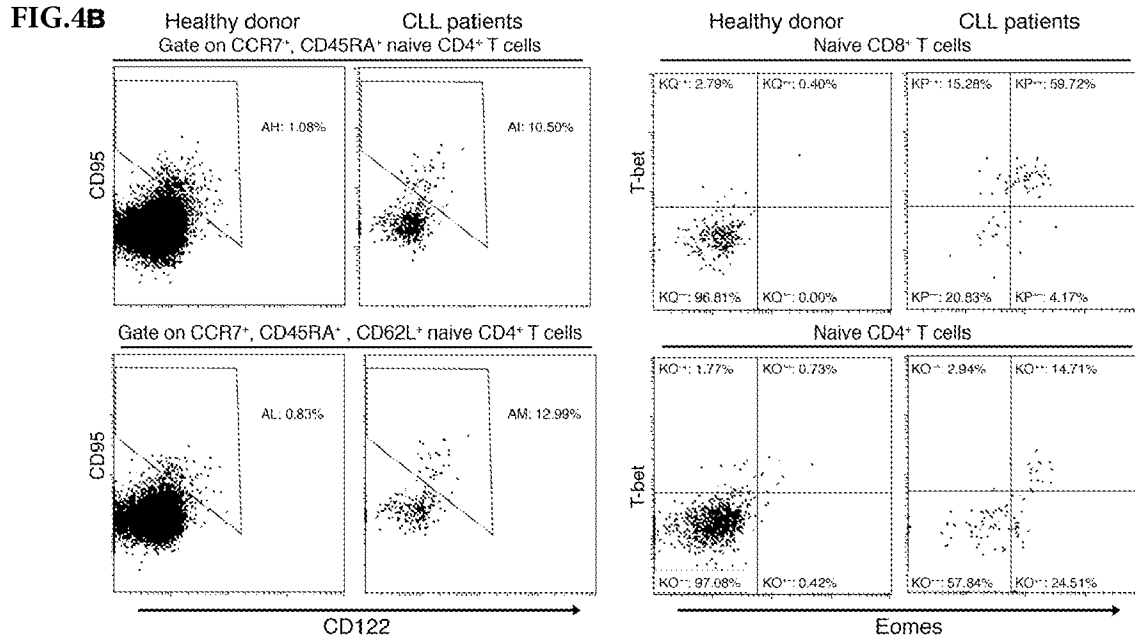
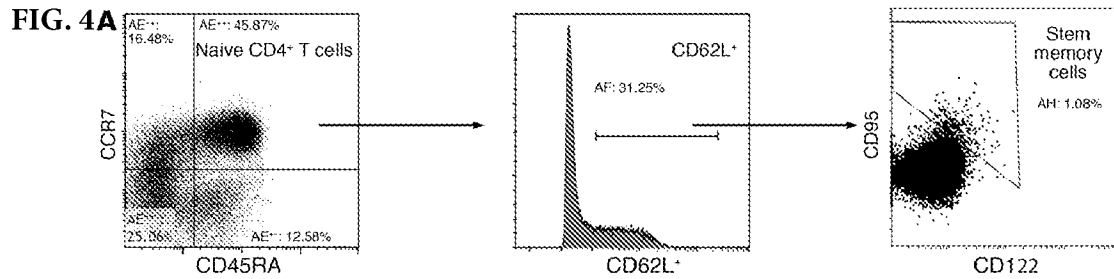


FIG. 4A, FIG. 4B, and FIG. 4C

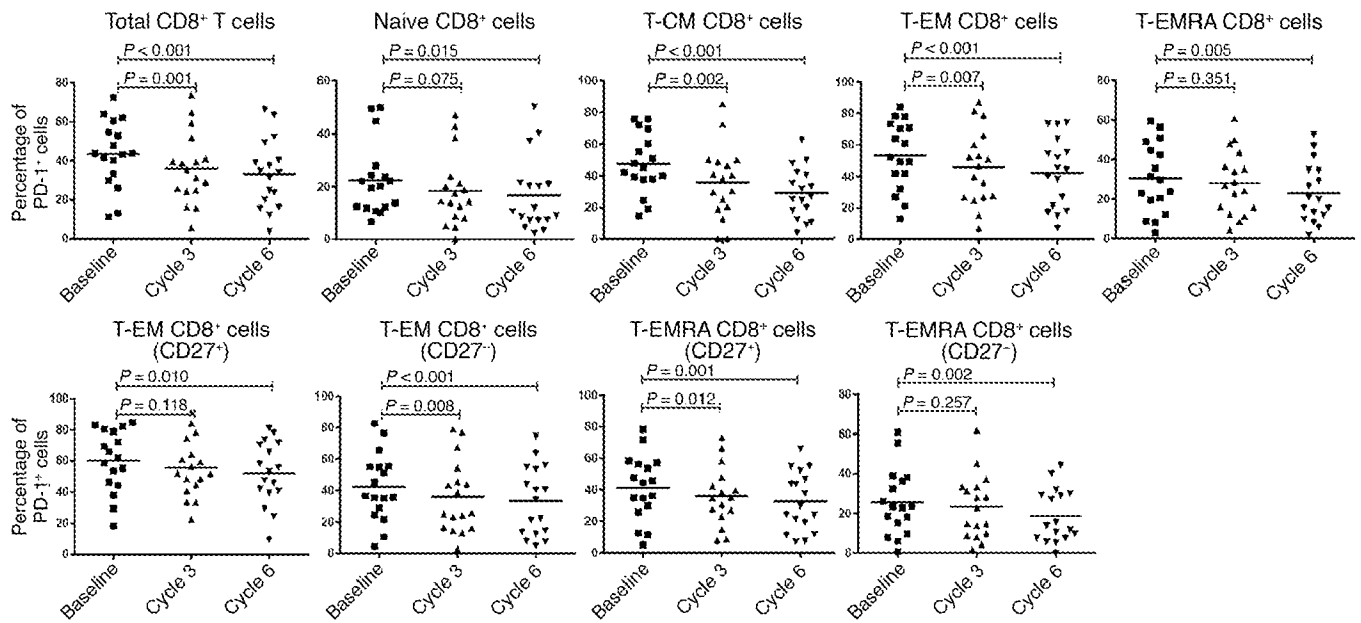
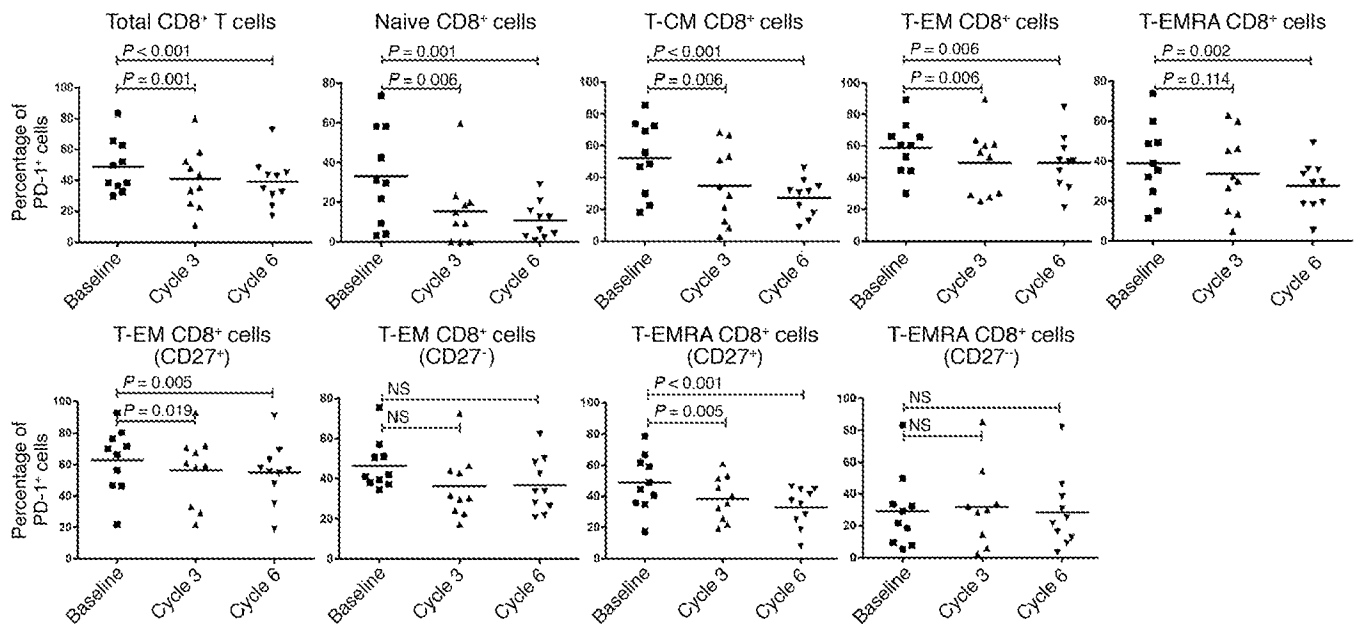
FIG. 5A Patients treated with ibrutinib**FIG. 5B** Patients treated with acalabrutinib**FIG. 5A and FIG. 5B**

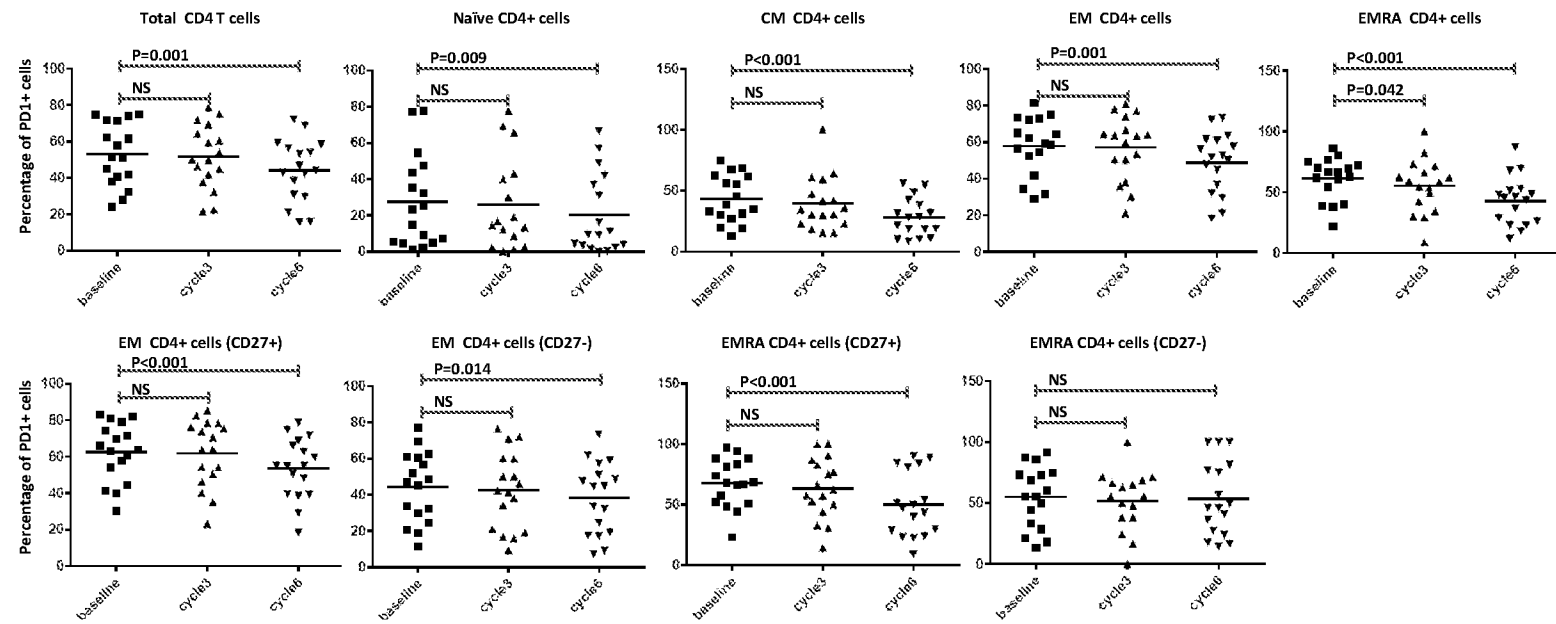
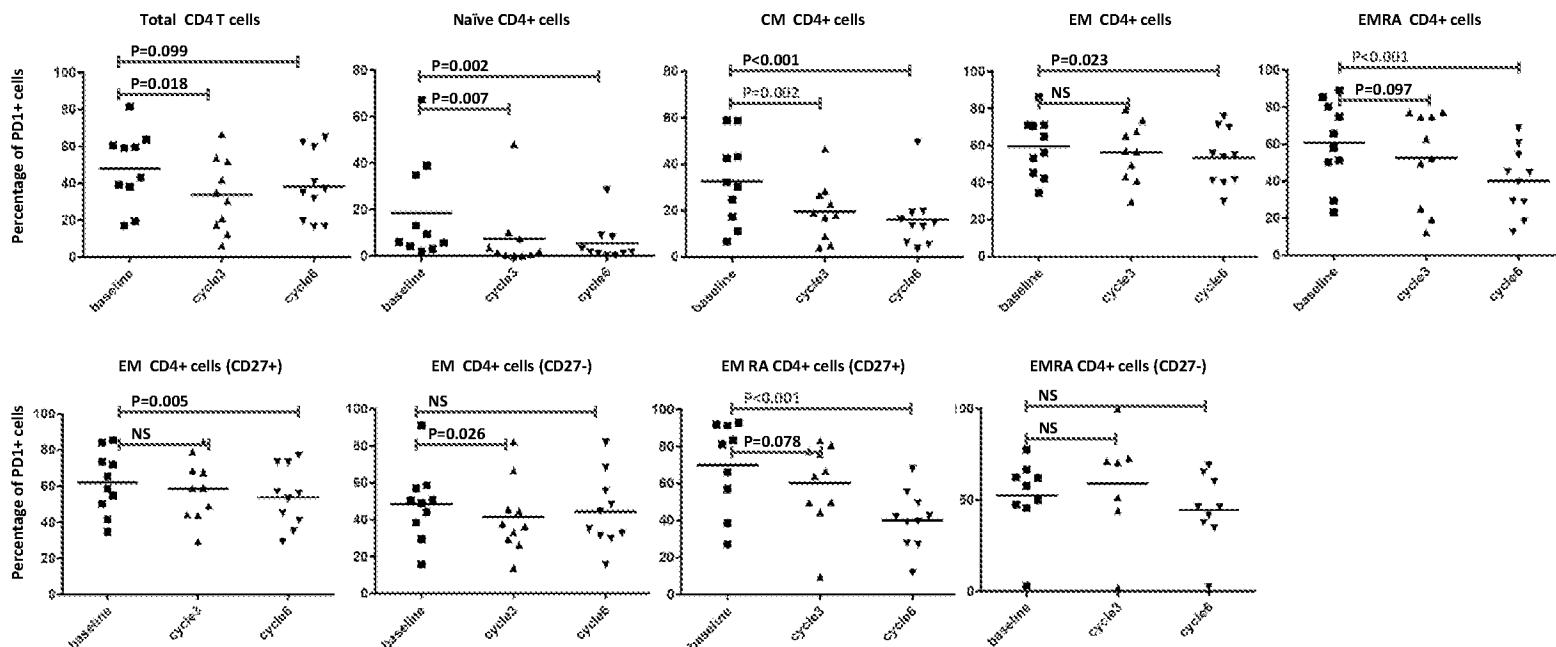
FIG. 6A. Patients treated with Ibrutinib**FIG. 6B.** Patients treated with acalabrutinib**FIG. 6A and FIG. 6B**

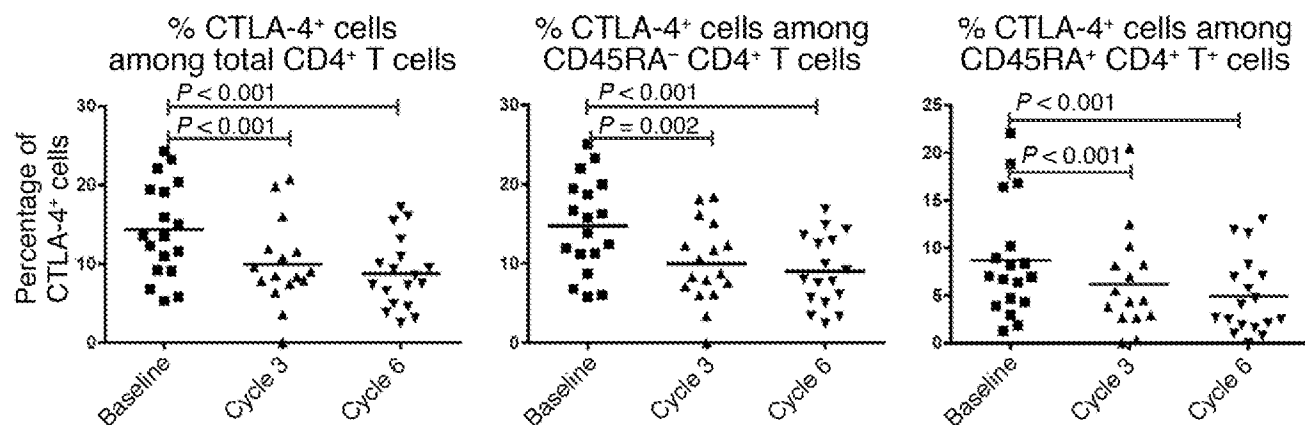
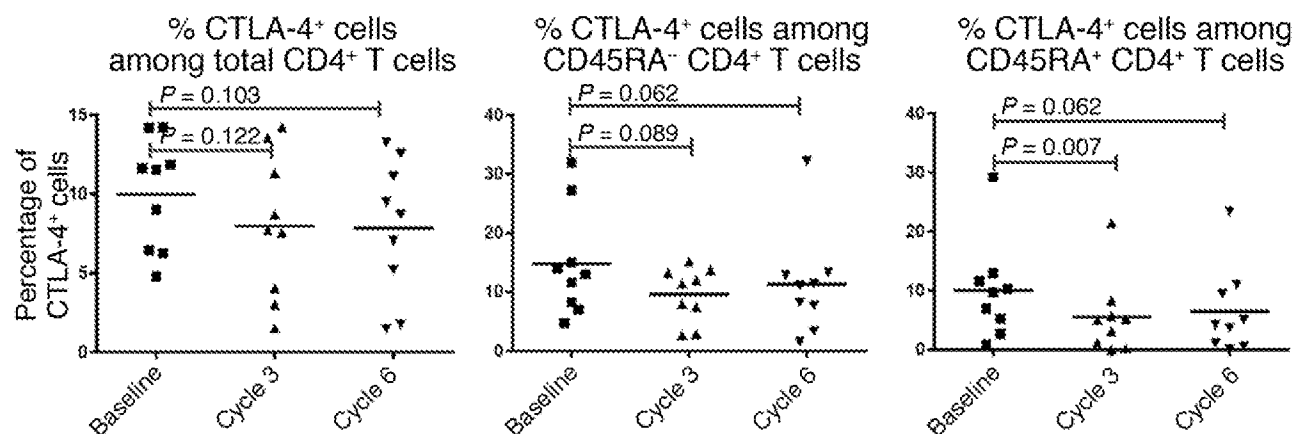
FIG. 7A Patients treated with ibrutinib**FIG. 7B** Patients treated with acalabrutinib**FIG. 7A and FIG. 7B**

FIG. 8A Patients treated with Ibrutinib

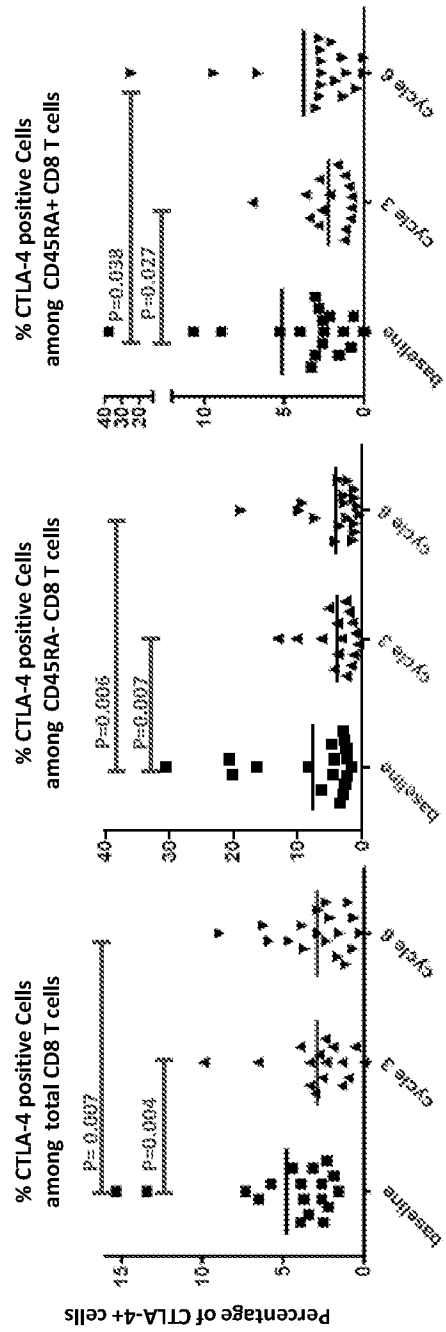


FIG. 8B Patients treated with acalabrutinib

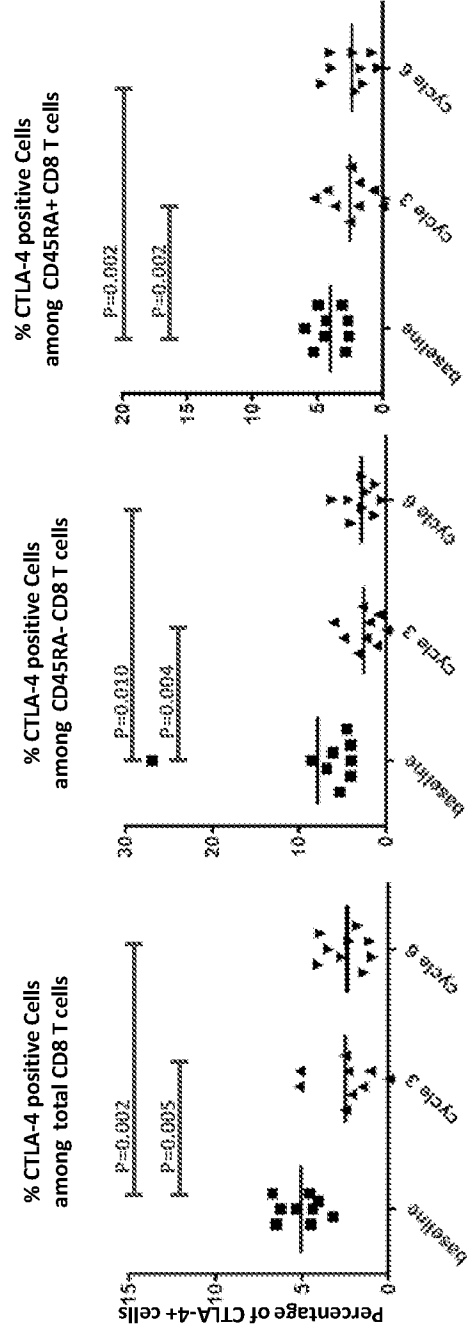


FIG 8A and FIG. 8B

FIG. 9A Patients treated with ibrutinib

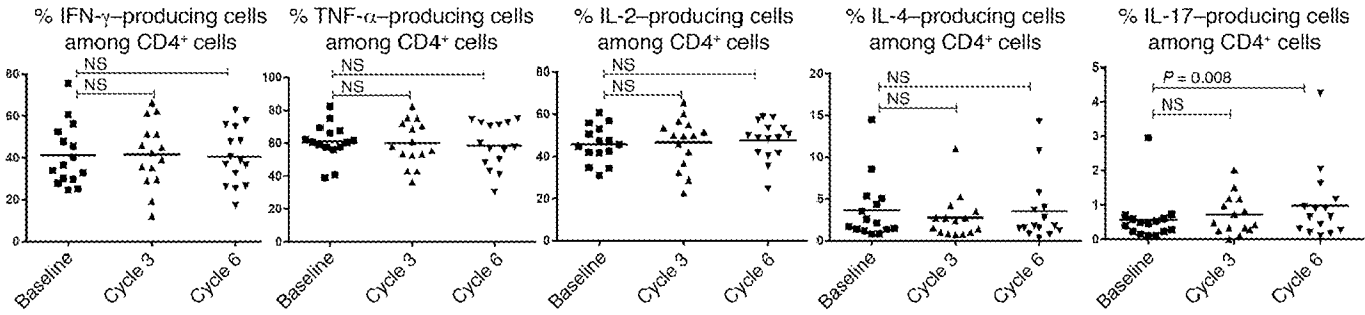


FIG. 9B Patients treated with acalabrutinib

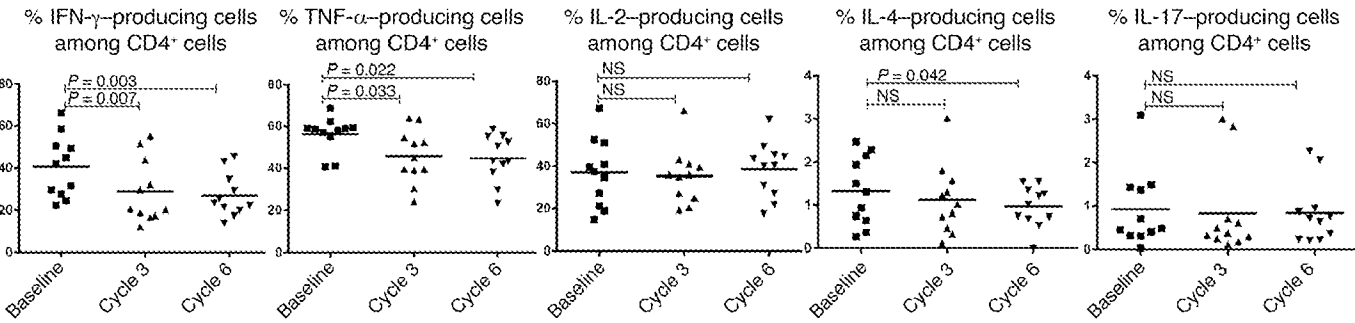


FIG. 9A and FIG. 9B

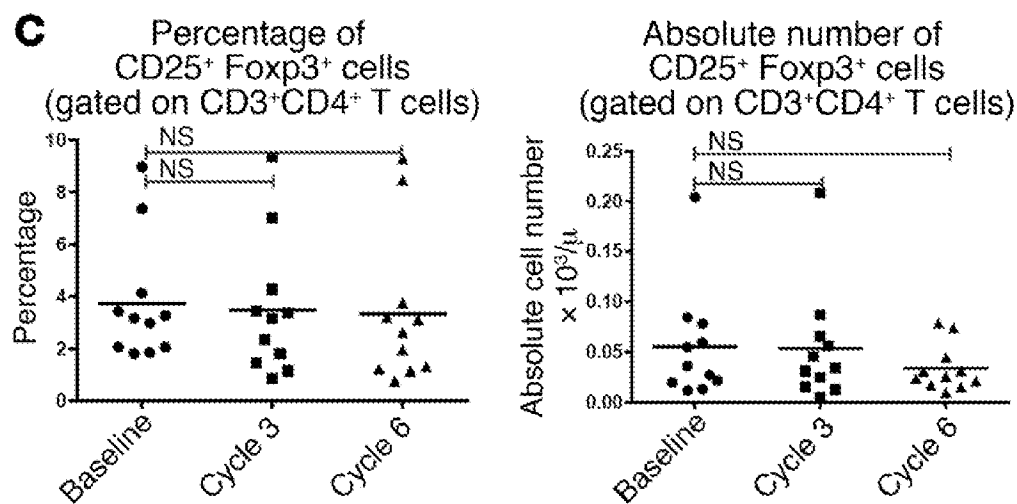
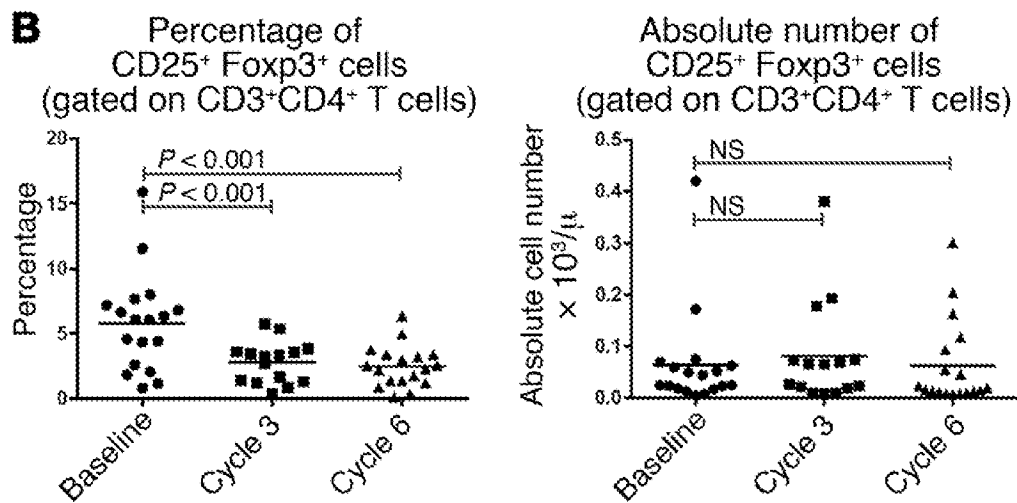
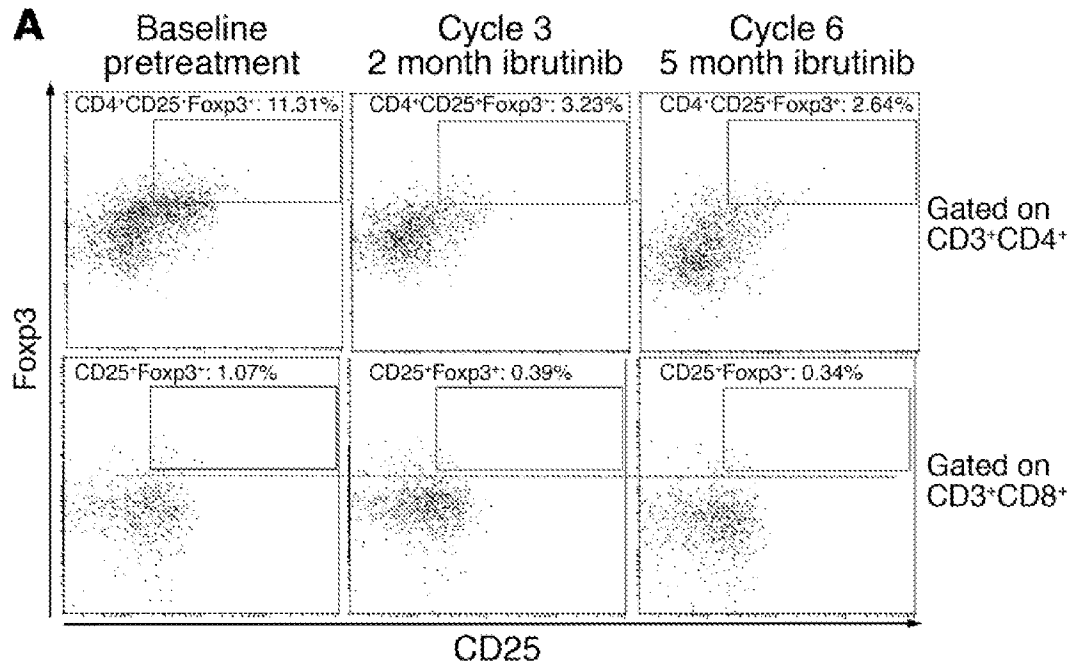
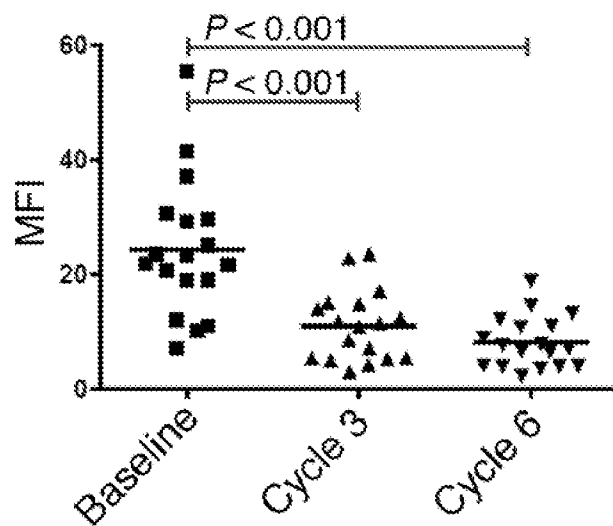


FIG. 10A, FIG. 10B, and FIG. 10C

CD200 expression level
FIG. 11A Ibrutinib treatment



BTLA expression level

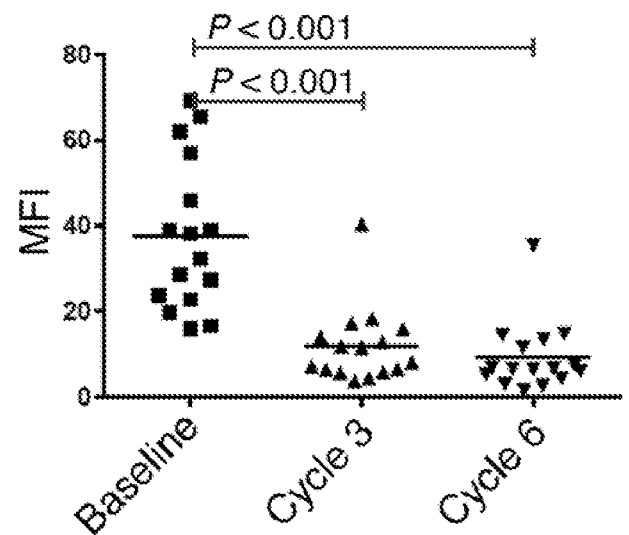


FIG. 11B Acalabrutinib treatment

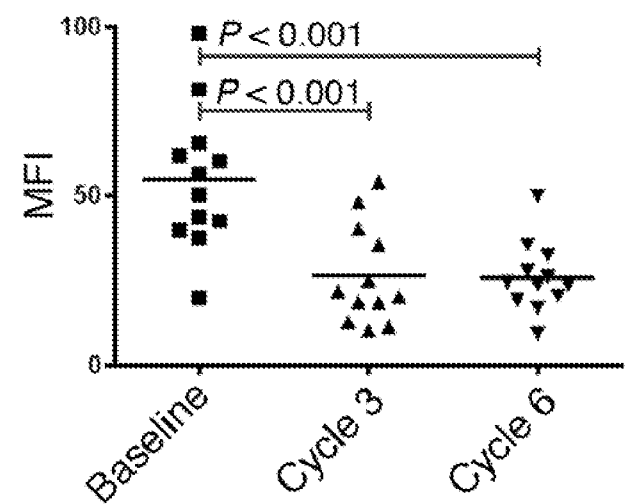
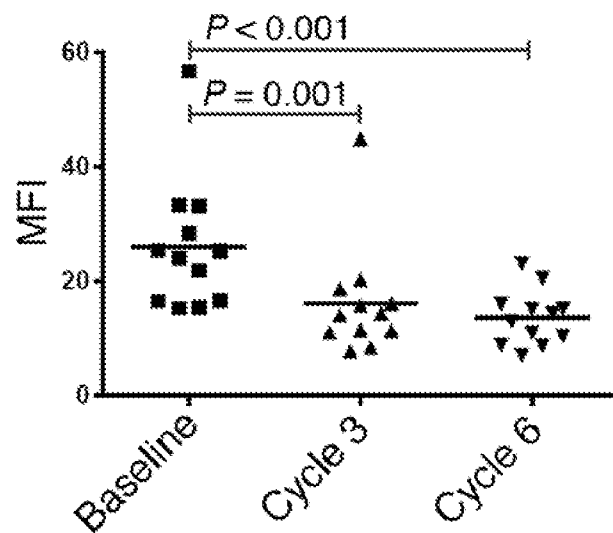


FIG. 11A and FIG. 11B

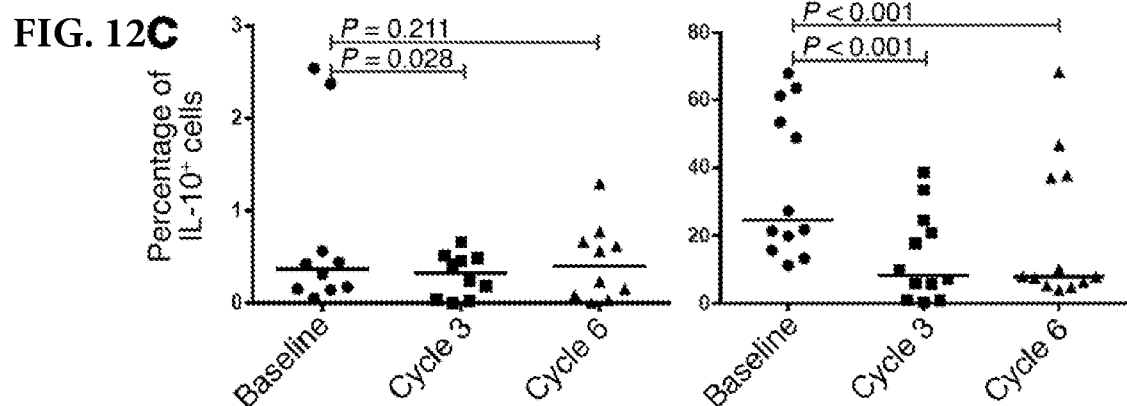
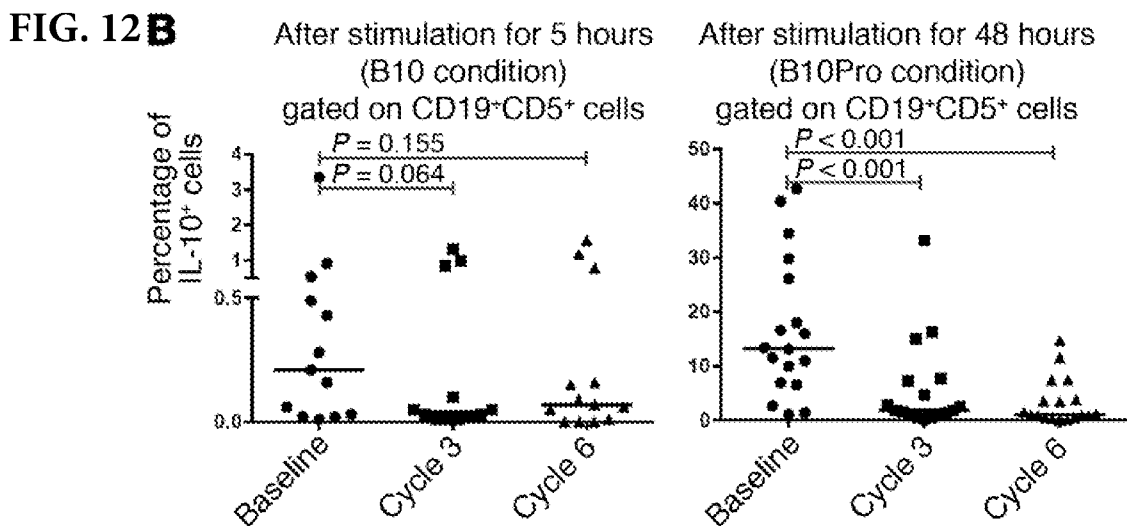
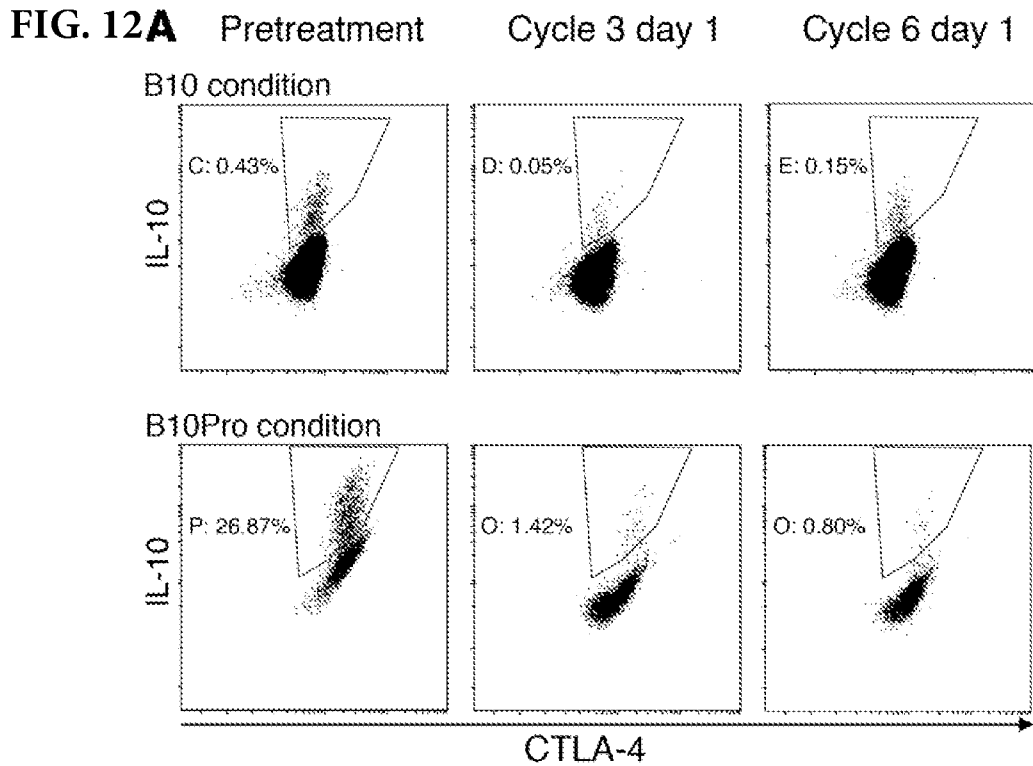


FIG. 12A, FIG. 12B, and FIG. 12C

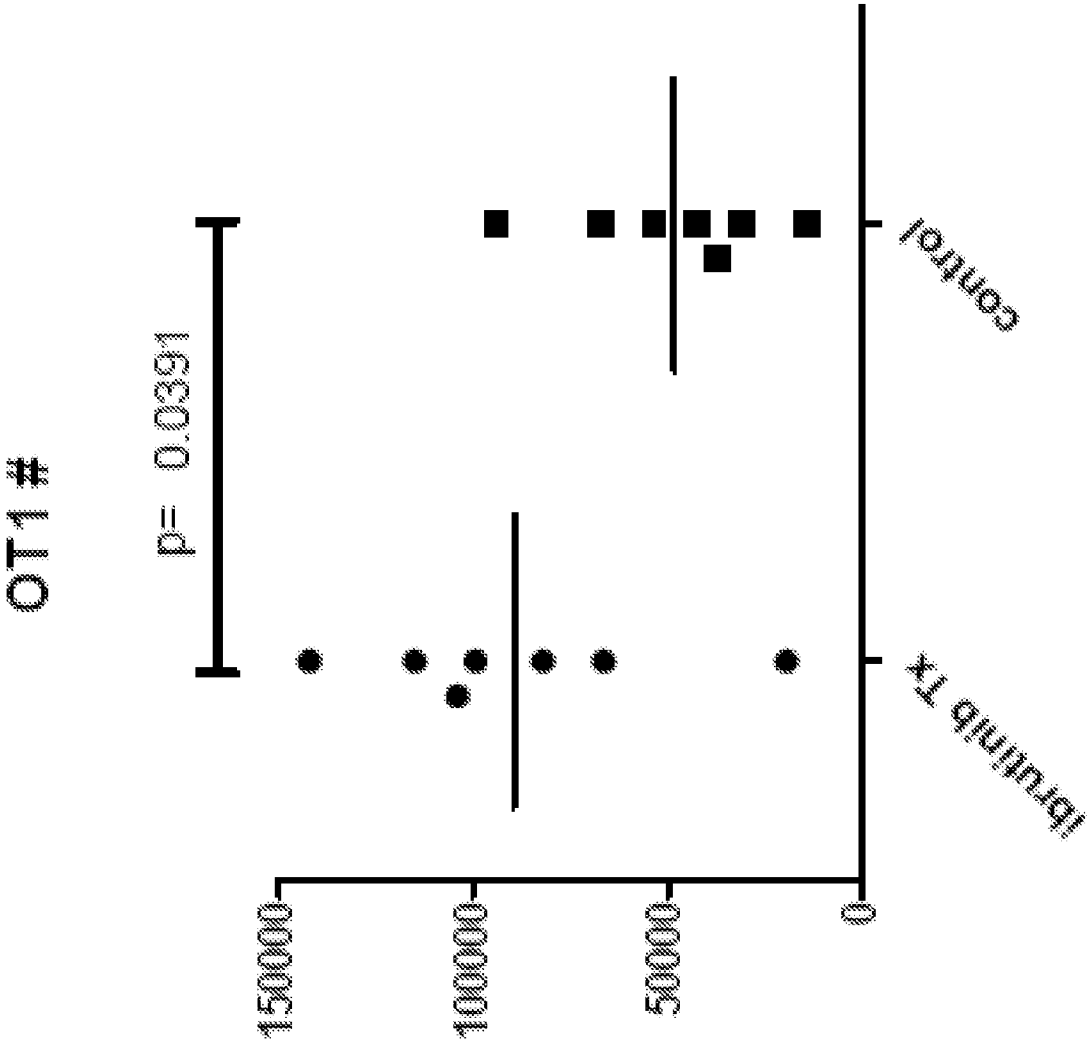


FIG. 13

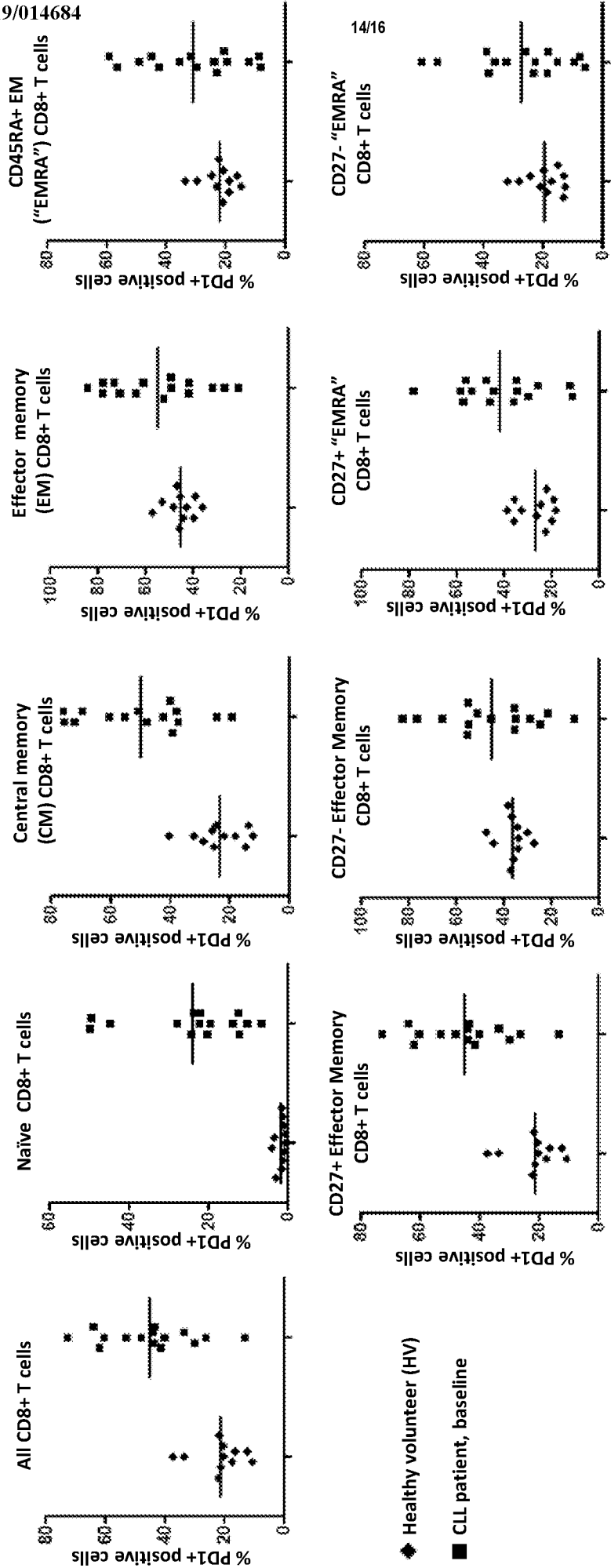


FIG. 14

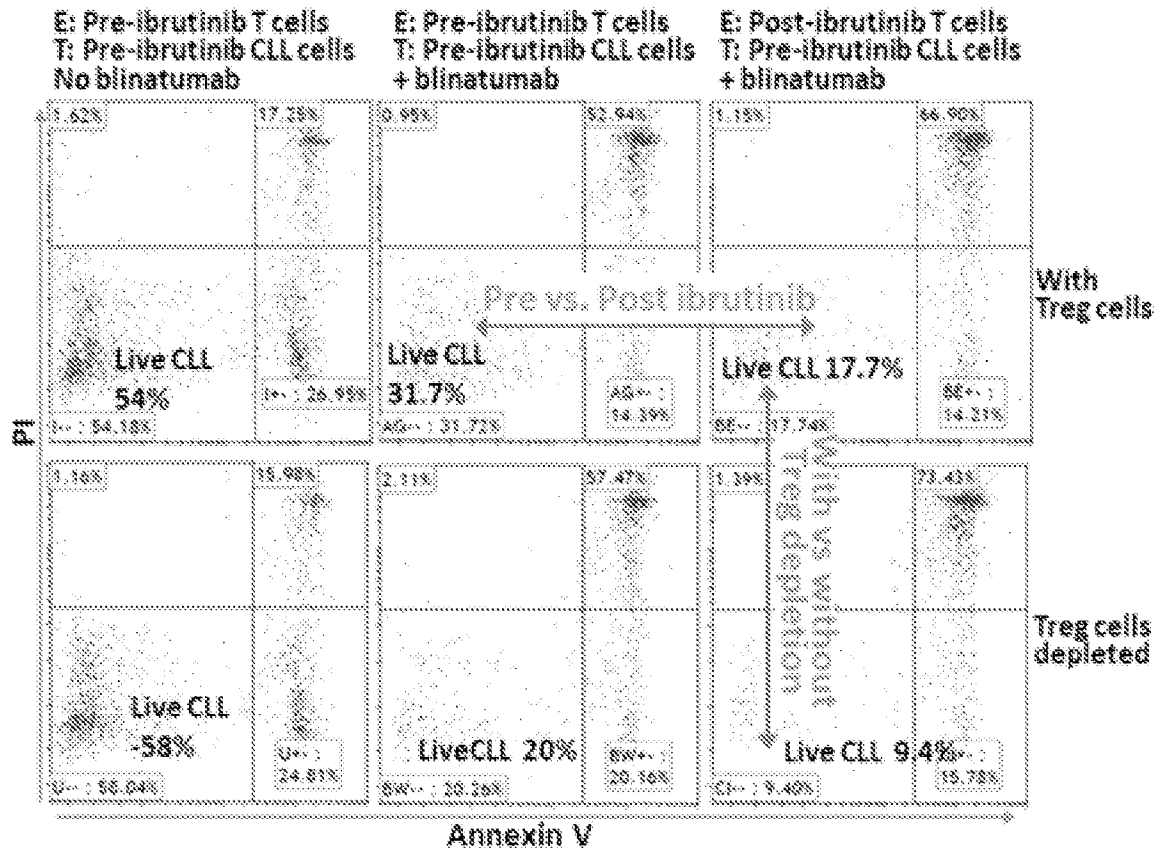


FIG. 15

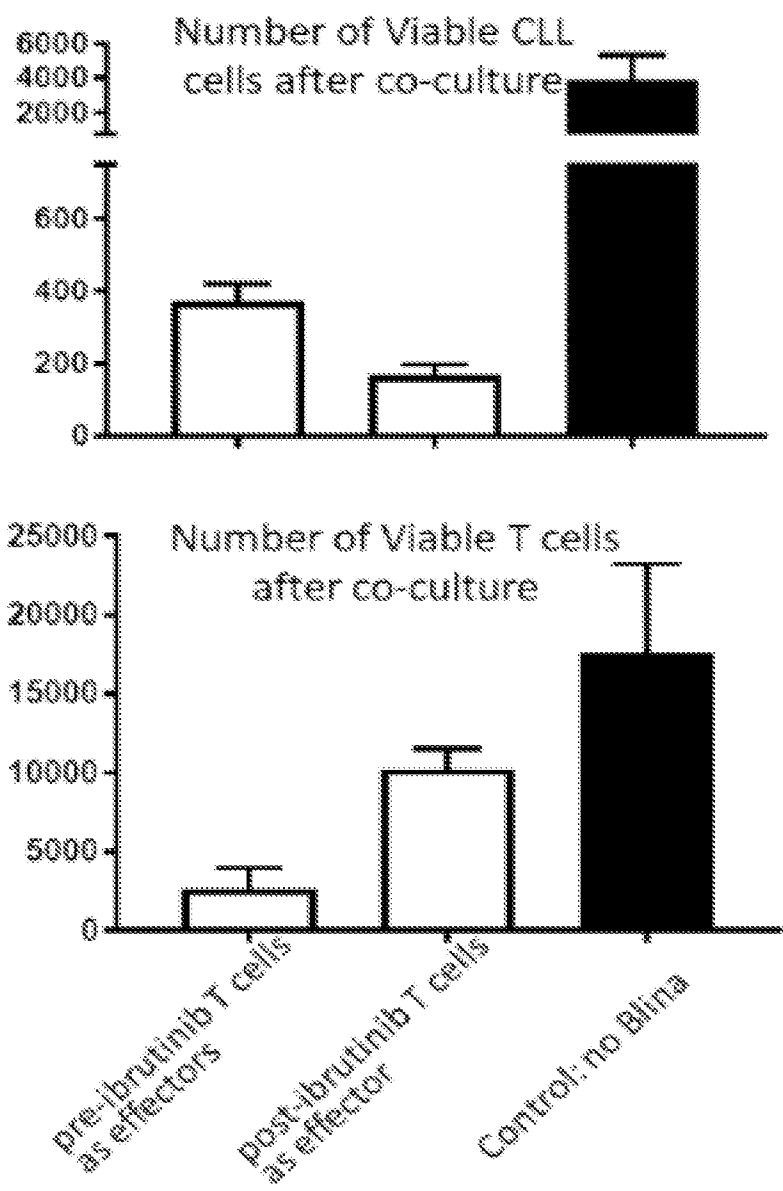


FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/42349

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/195, C12N 5/0789, C12N 5/09, C12N 5/00 (2018.01)

CPC - A61K 31/195, A61K 31/436, A61K 31/505, C12N 5/0647, C12N 5/0694, C12N 5/0093

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRAIETTA et al., Ibrutinib enhances chimeric antigen receptor T-cell engraftment and efficacy in leukemia. Blood, 3 March 2016, Vol. 127, No. 9, pp 1117-1127. Abstract; p1118, col 1, para 2; p1118, col 2, para 2; p1119, col 1, para 1-2; p1119, col 2, para 1-2; p1121, col 2, para 3; p1122, col 1, para 2; p1124, Figure 5 legend	10-12, 14, 17
Y		1-5, 13, 15
X	WO 2016/020901 A1 (ACERTA PHARMA BV) 11 February 2016 (11.02.2016) para [0081]; para [00189]	9
X	US 2014/0371241 A1 (PHARMACYCLICS INC.) 18 December 2014 (18.12.2014) para [0029]; para [0038]; para [0226]	16
Y		4
Y	KONDO et al., Ibrutinib Can Modulate the T Cell Response in Chronic Lymphocytic Leukemia By Reducing PD1/PDL1 Interactions. Blood, 2015, Vol. 126, No. 23, pp 1737. Abstract	1-4
Y	MILLER et al., Defective Fas ligand expression and activation-induced cell death in the absence of IL-2-inducible T cell kinase. J Immunol. 1 March 2002, Vol. 168, No. 5, pp 2163-72. Abstract	5



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

24 September 2018

Date of mailing of the international search report

19 NOV 2018

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/42349

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2016/044605 A1 (ENGELS et al.) 24 March 2016 (24.03.2016) Abstract; p260, ln 8-13; p261, ln 25-p262, ln 5; p290, ln 12-17	13, 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/42349

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

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

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

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

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

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

---Please see continuation in first extra sheet -----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5, 9-17

Remark on Protest

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

Continuation of Box No. III. Observations where unity of invention is lacking.

Group I, claims 1-5, 9-17, directed to methods of selectively expanding CD8 and CD4 T cells, inhibiting activation induced cell death of T cells, increasing the number of tumor infiltrating lymphocytes in a subject, increasing the cytotoxicity and survival of T cells, increasing the percentage of Th17 T cells and reducing checkpoint inhibition in a subject comprising administering to a subject an ITK inhibitor.

Group II, claims 6-8, directed to methods of expanding NK cells comprising contacting NK cells with a stimulatory molecule and ibrutinib and using expanded NK cells for treating cancer.

The inventions listed as Groups I-II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features:

Group I has the special technical feature of administering to a subject or cells therefrom an ITK inhibitor for selectively expanding T cell subsets, that is not required by Group II.

Group II has the special technical feature of contacting NK cells with a stimulatory molecule and ibrutinib, that is not required by Group I.

Common technical features:

Groups I-II share the common technical feature of expanding immune cells by contacting with a tyrosine kinase inhibitor, and treatment of cancer/tumor cells. However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is anticipated by the article entitled "Ibrutinib enhances chimeric antigen receptor T-cell engraftment and efficacy in leukemia" by Fraietta et al., (hereinafter 'Fraietta').

Fraietta teaches a method of selectively expanding CD8 and CD4 T cells in a subject comprising administering to a subject an agent that inhibits interleukin-2 inducible T cell kinase (ITK) improving treatment of cancer/tumor cells (Abstract - 'We found that .5 cycles of ibrutinib therapy improved the expansion of CD19-directed CAR T cells (CTL019)....Our collective findings indicate that ibrutinib enhances CAR T-cell function and suggest that clinical trials with combination therapy are warranted'; p1118, col 1, para 2 - 'For example, ibrutinib inhibits the interleukin (IL)-2 inducible T-cell kinase (ITK) in immunosuppressive T helper (Th)2-type CD4+ T cells'; p1119, col 1, para 1 - 'However, this proliferative defect was completely reversed after 5 to 11 cycles of ibrutinib therapy, and T-cell proliferation kinetics resembled those of young, healthy donors and were slightly improved compared with older multiple myeloma patients (Figure 2A). Similarly, T-cell activation and secretion of interferon (IFN)-g upon stimulation with CD3/CD28 beads and phorbolmyristate acetate/ionomycin were improved after 5 to 11 cycles of treatment, particularly in the CD8 T-cell compartment (Figure 2B).... Thus, prolonged ibrutinib treatment of CLL patients reverses the defects in T-cell function, particularly proliferation in response to T-cell receptor stimulation.').

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I-II inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.