DOI:
10.1182/blood.2023020554

Document Version
Peer reviewed version

American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
editorial@hematology.org

Ibrutinib-based therapy reinvigorates CD8 T cells compared to chemoimmunotherapy: immune-monitoring from the E1912 trial

Tracking no: BLD-2023-020554R1

Despoina Papazoglou (Lymphoma Immunology Group, School of Cancer and Pharmaceutical Sciences, Faculty of Life Sciences & Medicine, King's College London, United Kingdom) Victoria Wang (Dana-Farber Cancer Institute, United States) Tait Shanafelt (Stanford University School of Medicine, United States) Connie Lesnick (Mayo Clinic,) Nikolaos Ioannou (King's College, United Kingdom) Giulia De Rossi (University College London, United Kingdom) Sylvia Herter (Roche Glycart AG, Switzerland) Marina Bacac (Roche Pharmaceutical Research and Early Development, Switzerland) Christian Klein (Roche Glycart AG, Switzerland) Martin Tallman (Northwestern University Feinberg School of Medicine, Robert H. Lurie Comprehensive Cancer Center, United States) Neil Kay (Mayo Clinic, United States) Alan Ramsay (King's College London, United Kingdom)

Abstract:
Bruton’s tyrosine kinase Inhibitors (BTKis) that target B cell receptor signaling have led to a paradigm shift in CLL treatment. BTKis have been shown to reduce abnormally high CLL-associated T cell counts and the expression of immune checkpoint receptors concomitantly with tumor reduction. However, the impact of BTKi therapy on T cell function has not been fully characterized. Here, we performed longitudinal immunophenotypic and functional analysis of pre- and on-treatment (6- and 12-months) peripheral blood samples from patients in the phase 3 E1912 trial comparing ibrutinib-rituximab to fludarabine, cyclophosphamide and rituximab (FCR). Intriguingly, we report that despite reduced overall T cell counts, higher numbers of T cells including effector $CD8^+$ subsets at baseline and at the 6-month time-point associated with no infections and favorable progression-free survival (PFS) in the ibrutinib-rituximab arm. Assays demonstrated enhanced anti-CLL T cell killing function during ibrutinib-rituximab, including a switch from predominantly $CD4^+$ T-cell:CLL immune synapses at baseline to increased $CD8^+$ lytic synapses on-therapy. Conversely, in the FCR arm, higher T cell numbers correlated with adverse clinical responses and showed no functional improvement. We further demonstrate the potential of exploiting rejuvenated T cell cytotoxicity during ibrutinib-rituximab using the bispecific antibody glofitamab - supporting combination immunotherapy approaches.

Conflict of interest: COI declared - see note

COI notes: S.H., M.B. and C.K. are employees of and declare patents and stock ownership with Roche Glycart AG. A.G.R. has received research support from Roche Glycart AG to Institution as part of a research collaboration. The remaining authors have declared that no conflict of interest exists. This work received research support from the National Cancer Institute of the National Institutes of Health (NIH) under the following award: RO1CA193541.

Preprint server: No;

Author contributions and disclosures: Contributions: D.P. designed, performed experimental work, data analysis and wrote the manuscript. X.V.W. performed data and association analysis. C.E.L. and N.I. performed experimental work. G.D.R. contributed to image analysis. T.D.S., N.E.K., M.S.T. designed, supervised the study, and managed data. S.H., M.B. and C.K. provided the bispecific antibodies and advised on the study. A.G.R. designed and supervised the study and wrote the manuscript. Disclosures: S.H., M.B. and C.K. are employees of and declare patents and stock ownership with Roche Glycart AG. The remaining authors have declared that no conflict of interest exists. This work received research support from the National Cancer Institute of the National Institutes of Health (NIH) under the following award: RO1CA193541.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: Data sharing: For original data and protocols, please contact the corresponding author.

Clinical trial registration information (if any):

Ibrutinib-based therapy reinvigorates CD8$^+$ T cells compared to chemoimmunotherapy: immune-monitoring from the E1912 trial

Despoina Papazoglou,[1] Xin Victoria Wang, [2,3] Tait D. Shanafelt,[4] Connie E. Lesnick,[5] Nikolaos Ioannou,[1] Giulia De Rossi,[6] Sylvia Herter,[7] Marina Bacac,[7] Christian Klein,[7] Martin S. Tallman,[8] Neil E. Kay,[5] and Alan G Ramsay.[1*]

[1] School of Cancer and Pharmaceutical Sciences, Faculty of Life Sciences & Medicine, King's College London, London, UK

[2] Dana-Farber Cancer Institute, Boston, Massachusetts, USA

[3] Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA

[4] Stanford University, School of Medicine, Stanford, California, USA

[5] Mayo Clinic, Department of Immunology, Rochester, Minnesota, USA

[6] Institute of Ophthalmology, University College London, London, UK

[7] Roche Innovation Center Zürich, Schlieren, Switzerland

[8] Memorial Sloan-Kettering Cancer Center, New York, USA

***Corresponding author**:

Dr. Alan G. Ramsay,

Lymphoma Immunology,

Innovation Hub, Guy's Cancer Centre

Great Maze Pond

London, SE1 9RT, UK

Email: alan.ramsay@kcl.ac.uk

Phone: +44 77 192 295 78

Data sharing

For original data and protocols, please contact the corresponding author.

Presented during the 60th annual meeting of the American Society of Hematology, San Diego, CA, 1-4 December 2018 (CLL: Biology and Pathophysiology, excluding Therapy: Overcoming Immunodeficiency in CLL).

Running title: Ibrutinib-based therapy reinvigorates $CD8^+$ T cells

Abstract word count: **200**

Brief Report Manuscript (Introduction, Methods, Results and Discussion) word count: **1200**

Key Points:

- Higher effector T cell numbers and their rejuvenated cytotoxic function accompanies favorable clinical responses to ibrutinib-rituximab.
- Enhanced $CD8^+$ T cell lytic synapse activity during ibrutinib-rituximab can be exploited using the bispecific antibody glofitamab.

Abstract

Bruton's tyrosine kinase Inhibitors (BTKis) that target B cell receptor signaling have led to a paradigm shift in CLL treatment. BTKis have been shown to reduce abnormally high CLL-associated T cell counts and the expression of immune checkpoint receptors concomitantly with tumor reduction. However, the impact of BTKi therapy on T cell function has not been fully characterized. Here, we performed longitudinal immunophenotypic and functional analysis of pre- and on-treatment (6- and 12-months) peripheral blood samples from patients in the phase 3 E1912 trial comparing ibrutinib-rituximab to fludarabine, cyclophosphamide and rituximab (FCR). Intriguingly, we report that despite reduced overall T cell counts, higher numbers of T cells including effector $CD8^+$ subsets at baseline and at the 6-month time-point associated with no infections and favorable progression-free survival (PFS) in the ibrutinib-rituximab arm. Assays demonstrated enhanced anti-CLL T cell killing function during ibrutinib-rituximab, including a switch from predominantly $CD4^+$ T-cell:CLL immune synapses at baseline to increased $CD8^+$ lytic synapses on-therapy. Conversely, in the FCR arm, higher T cell numbers correlated with adverse clinical responses and showed no functional improvement. We further demonstrate the potential of exploiting rejuvenated T cell cytotoxicity during ibrutinib-rituximab using the bispecific antibody glofitamab - supporting combination immunotherapy approaches.

Introduction

E1912 was the first frontline phase 3 study to compare ibrutinib-based therapy (ibrutinib with rituximab) to the chemoimmunotherapy fludarabine, cyclophosphamide and rituximab (FCR).[1] Long-term follow-up has demonstrated superior PFS and OS for ibrutinib-rituximab relative to FCR.[2] Nevertheless, clinical challenges remain including the current need for continuous therapy, tolerability, residual and progressive disease. Immunotherapy represents a powerful combination or alternative therapy to tackle resistant disease and deepen responses.[3,4] However, T cell 'exhaustion' is a major barrier for optimal immunotherapy.[5,6] Dysfunctionality of T cells is characterized by increased CLL-associated T cell subsets expressing inhibitory checkpoint molecules.[7] Furthermore, helper $CD4^+$ T cells have a tumor-promoting capacity, while impaired immune synapse formation contributes to suppressed $CD8^+$ T cell cytotoxicity.[8] Correlative studies have revealed that BTKis reduce abnormally high T cell numbers and checkpoint receptor expression, while reducing malignant B cells.[9-13] However, the impact of BTKis on T cell function and association with clinical response is less well defined. Here, we leverage pre-, and on-treatment peripheral blood patient samples serially collected from the E1912 trial and report on the impact of ibrutinib-rituximab versus FCR on T cells utilizing immune-monitoring functional assays.

Methods

CLL patient samples. Viable PBMCs at baseline, 6-, 12- and 18-month time-points from ECOG-ACRIN-E1912 (**supplemental Figure 1**) were biobanked for longitudinal comparative immune analysis. **Supplemental Table 1**,**2**[1,2] summarize the samples studied (ibrutinib-rituximab, n=89; FCR, n=62).

Immune-monitoring and functional assays are detailed in supplemental Methods.

Results and discussion

T cell monitoring and correlation with PFS, measurable residual disease (MRD) and infections

We initially investigated the impact of therapy on T cells and explored the association with clinical outcome (**Figure 1A**). Flow cytometry measured the absolute numbers of naïve (T_{naive}, $CD45RA^+/CCR7^+$), central memory (T_{CM}, $CD45RA^-/CCR7^+$), effector memory (T_{EM}, $CD45RA^-/CCR7^-$) and terminally differentiated effector memory (T_{EMRA}, $CD45RA^+/CCR7^-$) subsets in patients at baseline, 6-month and 12-month treatment time-points (**supplemental Table 3**). This analysis revealed a reduction in the majority of $CD4^+$ and $CD8^+$ T cell subsets during ibrutinib-rituximab including naive and effectors (**Figure 1B**; **supplemental Figure 2A**,**C**) - consistent with T cell normalization as previously reported for monotherapy.[10,13] Expectedly, we observed a marked decrease of subsets following FCR, with evidence of immune reconstitution at 12-months (**Figure 1B**; **supplemental Figure 2B**,**D**).[14] The frequencies of subsets remained relatively stable during ibrutinib-rituximab, whereas FCR caused T_{naive} and T_{CM} to contract, while T_{EM} expanded (**Supplemental Figure 2E**,**F**). Both therapies reduced the number of T_{Reg}, T_H17 cells and NK cells compared to baseline, but an increased T_{Reg}/CD4 ratio post-FCR was observed (**supplemental Figure 3A-C**).[15] Strikingly, patients on ibrutinib-rituximab with higher T cell numbers including PD-1^+ effector $CD8^+$ and $CD4^+$ subsets at baseline had longer PFS (**Figure 1D** and **F**), suggesting the importance of an existent but exhausted immune response before therapy. Interestingly, higher levels of PD-L1-expressing CLL cells at baseline correlated with favorable PFS (**Figure 1D** and **F,** and multivariable analysis in **Supplemental Table 4**). Furthermore, an elevated frequency of effector $CD8^+$ T cells at the 6-month ibrutinib-rituximab time-point associated with favorable PFS, whereas no association was detectable at 12-months (**Figure 1D** and **F**). Conversely, higher T cell numbers correlated with worse PFS in the FCR arm, whereas increased NK cell frequency at baseline associated with favorable outcome (**supplemental Figure 4A**). Consistent with tumor-mediated exhaustion, greater numbers of PD-1^+ and PD-$L1^+$ $CD8^+$ T cell subsets associated with higher MRD during ibrutinib-rituximab (**supplemental Figure 5A**). In contrast, elevated frequencies of T cell

subsets not expressing checkpoint molecules including $CD8^+$ T_{EMRA} and NK cells correlated with low MRD during ibrutinib-rituximab, in keeping with reduced exhaustion. An association between T cells and MRD was less evident in the FCR arm, except for increased checkpoint-expressing T cells at 12-months that correlated with higher MRD (**supplemental Figure 5B**). Ibrutinib's inhibition of ITK enhanced T_H1 polarization,[16,17] but both therapeutic arms reduced T_H1 and T_H2 numbers and T_H1/T_H2 ratios (**supplemental Figure 6A**,**B**). Nevertheless, an increased frequency of T_H2 and CD4/CD8 ratio (baseline and 6-months) associated with unfavorable PFS and incidence of infection respectively during ibrutinib-rituximab (**Figure 1F**,**G**). Whereas increased effector $CD8^+$ T cell numbers and $CD16^+$ NK cells at 6-months was associated with no infections during ibrutinib-rituximab (**Figure 1G**). Conversely, increased T cells post-FCR correlated with infections (**supplemental Figure 4B**). In sum, higher $CD8^+$ T cell numbers at baseline and early on-therapy, associated with favorable clinical responses, while PD-1^+, PD-$L1^+$ subsets associated with greater MRD during ibrutinib-rituximab.

Ibrutinib-rituximab promotes $CD8^+$ synapses and immunotherapy-triggered killing function

Next, we characterized the cytolytic function of therapy-reshaped T cells against baseline CLL cells (**Figure 2A**,**B**). T cells from both 6- and 12-month ibrutinib-rituximab time-points showed enhanced killing function compared to pre-treatment levels. In contrast, T cells post-FCR showed no cytolytic improvement. Notably, patients who experienced grade 3 infections during ibrutinib-rituximab showed lower anti-CLL T cell cytotoxic function (**Figure 2C**). Hypothesizing altered T cell:CLL interactions, we next performed conjugation assays. T cells during ibrutinib-rituximab showed augmented formation of polarized F-actin synapses with baseline CLL cells (**Figure 2D**; **supplemental Figure 7A**). In comparison, T cells post-FCR exhibited distinctly non-polarized synapses (**Figure 2E**). Given the opposing roles of patient $CD4^+$ and $CD8^+$ T cells,[8] we examined these subsets and detected an increased frequency of granzyme B^+ $CD8^+$ T cell:CLL synapses at both ibrutinib-rituximab time-points compared to baseline, where $CD4^+$ T cell:CLL synapses dominated. This switch in the $CD4^+/CD8^+$ synapse balance was not detected post-FCR (**Figure 2F**,**G**; **supplemental Figure 7B-D**). Interestingly, in

keeping with pro-tumoral $CD4^+$ T cells, increased formation of $CD4^+$ T cell:CLL F-actin$^+$ synapses at baseline correlated with unfavorable PFS and grade 3 infections during ibrutinib-rituximab (**Figure 2H**; **supplemental Figure 7E).** Together, these data demonstrate that ibrutinib-rituximab promotes previously exhausted $CD8^+$ T cell activity, that could provide a gateway for immunotherapy.

Ibrutinib is known to reduce PD-1 expression on patient T cells.[9,10,18] Here, we detected a reduced frequency of PD-1-expressing T cell subsets during ibrutinib-rituximab, as well as PD-L1-expressing T cells except for $CD8^+$ T_{EM} at 6-months (**Figure 1D,E**; **Supplemental Figure 8**). In contrast, T cell PD-1/PD-L1 expression was relatively unaffected following FCR. This prompted us to investigate checkpoint blockade in our cytotoxicity assay (**Figure 2I-K**; **Supplemental Figure 9**). Both ibrutinib-rituximab- and FCR-exposed T cells were insensitive to anti-PD-1. Whereas anti-PD-L1[19] increased anti-CLL T cell cytotoxicity at the 6-month ibrutinib-rituximab time-point only, suggesting a narrow window for checkpoint blockade activity.[17] This led us to investigate whether the T cell-engaging bispecific antibody glofitamab (CD20xCD3)[20] could trigger improved cytolytic responses. T cells from all ibrutinib-rituximab time-points tested up to 18-months showed increased anti-CLL T cell killing following glofitamab treatment, compared to baseline (**Figure 2L-N**). However, T cells post-FCR did not respond to glofitamab, including the later time-point. Overall, these data support the ability of ibrutinib-based therapy to enhance T cell-mediated cytotoxicity induced by bispecific immunotherapy.

In summary, our data highlights the importance of T cells during ibrutinib-rituximab, with higher T cell numbers and rejuvenated cytotoxicity accompanying favorable clinical responses. Our exploratory findings that increased levels of PD-1-expressing T cells, as well as PD-L1-expressing CLL cells prior to therapy associate with longer PFS, suggests that ibrutinib-rituximab appears to capitalize on T cell-mediated immune surveillance in patients. Strikingly, opposing associations were found in the chemoimmunotherapy arm and T cells showed no functional improvement post-FCR. Previous studies

have reported $CD8^+$ T clonotype expansion during ibrutinib therapy,[21,22] likely reflecting active immunosurveillance. Taken together, tumor debulking and alleviation of T cell exhaustion during BTKi-based therapy,[9-13] may promote $CD8^+$ T cell activity. The switch from $CD4^+$ T cell:CLL interactions at baseline to $CD8^+$ lytic synapses during ibrutinib-rituximab supports this concept. Although ibrutinib-rituximab did not increase T_H1 numbers, we do not exclude ITK inhibition contributing to beneficial immunomodulation.[18] Furthermore, our longitudinal assays designed to evaluate changes in T cell cytolytic function with therapy, revealed that revitalized cytotoxicity during ibrutinib-rituximab could be maximized with glofitamab, further supporting combination immunotherapy approaches.[23-25] Overall, this report underscores the importance of trial-associated science to understand how BTKis modulate T cells and supports the development of immunotherapy-based therapies.

Acknowledgements

The authors thank the Nikon Imaging Facility at King's College London. This study was coordinated in part by the ECOG-ACRIN Cancer Research Group (Peter J. O'Dwyer MD and Mitchell D. Schnall, MD, PhD, Group Co-Chairs) and supported by the National Cancer Institute of the National Institutes of Health (NIH) under the following award numbers: RO1CA193541, U10CA180820, UG1CA232760, UG1CA233290, U10CA180794 and RO1CA251801. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Authorship contributions

D.P. designed, performed experimental work, data analysis and wrote the manuscript. X.V.W. performed data and association analysis. C.E.L. and N.I. performed experimental work. G.D.R. contributed to image analysis. T.D.S., N.E.K., M.S.T. designed, supervised the study, and managed data. S.H., M.B. and C.K. provided the bispecific antibodies and advised on the study. A.G.R. designed and supervised the study and wrote the manuscript.

Disclosure of Conflicts of Interest

S.H., M.B. and C.K. are employees of and declare patents and stock ownership with Roche Glycart AG. A.G.R. has received research support from Roche Glycart AG to Institution as part of a research collaboration. The remaining authors have declared that no conflict of interest exists. This work received research support from the National Cancer Institute of the National Institutes of Health (NIH) under the following award: RO1CA193541.

References

1. Shanafelt TD, Wang XV, Kay NE, et al. Ibrutinib-Rituximab or Chemoimmunotherapy for Chronic Lymphocytic Leukemia. N Engl J Med. 2019;381(5):432-443.
2. Shanafelt TD, Wang XV, Hanson CA, et al. Long-term Outcomes for Ibrutinib-Rituximab and Chemoimmunotherapy in CLL: Updated Results of the E1912 Trial. Blood. 2022.
3. Skånland SS, Mato AR. Overcoming resistance to targeted therapies in chronic lymphocytic leukemia. Blood Adv. 2021;5(1):334-343.
4. Mhibik M, Wiestner A, Sun C. Harnessing the Effects of BTKi on T Cells for Effective Immunotherapy against CLL. Int J Mol Sci. 2019;21(1).
5. Ding W, LaPlant BR, Call TG, et al. Pembrolizumab in patients with CLL and Richter transformation or with relapsed CLL. Blood. 2017;129(26):3419-3427.
6. Younes A, Brody J, Carpio C, et al. Safety and activity of ibrutinib in combination with nivolumab in patients with relapsed non-Hodgkin lymphoma or chronic lymphocytic leukaemia: a phase 1/2a study. Lancet Haematol. 2019;6(2):e67-e78.
7. Palma M, Gentilcore G, Heimersson K, et al. T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers. Haematologica. 2017;102(3):562-572.
8. Roessner PM, Seiffert M. T-cells in chronic lymphocytic leukemia: Guardians or drivers of disease? Leukemia. 2020;34(8):2012-2024.
9. Long M, Beckwith K, Do P, et al. Ibrutinib treatment improves T cell number and function in CLL patients. J Clin Invest. 2017;127(8):3052-3064.
10. Solman IG, Blum LK, Hoh HY, et al. Ibrutinib restores immune cell numbers and function in first-line and relapsed/refractory chronic lymphocytic leukemia. Leuk Res. 2020;97:106432.
11. Byrd JC, Wierda WG, Schuh A, et al. Acalabrutinib monotherapy in patients with relapsed/refractory chronic lymphocytic leukemia: updated phase 2 results. Blood. 2020;135(15):1204-1213.
12. Woyach JA, Blachly JS, Rogers KA, et al. Acalabrutinib plus Obinutuzumab in Treatment-Naïve and Relapsed/Refractory Chronic Lymphocytic Leukemia. Cancer Discov. 2020;10(3):394-405.
13. Solman IG, Blum LK, Burger JA, et al. Impact of long-term ibrutinib treatment on circulating immune cells in previously untreated chronic lymphocytic leukemia. Leuk Res. 2021;102:106520.
14. Ysebaert L, Gross E, Kuhlein E, et al. Immune recovery after fludarabine-cyclophosphamide-rituximab treatment in B-chronic lymphocytic leukemia: implication for maintenance immunotherapy. Leukemia. 2010;24(7):1310-1316.
15. Gauthier M, Durrieu F, Martin E, et al. Prognostic role of CD4 T-cell depletion after frontline fludarabine, cyclophosphamide and rituximab in chronic lymphocytic leukaemia. BMC Cancer. 2019;19(1):809.
16. Dubovsky JA, Beckwith KA, Natarajan G, et al. Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. Blood. 2013;122(15):2539-2549.
17. Sagiv-Barfi I, Kohrt HE, Czerwinski DK, Ng PP, Chang BY, Levy R. Therapeutic antitumor immunity by checkpoint blockade is enhanced by ibrutinib, an inhibitor of both BTK and ITK. Proc Natl Acad Sci U S A. 2015;112(9):E966-972.
18. Niemann CU, Herman SE, Maric I, et al. Disruption of in vivo Chronic Lymphocytic Leukemia Tumor-Microenvironment Interactions by Ibrutinib--Findings from an Investigator-Initiated Phase II Study. Clin Cancer Res. 2016;22(7):1572-1582.
19. Ioannou N, Hagner PR, Stokes M, et al. Triggering interferon signaling in T cells with avadomide sensitizes CLL to anti-PD-L1/PD-1 immunotherapy. Blood. 2021;137(2):216-231.
20. Dickinson MJ, Carlo-Stella C, Morschhauser F, et al. Glofitamab for Relapsed or Refractory Diffuse Large B-Cell Lymphoma. N Engl J Med. 2022.

21. Baptista MJ, Baskar S, Gaglione EM, et al. Select Antitumor Cytotoxic CD8(+) T Clonotypes Expand in Patients with Chronic Lymphocytic Leukemia Treated with Ibrutinib. Clin Cancer Res. 2021;27(16):4624-4633.
22. Vardi A, Vlachonikola E, Papazoglou D, et al. T-Cell Dynamics in Chronic Lymphocytic Leukemia under Different Treatment Modalities. Clin Cancer Res. 2020;26(18):4958-4969.
23. Gill SI, Vides V, Frey NV, et al. Anti-CD19 CAR T Cells in Combination with Ibrutinib for the Treatment of Chronic Lymphocytic Leukemia. Blood Adv. 2022.
24. Mhibik M, Gaglione EM, Eik D, et al. BTK inhibitors, irrespective of ITK inhibition, increase efficacy of a CD19/CD3-bispecific antibody in CLL. Blood. 2021;138(19):1843-1854.
25. Mhibik M, Gaglione EM, Eik D, et al. Cytotoxicity of the CD3xCD20 bispecific antibody epcoritamab in CLL is increased by concurrent BTK or Bcl-2 targeting. Blood Adv. 2023.

Figure Legends

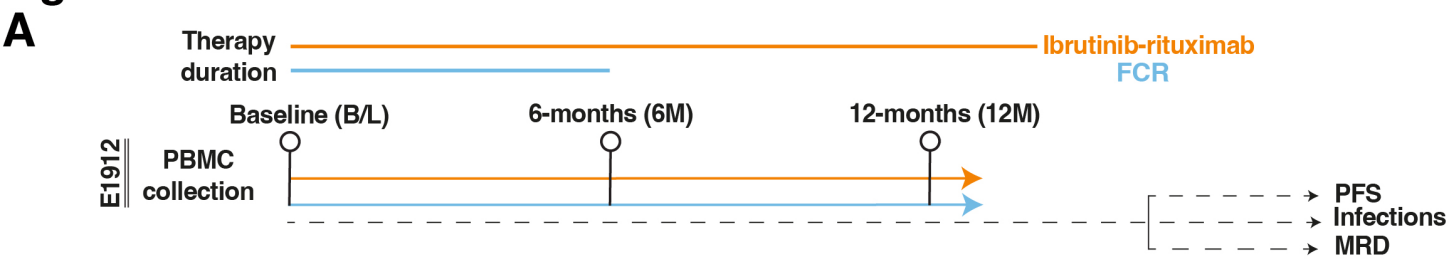
Figure 1. Higher $CD8^+$ T cell numbers at baseline and early on-therapy associate with favorable PFS and no infections during ibrutinib-rituxumab. (**A**) Schematic of the E1912 trial and biobanked PBMC samples collected at baseline (B/L), 6 (6M) and 12 (12M) month time-points for correlative T cell analysis. PFS, infection and MRD clinical outcome data collected. (**B**) Absolute numbers of $CD8^+$ T_{EM} cell subsets ($CD45RA^-$ $CCR7^-$) for ibrutinib-rituximab (n=86 patients) and FCR (n=50) at the time-points indicated. Patient data are presented as Box and whiskers (10-90 percentile) (log scale) plots. (**C**) Percentage of PD-1^+ $CD8^+$ T_{EM} subsets during ibrutinib-rituximab (n=86) or FCR (n=50). (**B**,**C**) Data are the mean ± SEM, statistical analysis between time-points assessed using the Wilcoxon signed-rank test. (**D**) Table schematic summarizes the significant correlations (Cox model) between higher immune subset levels (flow cytometry, median values used as cut-off point) and PFS for patients on ibrutinib-rituximab (n=88 patients with 13 experiencing disease progression). Green rows (correlations with Hazard Ratio (HR) values < 1) indicate higher immune subsets associated with longer PFS, while higher immune subsets associating with shorter PFS (HR > 1) are highlighted in blue rows. Confidence intervals (95%) and *P* values shown. (**E**) Schematic summarizing the significant correlations (Wilcoxon test) between immune subsets and infection (any infection) during ibrutinib-rituximab (n=88 patients). Negative t-statistics (t.stat) indicate higher immune subset levels in patients who did not develop infection (green rows). In contrast, correlations with a positive t.stat indicate higher immune subset levels in patients who developed infection (blue rows). (**F**) Kaplan-Meier curves of immune subsets associated to good prognosis for the ibrutinib-rituximab arm. Higher levels of % PD-$L1^+$ $CD19^+$ cells (High: 12 progression events/43 patients, Low: 1 progression event/43 patients), absolute number of PD-1^+CD8^+ T_{EM} (High: 3 progression events/43 patients, Low: 10 progression event/43 patients) and PD-1^+CD4^+ T cells (High: 3 progression events/43 patients, Low: 10 progression event/43 patients) at baseline associate with longer PFS. Higher percentage of $CD8^+$ T_{EM} (High: 3 progression events/42 patients, Low: 10 progression event/43 patients) at the 6-month time-

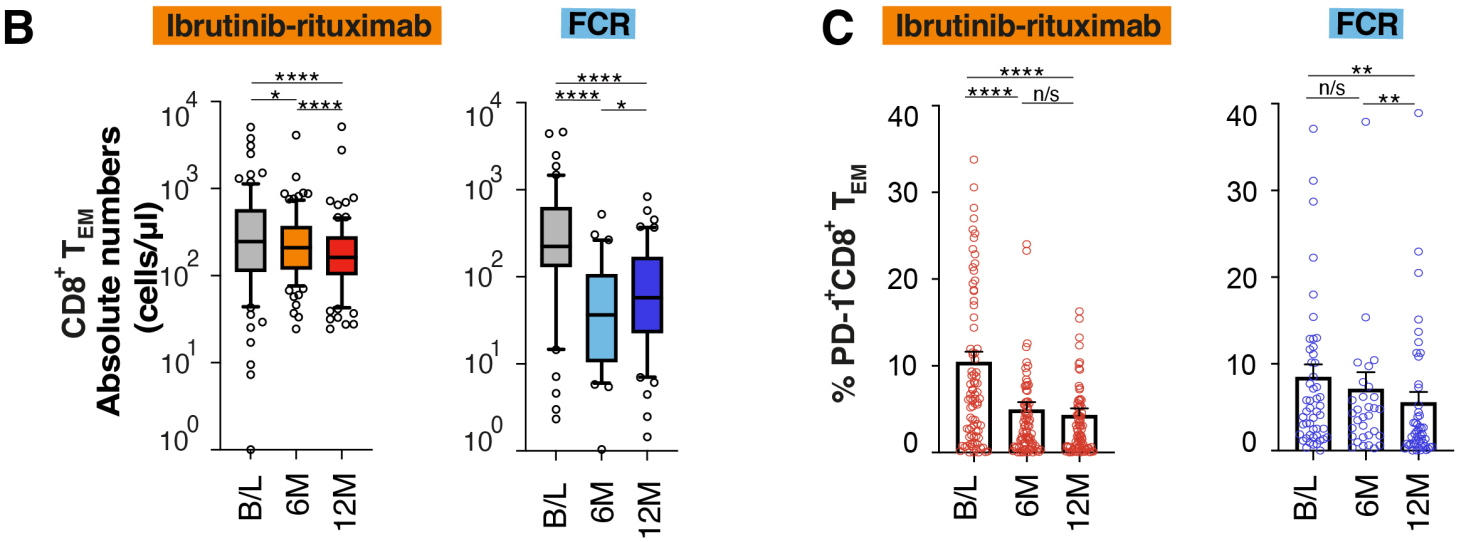
point associate with longer PFS. Absolute number data is referred to as "ab". *P* values indicated. **P*<.05; ***P*<.01; ****P*<.001; *****P*<.0001; n/s, not significant.

Figure 2. Ibrutinib-rituximab promotes CD8$^+$ T cell lytic synapse activity and supports immunotherapy-triggered anti-CLL killing function. (**A**) Illustration of the autologous cytotoxicity assay using anti-CD3/-CD28 activated T cells (cytolytic T lymphocytes, "CTLs") from B/L, 6M and 12M time-points mixed with target B/L CLL B cells (pulsed with superantigen as a model antigen) with flow-based quantification of T cell killing function. (**B**) T cell-mediated CLL cell death comparing T cells purified from B/L, 6M and 12M time-point samples (n=30 patients per treatment arm). Data at 6M and 12M was normalized to B/L levels to generate fold change values for each patient. (**C**) The association between patient's T cell killing function (12M ibrutinib-rituximab time-point, n=30) and infection status during ibrutinib-rituximab (no infections versus grade 2 or 3 infections) (Wilcoxon test, *P*=.01). (**D**, **E**) Representative confocal medial optical section and 3D volume rendered images of T cell:CLL conjugates formed between patient T cells (B/L, 6M and 12M on-ibrutinib-rituximab (**D**) or FCR (**E**)) interacting with autologous B/L CLL B cells (blue, CMAC dyed). Bar charts: quantitative relative recruitment index (RRI) analysis of F-actin polarization (red, rhodamine phalloidin) in T cell:CLL conjugates (n=50 patients per treatment arm). (**F**) Box and violin plots (Min-Max) showing the % of CD4$^+$ or CD8$^+$ T cell:CLL conjugates formed from the total/all T cell:CLL conjugates in B/L, 6M and 12M ibrutinib-rituximab time-point samples (n=15 patients). Representative confocal images of CD8$^+$ (white) and CD4$^+$ (green) T cell conjugates with CLL B cells (blue) at B/L versus on ibrutinib-rituximab therapy. (**G**) Representative confocal 3D volume rendered images of Granzyme B (GrB, white) expression at CD8$^+$ T cell synapses comparing ibrutinib-rituximab and FCR 12M time-point samples. (**H**) Kaplan Meier curve showing the association between the strength of polarized F-actin CD4$^+$ T cell:CLL immune synapse interactions in patient B/L samples and their PFS outcomes during ibrutinib-rituximab. Median F-actin RRI values were used as a cut-off point to determine "weak" (< median RRI) versus "strong" (> median RRI) CD4$^+$ T cell synapses (n=52 patients). Patients' showing strong CD4$^+$ T cell:CLL immune synapses

at B/L showed significantly adverse PFS (9 progression events/29 patients) compared to patients showing weak $CD4^+$ T cell:CLL interactions (1 progression event/23 patients). (Cox model, *P*=.01, HR=9.14 (95% CI:1.15-72.47). Bar chart: F-actin RRI analysis of $CD4^+$ T cell:CLL conjugates at B/L and representative 3D volume rendered confocal images comparing patients who progressed (n=7) or not (progression-free, n=7) during ibrutinib-rituximab. (**I**) Illustration of the cytotoxicity assay following *ex vivo* treatment of purified T cells (BL, 6M and 12M time-points) and B/L CLL cells with anti-PD-1 (αPD-1) or anti-PD-L1 (αPD-L1) blocking antibodies (10 μg/ml) or isotype controls. (**J**, **K**) T cell killing function against autologous B/L CLL cells examining T cells at B/L or at the 6-month ibrutinib-rituximab (orange) or FCR (blue) time-points following *ex vivo* treatment with (**J**) αPD-1 or isotype control (indicated using "-") (B/L: n=6, ibrutinib-rituximab: n=13, FCR: n=15) or (**K**) αPD-L1 or isotype control (-) (B/L: n=6, ibrutinib-rituximab: n=23, FCR: n=13). **(L)** Illustration of the autologous cytotoxicity assay incorporating the CD20xCD3 glofitamab or a non-binding antibody control. (**M, N**) T cell-mediated CLL cell death using purified T cells from B/L, 6M, 12M or 18M time-points ibrutinib-rituximab (**M**) or FCR (**N**) against target B/L CLL B cells following *ex vivo* treatment with glofitamab (0.01 μg/ml) or non-binding antibody control (indicated as "-") (B/L n=13; ibrutinib-rituximab 6M, 12M n=6, 18M n=5; FCR 6M, 12M n=7, 18M n=5 patient samples). Data for all cytotoxicity assay time-points were normalized to isotype antibody control (J, K) or non-binding antibody control (M, N) treated sample levels and presented as fold change data for each immunotherapy treated patient sample. **P*<.05; ***P*<.01; ****P*<.001; *****P*<.0001; n/s, not significant. Wilcoxon signed-rank test (B, D, E, J, K, M, N), multiple comparisons mixed effect ANOVA (F) and Mann-Whitney U test (H). Mann-Whitney U test was used to compare cell death between CD20-TCB-treated conditions at B/L, 6M, 12M and 18M. Original image magnification of x63, scale bars: 10 μm. Bar chart data presented as mean ± SEM.

Figure 1





D

PFS

B/L

Immune Subset	HR	Lower.95	Upper.95	*P*-value
PD-1+ CD8+ T_{EM} ab	0.266	0.0731	0.970	0.0448
CD4+ T_{EM} ab	0.263	0.0723	0.957	0.0427
T_{Regs} of CD4+ ab	0.270	0.0742	0.983	0.0471
PD-1+ CD4+ ab	0.269	0.074	0.980	0.0465
% PD-L1+ CD19+	0.070	0.009	0.541	0.0108
% CD8+ T_{naive} of CD8+	6.570	1.45	29.7	0.0144
% T_H2 of CD3+	3.860	1.06	14	0.0407

6M

Immune Subset	HR	Lower.95	Upper.95	p-value
% T_{EM} of CD8+	0.267	0.0734	0.97	0.0449
CD8+ T_{CM} ab	4.240	1.15	15.6	0.0296
% T_H2 of CD3+	3.860	1.06	14.1	0.0411

Ibrutinib-rituximab

Higher levels associated with longer PFS
Higher levels associated with shorter PFS

E

Infection

B/L

Immune Subset	t.stat	*P*-value
CD4+/CD8+ Ratio	2.089	0.0397
% T_H2 of CD3+	2.021	0.0467

6M

Immune Subset	t.stat	p-value
CD8+ T_{EM} ab	-2.057	0.0432
CD56+ CD16+ ab	-2.450	0.0171
CD4+/CD8+ Ratio	2.108	0.0380

12M

Immune Subset	t.stat	p-value
PD-L1+ CD4+ T_{EMRA} ab	-2.002	0.0494

Ibrutinib-rituximab

Higher levels associated with no infections
Higher levels associated with infections

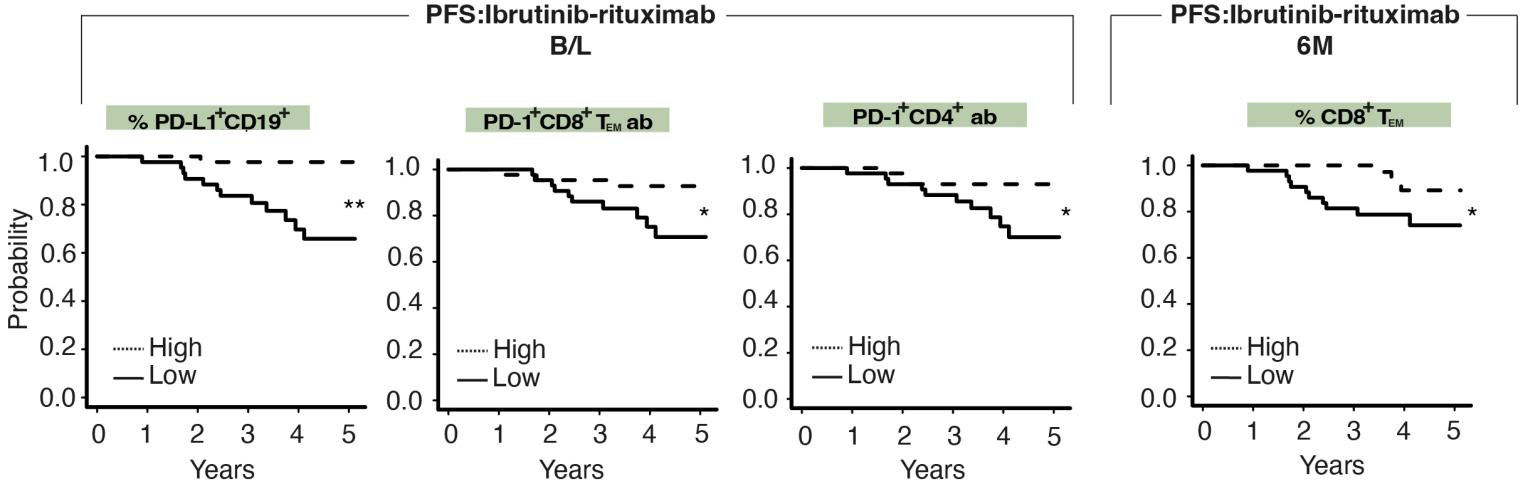
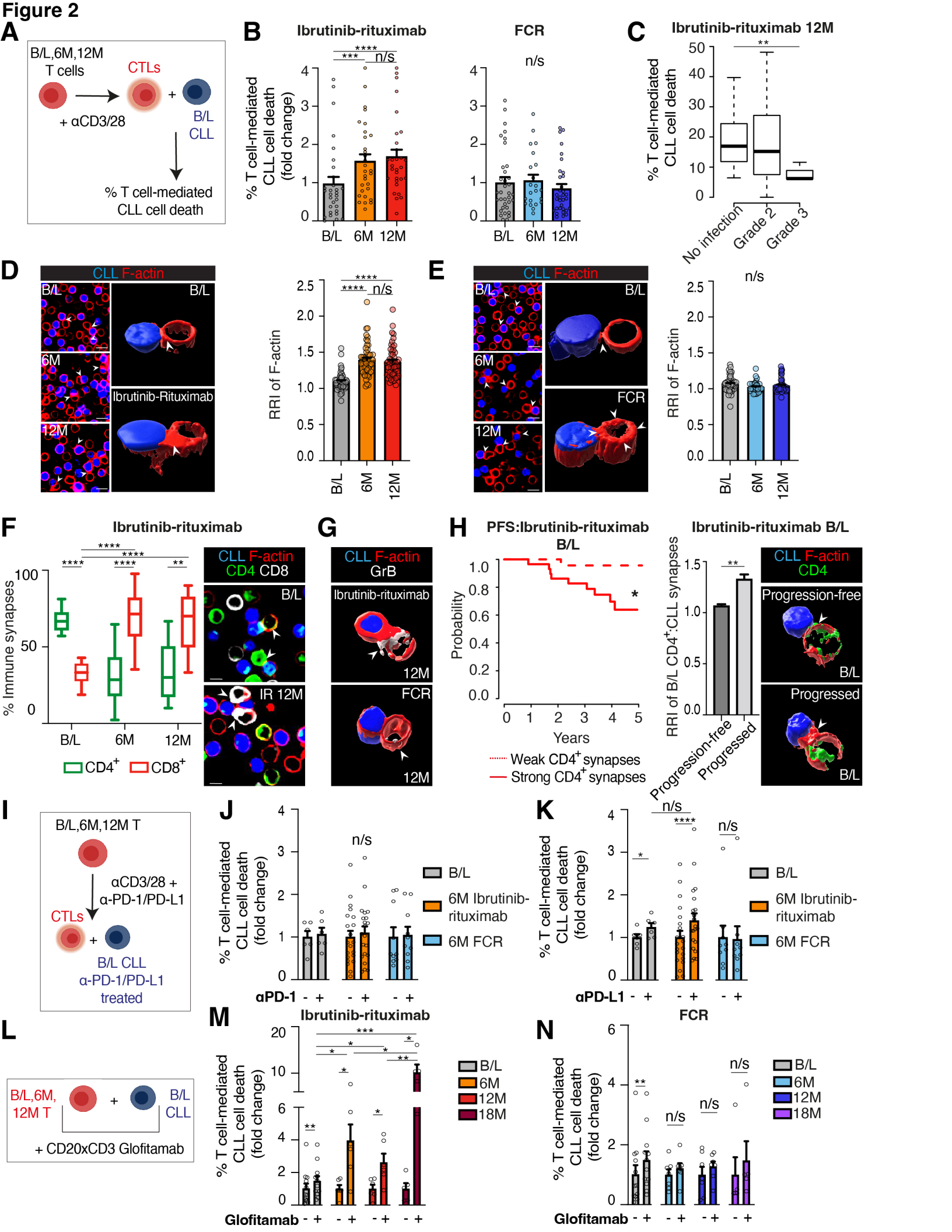


Figure 2


A
B/L,6M,12M T cells
CTLs
+ αCD3/28
B/L CLL
% T cell-mediated CLL cell death
B
Ibrutinib-rituximab
FCR
% T cell-mediated CLL cell death (fold change)
n/s
B/L 6M 12M
C
Ibrutinib-rituximab 12M
% T cell-mediated CLL cell death
No infection
Grade 2
Grade 3
D
CLL F-actin
B/L
6M
12M
Ibrutinib-Rituximab
RRI of F-actin
E
FCR
F
Ibrutinib-rituximab
% Immune synapses
CD4+ CD8+
G
CLL F-actin GrB
Ibrutinib-rituximab
H
PFS:Ibrutinib-rituximab B/L
Probability
Years
Weak CD4+ synapses
Strong CD4+ synapses
Ibrutinib-rituximab B/L
RRI of B/L CD4+:CLL synapses
CLL F-actin CD4
Progression-free
Progressed
I
B/L,6M,12M T
αCD3/28 + α-PD-1/PD-L1
CTLs
B/L CLL α-PD-1/PD-L1 treated
J
αPD-1
B/L
6M Ibrutinib-rituximab
6M FCR
K
αPD-L1
L
B/L,6M, 12M T
CD20xCD3 Glofitamab
M
Glofitamab
6M
12M
18M
N