Supplemental methods

BTK target occupancy ELISA

Cryopreserved peripheral blood mononuclear cells (PBMCs) were thawed (37°C water bath) and washed in RPMI + 1% fetal bovine serum. Five million cells per sample/time point were washed with 1 mL cold phosphate buffered saline (PBS) and cell pellets were snap frozen in liquid nitrogen. Ninety-six-well Optiplate (PerkinElmer) plates were coated overnight with 125 ng/well anti-Bruton tyrosine kinase (BTK) antibody (BD Biosciences) and blocked with bovine serum albumin (BSA). Frozen cell pellets were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM MgCl2, 1 mM dithiothreitol, 0.05% digitonin and protease inhibitor cocktail (Sigma-Aldrich). Lysates were incubated for 1 hour on ice in the presence or absence of acalabrutinib (1 μM). At this concentration, BTK has been shown to be saturated and completely bound by acalabrutinib. The cell lysates were incubated for 1 hour on ice with a biotinylated derivative of acalabrutinib (10^{-7} M). The equivalent of cells of lysate/well, in replicates of 4, were incubated for 2 hours at ambient temperature on an anti-BTK coated 96-well Optiplate. Plates were washed 4 times with PBS + 0.05% Tween. Streptavidin-HRP (Invitrogen; ELISA grade) was added at 100 μL/well (120 ng/mL) and incubated for 1 hour at room temperature. Plates were washed 3 times with PBS + 0.05% Tween and then washed twice with PBS. SuperSignal ELISA Femto Substrate (ThermoFisher Scientific) was added (100 μL/well) and then chemiluminescence was measured after 1 minute (EnVision® plate reader; PerkinElmer). The percentage of BTK occupancy for each sample time point was calculated relative to the day 1 predose sample for each patient. The signal from the day 1 predose sample without exogenous acalabrutinib represents 100% free BTK (or 0% occupied BTK), whereas the signal from the day 1 predose sample with exogenous acalabrutinib represents 0% free BTK (or 100% occupied BTK). The incubation of each cell lysate with 1 μM acalabrutinib was used to correct for background signal not related to free BTK.

$$\%\ \text{Free BTK sample X} = [(\text{Sample X} - \text{Sample X}^{+\text{ACP196 [1μM]}}) \ / \ (\text{Day1 Predose} - \text{Day1 Predose}^{+\text{ACP196 [1μM]}})] \times 100$$

$$\%\ \text{Occupied BTK} = 100\% - \%\ \text{Free BTK}$$

IFNɣ-producing T cells in stimulated PBMC cultures

PBMCs were incubated in RPMI + 1% FBS with PMA (50ng/mL; Sigma-Aldrich), ionomycin (1μg/mL; Sigma-Aldrich) and GolgiStop (30μM; BD Biosciences) for 6 hours at 37°C to induce IFNɣ production. Cells were washed with PBS + 0.5% BSA and stained with an antibody cocktail consisting of fluorescently labeled mouse antibodies against human surface markers CD3, CD4, CD8, CD20 and CD45 (all from BD Biosciences) for 15 minutes at 4°C. Cells were washed with PBS + 0.5% BSA, fixed with paraformaldehyde (1.6%; Electron Microscopy Sciences) for 10 minutes at 37°C and permeabilized with Perm/Wash buffer (1x; BD Biosciences). Cells were then stained with fluorescently labeled mouse antibody against intracellular IFNɣ (BD Biosciences) for 30 minutes at 4°C. Cells were washed twice with Perm/Wash buffer and acquired on a FACSVerse flow cytometer (BD Biosciences). The percentage of cells staining positive for IFNɣ were measured in T-cell subsets. All conditions were run in triplicate and the results were reported as an average of the 3 wells.

PD-1 cell surface expression

PBMCs were stained with an antibody cocktail consisting of fluorescently labeled mouse antibodies against human surface markers CD3, CD4, CD8, CD19, CD279 (PD-1), and CD45 (BD Biosciences) for 30 minutes at 4°C. Data were acquired on a FACSVerse flow cytometer (BD Biosciences) and analyzed using FCS Express Software. The percentage of PD-1+ cells was measured in the CD4+ T cell subset, provided that the subset was >1% of all T cells, and the percentage of total CD3+ T cells was >1% of viable CD45+ cells.

Genomic analysis

Whole exome analysis was processed through the bcbio pipeline: (https://bcbio-nextgen.readthedocs.io/en/latest/), using human hg38 reference genome and hg38 Ensembl transcripts (Ensembl version 92). Whole exome samples were processed using BWA aligner (ver. 0.7.17).[1] Variants were called using VarDictJava (https://github.com/AstraZeneca-NGS/VarDictJava) (ver. 1.5.2).[2] Structural variants were called with seq2c (ver. 2016.03.23).[3] Raw data values and screen shots were extracted using IGV.[4]

In brief, BTK C481X ddPCR hotspot assay was custom designed with primer and probe pairs, optimizing them for annealing temperature and cycling condition using serial dilutions of mutant DNA. The assay detects any amino acid alteration at the position that is not WT.

Supplemental Table 1. Prior systemic therapies

Prior systemic therapy, n (%)	(N = 134)
Anti-CD20	127 (95)
Alkylating agent	93 (69)
Nucleoside analog	78 (58)
Steroid	38 (28)
Other	25 (19)
PI3K inhibitor	20 (15)
Lenalidomide	17 (13)
Chemotherapy	16 (12)
Anti-CD52	12 (9)
Stem cell transplant	3 (2)
BCL2 inhibitor	2 (1)
BTK inhibitor	0

Supplemental Table 2. Adverse events in ≥10% of patients

	Relapsed/refractory (N = 134)					
Preferred term	**All Grades**	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
Patients with an adverse event, n (%)	134 (100.0)	4 (3.0)	41 (30.6)	49 (36.6)	29 (21.6)	11 (8.2)*
Diarrhea	70 (52.2)	41 (30.6)	22 (16.4)	7 (5.2)	0	0
Headache	68 (50.7)	59 (44.0)	9 (6.7)	0	0	0
Upper respiratory tract infection	50 (37.3)	14 (10.4)	35 (26.1)	1 (0.7)	0	0
Fatigue	45 (31.3)	23 (17.2)	18 (13.4)	4 (3.0)	0	0
Arthralgia	43 (29.1)	31 (23.1)	11 (8.2)	1 (0.7)	0	0
Nausea	42 (31.3)	32 (23.9)	9 (6.7)	1 (0.7)	0	0
Cough	40 (29.9)	26 (19.4)	14 (10.4)	0	0	0
Contusion	39 (29.1)	35 (26.1)	4 (3.0)	0	0	0
Pyrexia	35 (26.1)	21 (15.7)	12 (9.0)	2 (1.5)	0	0
Constipation	34 (25.4)	32 (23.9)	2 (1.5)	0	0	0
Weight increased	32 (23.9)	15 (11.2)	12 (9.0)	5 (3.7)	0	0
Petechiae	28 (20.9)	25 (18.7)	3 (2.2)	0	0	0
Sinusitis	28 (20.9)	5 (3.7)	22 (16.4)	1 (0.7)	0	0
Vomiting	28 (20.9)	20 (14.9)	6 (4.5)	2 (1.5)	0	0
Pneumonia	25 (18.7)	1 (0.7)	9 (6.7)	8 (6.0)	2 (1.5)	5 (3.7)
Hypertension	24 (17.9)	2 (1.5)	12 (9.0)	10 (7.5)	0	0
Edema peripheral	24 (17.9)	19 (14.2)	4 (3.0)	1 (0.7)	0	0
Weight decreased	24 (17.9)	16 (11.9)	8 (6.0)	0	0	0
Dyspnea	22 (16.4)	11 (8.2)	6 (4.5)	3 (2.2)	2 (1.5)	0
Night sweats	21 (15.7)	16 (11.9)	4 (3.0)	1 (0.7)	0	0
Decreased appetite	20 (14.9)	15 (11.2)	5 (3.7)	0	0	0
Oropharyngeal pain	20 (14.9)	15 (11.2)	5 (3.7)	0	0	0
Pain in extremity	20 (14.9)	16 (11.9)	4 (3.0)	0	0	0
Productive cough	20 (14.9)	14 (10.4)	6 (4.5)	0	0	0

	Relapsed/refractory (N = 134)					
Preferred term	**All Grades**	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
Rash	20 (14.9)	18 (13.4)	2 (1.5)	0	0	0
Neutropenia	19 (14.2)	0	0	4 (3.0)	15 (11.2)	0
Abdominal pain	18 (13.4)	10 (7.5)	5 (3.7)	3 (2.2)	0	0
Anemia	18 (13.4)	5 (3.7)	4 (3.0)	8 (6.0)	1 (0.7)	0
Dizziness	18 (13.4)	17 (12.7)	0	1 (0.7)	0	0
Ecchymosis	18 (13.4)	17 (12.7)	1 (0.7)	0	0	0
Nasal congestion	18 (13.4)	11 (8.2)	7 (5.2)	0	0	0
Back pain	17 (12.7)	8 (6.0)	8 (6.0)	1 (0.7)	0	0
Fall[†]	16 (11.9)	9 (6.7)	6 (4.5)	0	0	0
Insomnia	16 (11.9)	11 (8.2)	5 (3.7)	1 (0.7)	0	0
Myalgia	16 (11.9)	13 (9.7)	3 (2.2)	0	0	0
Paresthesia	16 (11.9)	16 (11.9)	0	0	0	0
Epistaxis	15 (11.2)	9 (6.7)	5 (3.7)	1 (0.7)	0	0
Increased tendency to bruise	15 (11.2)	14 (10.4)	1 (0.7)	0	0	0
Rhinorrhea	15 (11.2)	13 (9.7)	2 (1.5)	0	0	0
Anxiety	14 (10.4)	8 (6.0)	6 (4.5)	0	0	0
Chills	14 (10.4)	13 (9.7)	1 (0.7)	0	0	0
Rash maculopapular	14 (10.4)	13 (9.7)	1 (0.7)	0	0	0
Skin lesion	14 (10.4)	11 (8.2)	3 (2.2)	0	0	0

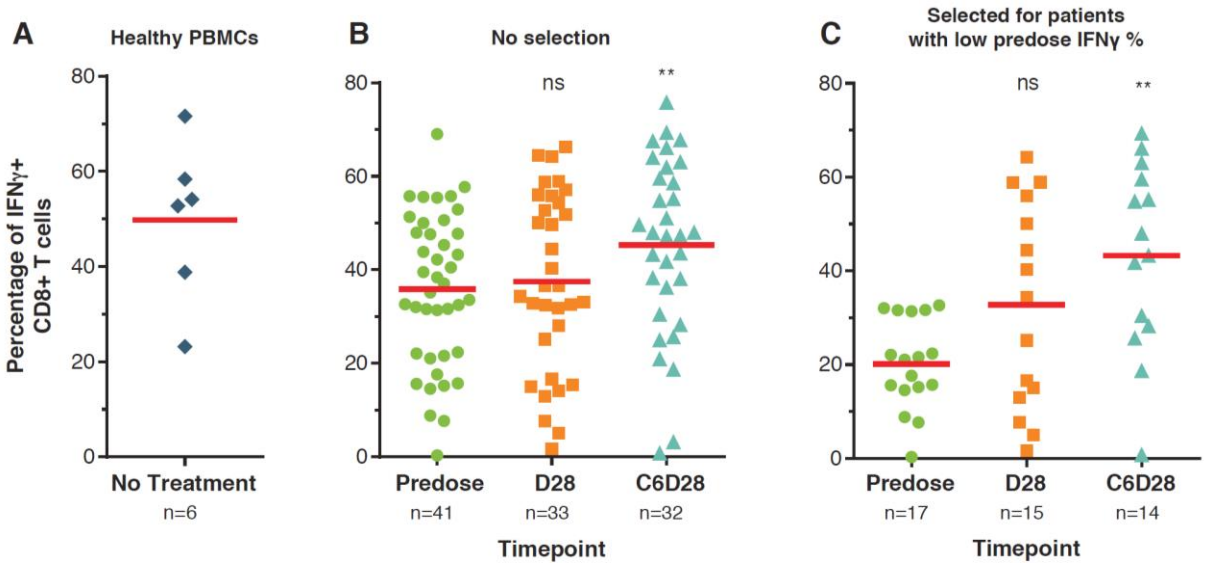
*A case of plasmablastic lymphoma was initially reported as a Grade 5 AE but subsequently updated to death due to disease progression.
[†]Grade information missing for 1 event.

Supplemental Table 3. Serious adverse events in ≥2 patients

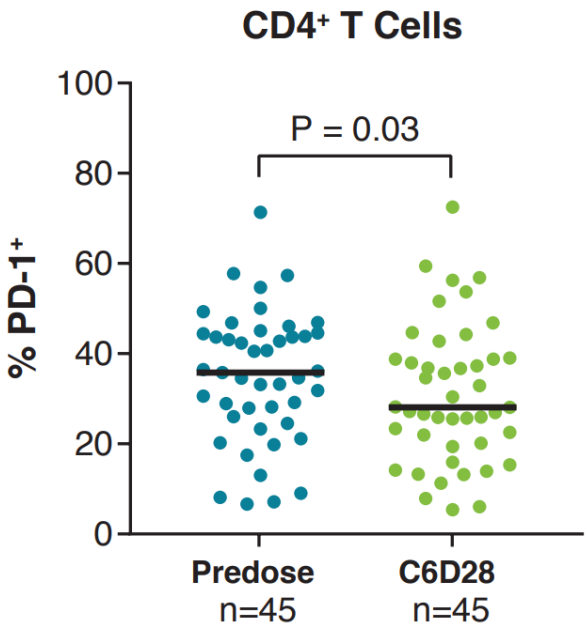
	Relapsed/refractory (N = 134)					
Preferred term	**All Grades**	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
Patients with a serious adverse event, n (%)	71 (53.0)	1 (0.7)	6 (4.5)	38 (28.4)	15 (11.2)	11 (8.2)
Pneumonia	15 (11.2)	0	0	8 (6.0)	2 (1.5)	5 (3.7)
Anemia	4 (3.0)	0	1 (0.7)	2 (1.5)	1 (0.7)	0
Atrial fibrillation	4 (3.0)	0	1 (0.7)	3 (2.2)	0	0
Abdominal pain	3 (2.2)	0	1 (0.7)	2 (1.5)	0	0
Acute kidney injury	3 (2.2)	1 (0.7)	1 (0.7)	0	1 (0.7)	0
Diarrhea	3 (2.2)	0	1 (0.7)	2 (1.5)	0	0
Febrile neutropenia	3 (2.2)	0	0	1 (0.7)	2 (1.5)	0
Hypercalcemia	3 (2.2)	0	0	0		
Acute sinusitis	2 (1.5)	0	0	2 (1.5)	0	0
Atrioventricular block complete	2 (1.5)	0	0	0	2 (1.5)	0
Autoimmune hemolytic anemia	2 (1.5)	0	0	2 (1.5)	0	0
Chest pain	2 (1.5)	0	2 (1.5)	0	0	0
Hip fracture	2 (1.5)	0	0	2 (1.5)	0	0
Lung infection	2 (1.5)	0	0	1 (0.7)	1 (0.7)	0
Pyrexia	2 (1.5)	0	2 (1.5)	0	0	0
Respiratory failure	2 (1.5)	0	0	0	1 (0.7)	1 (0.7)
Sepsis	2 (1.5)	0	0	1 (0.7)	1 (0.7)	0
Syncope	2 (1.5)	0	1 (0.7)	1 (0.7)	0	0
Thrombocytopenia	2 (1.5)	0	0	0	2 (1.5)	0
Upper respiratory tract infection	2 (1.5)	0	1 (0.7)	1 (0.7)	0	0
Urinary tract infection	2 (1.5)	0	2 (1.5)	0	0	0

Supplemental Table 4. Allele Fractions (AF) of BTK C481 mutation for 6 patients that progressed on acalabrutinib with a detectable BTK C481X mutation. Raw NGS AF reported before bioinformatic filters as in Supplemental methods.

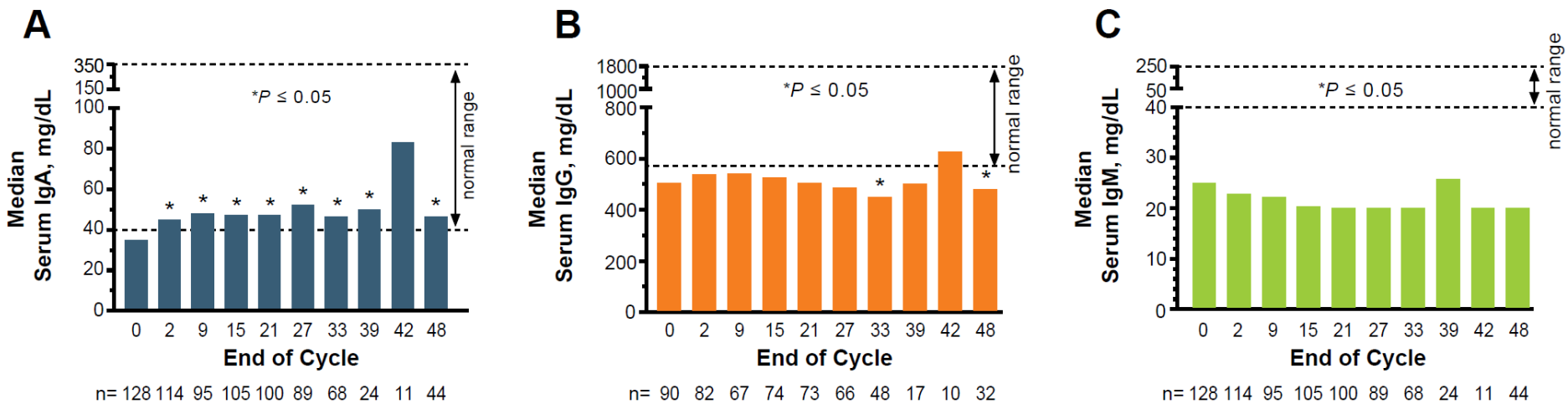
Patient	Mutation	Cycle	Days	ddPCR AF%	WES AF%
1	C481Y	C1D1 Pre	0	0	0
		C1D8 Pre	7	0	0
		Follow-up	552	49	15.65
	C481R	C1D1 Pre	0		0
		C1D8 Pre	7		0
		Followup	552		31.68
2	C481S	C1D2 Pre	0	0	0
		C1D28 Pre	27	0	0
		Follow-up	1015	36	15.2
	C481S	C1D2 Pre	0		0
		C1D28 Pre	27		0
		Follow-up	1015		16.18
3	C481S	C1D1 Pre	0	0	0
		C1D8 Pre	7	0	0
		DE Pre	449	0.02	0
		DE2 Pre	695	1	0
4	C481S	C1D1 Pre	0	0	0
		C1D23 Pre	22	0	0
		Progressed	495	43	30.71
5	C481S	C1D2 Pre	0	0	0
		C6D28 Pre	167	0	0
		DE Pre	902	2	1.46
6	C481S	C1D1 Pre	0	0	0
		C6D28 Pre	169	0.26	0
		Follow-up	254	57	44.05



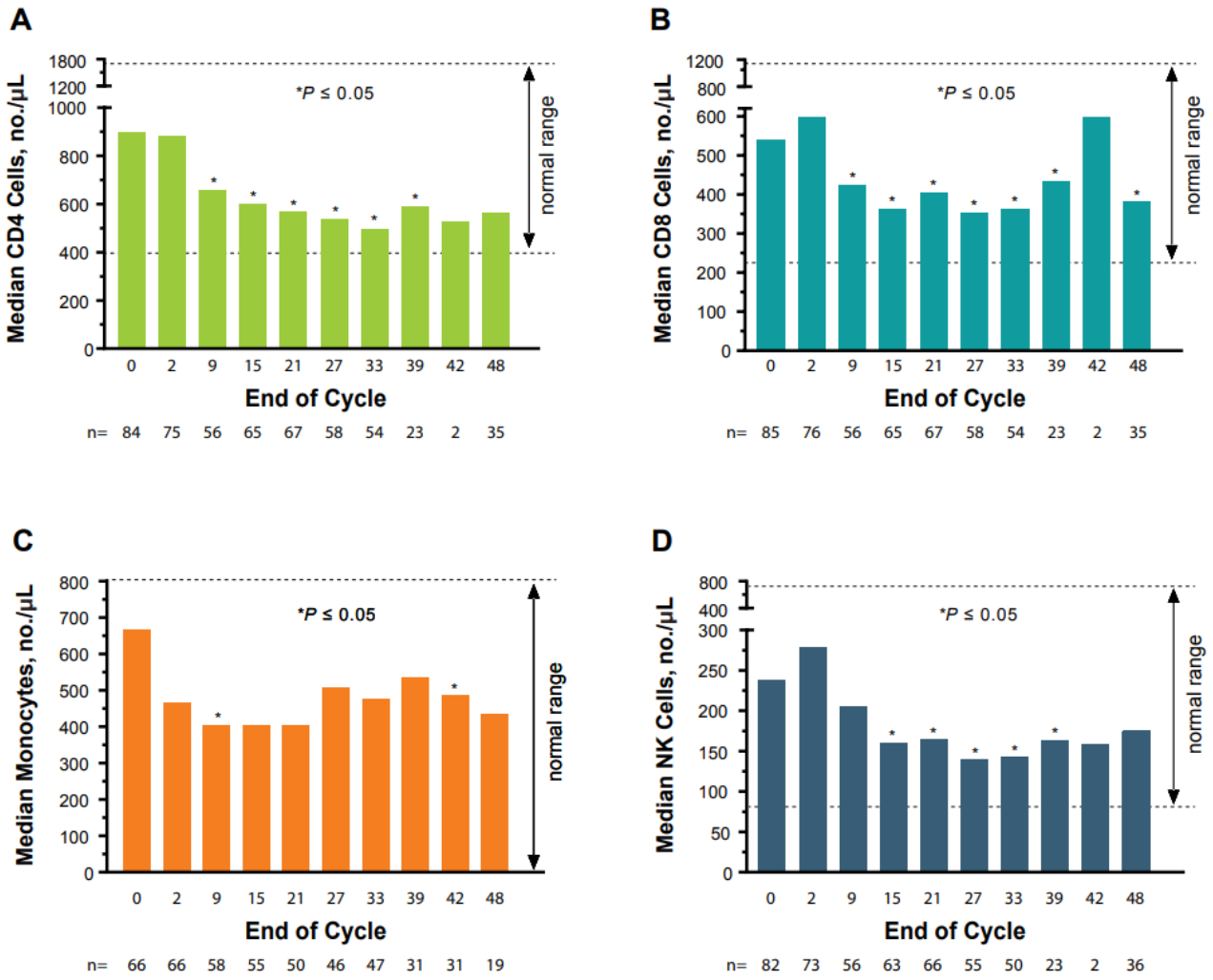
Supplemental Figure 1. Acalabrutinib treatment increases CD8+ T cell effector function. Interferon gamma (IFNɣ) production was induced in ex vivo peripheral blood mononuclear cell (PBMC) cultures by stimulation with PMA/ionomycin and measured by intracellular flow cytometry. The percentage of CD8+CD3+ T cells that stained positive for Interferon-gamma (IFNɣ) is shown. (A) Healthy PBMCs shown as a reference. (B) PBMCs from patients with relapsed/refractory chronic lymphocytic leukemia (R/R CLL) PBMCs. (c) R/R CLL PBMCs selected with low IFNɣ+ cells at D1 predose (1 standard deviation below healthy controls). ***P* < .01. ns, nonsignificant; D, day; C, Cycle (4 weeks).



Supplemental Figure 2. Acalabrutinib treatment decreases PD-1 expression on CD4+ T cells. Percentage of PD-1+ cells of CD4+ T cells from peripheral blood mononuclear cells (PBMCs) of patients with relapsed or refractory chronic lymphocytic leukemia treated with acalabrutinib. Line denotes the median. Significance was determined using a paired, 2-tailed, parametric t test. D, day; C, Cycle (4 weeks); PD-1, programmed cell death protein-1.



Supplemental Figure 3. Median serum IgA (A), IgG (B) and IgM (C) levels over time. Patients receiving intravenous immunoglobulin therapy were excluded from analysis. The vertical dashed lines represent normal laboratory ranges. All plots include patients having assessments at baseline and the respective visit. *P* values are from the Wilcoxon signed-rank test. Ig, immunoglobulin.



Supplemental Figure 4. T cells, monocyte and natural killer cell counts over time. Absolute counts of CD4+ (A) and CD8+ (B) T cells, CD14+ monocytes (C), and natural killer cells (D) in peripheral blood from acalabrutinib-treated patients. Cells were measured by flow cytometry. The normal reference range for each subset is shown with dashed lines. All plots include patients having assessments at baseline assessment and the respective visit. *P* values are from the Wilcoxon signed-rank test.

References

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