Materials and methods

Kinase activity assay:

Vecabrutinib interaction with the kinase domain of BTK was characterized by X-crystallography and mass spectrometry. Vecabrutinib protein kinase selectivity and kinase inhibitory activities were evaluated in the KinaseProfiler™ panel of 234 individual kinases (Eurofins Pharma Discovery Services, Dundee, UK). Vecabrutinib was also evaluated in a direct kinase assay for activity against BTK wild-type (WT) or BTK with a C481S mutation. Briefly , 170 pM WT His-BTK full-length protein (Thermo Fischer) or 1nM His-BTK C481S (expressed in Sf9 cells and purified using Ni-NTA affinity resin and size exclusion chromatography) were incubated with vecabrutinib or DMSO for 30 min. Kinase reactions were conducted in 10mM Tris/HCl, pH7.5, 10mM $MgCl_2$, 0.01% (v/v) Triton X-100, and 1mM DTT containing 15µM fluorescein-poly-Glu-Tyr (Thermo Fisher) and 50µM ATP. One hour later, reactions were stopped upon addition of 10mM EDTA and 3nM Tb-labelled pY20 antibody (Thermo Fisher). After 30 minutes, fluorescence was measured at 485nm and 520nm after excitation at 340nm using a Tecan Infinite F500 plate reader. Titration curves were generated using average FRET ratios from triplicate data points and fit with GraphPad Prism to determine the IC_{50} values.

Cell line models:

Chicken DT40 B cells (Cell Engineering Division, RIKEN BioResource Center, Japan) were cultured at 37 °C in a humidified atmosphere of 90 % air and 10 % CO_2 in RPMI 1640 medium (Gibco), supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS), 1 % heat-inactivated chicken serum, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 100 units/ml penicillin, and 100 µg/ml streptomycin. COS-7 cells (DSMZ, Germany) were maintained at 37 °C in a humidified atmosphere of 90 % air and 10 % CO_2 in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10 % (v/v) FCS (Invitrogen), 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Jeko-1 cells (DSMZ) and murine primary Eµ-TCL1 tumors used *in vitro* for drug sensitivity assays were cultured at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2 in RPMI 1640 containing 20 % and 10 % FCS, respectively, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin.

<u>Primary CLL patient samples:</u>

Patient cohort given in Suppl. table 3 which included n=8 BTK wt samples and n=4 BTK C481S mutant samples were included for the analysis of drug impact on viability and apoptotic priming by BH3 profiling. The patient cohort in suppl. table 4 was used for studying drug impact on T-cells. Genomic aberrations were analyzed using FISH[1]. IGHV gene mutations status was analyzed as previously described[2]. The analyses were conducted in accordance with the Declaration of Helsinki, approved by our institutional review board, and informed consent was obtained from all patients.

<u>Cloning of cDNAs encoding WT and mutant BTK and PLCγ2 and lentiviral transduction:</u>

Complementary DNAs encoding WT and C481S mutant human BTK were cloned into the pSFLV-GFP vector, while the cDNAs encoding WT and R665W mutant human PLCγ2 were cloned into the pLVX-EF1α-IRES-Puro vector. Production of lentivirus was performed as described using the lentiX lentiviral expression system (Clontech) following the manufacturer´s instructions. For packaging of the virus, a viral packaging vector (pAX2) and an envelope vector for the virus (pMD2) were used. Viruses harvested 24 and 48 h after transfection were used for transduction of JeKo-1 cells. The cells were infected for 12 h, after which the virus was removed and the cells were allowed to recover for 2 days. Selection of WT- and mutant-PLCγ2-transduced cells was performed by using 0.25 µg/ml puromycin, followed by seeding single cells in a 96-well plate. Clones resistant to puromycin were screened for PLCγ2 protein expression using Western blotting and used for further experiments. BTK-WT- and -mutant-transduced cells were expanded and subsequently selected by sorting for GFP expression using a BD FACS Aria II cell sorter.

<u>Analysis of inositol phosphate formation in transfected COS-7 cells and generation of stably transfected DT40 cells:</u>

Transfection of COS-7 cells, radiolabelling of inositol phospholipids and analysis of inositol phosphate formation was done as described in [Wist et al., 2020]. The generation of stably transfected DT40 cell clones expressing WT or C481S mutant

human BTK was essentially done as described in [Walliser et al., 2015] except that BTK-deficient DT40 cells (J. Wienands, Institute of Cellular and Molecular Immunology, University of Göttingen) were used as recipients. Proteomic analysis of whole cell extract proteins, fractionated by SDS-PAGE, was used to verify the expression of WT and C481S variant BTK in the respective cell clones. MS/MS spectra were correlated with the UniProt chicken database complemented with the sequences of human WT and C481S variant BTK. Carbamidomethylated cysteine was considered as a fixed modification along with oxidation (M), and acetylated protein N-termini as variable modifications. False discovery rates were set, both on peptide and protein levels, to 0.01.

Analysis of cytosolic Ca^{2+} by flow cytometry:

Two cell populations were analyzed simultaneously by staining half of the cells (1.8 × 10^7 cells) for 10 min at 37 °C and 10 % CO_2 at a density of 10^7 cells/ml with 20 nM CFSE in PBS, followed by washing of the cells once with 10 ml of PBS[3]. For BTK inhibitor treatment, unstained and CFSE- labeled DT40 cells were treated for 1 h at 37 °C with continuous shaking at a density of 10^7 cells/ml with 1 µM of the inhibitor or solvent in RPMI medium containing supplements. The irreversible inhibitors were washed out before adding the Ca^{2+} indicator, whereas the reversible inhibitors were continuously present. Cells were then suspended at a density of 10^7 cells/ml in FACS buffer (143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 20 mM HEPES/NaOH, pH 7.4, 1 mM $CaCl_2$, and 5.6 mM glucose) and incubated for 45 min at 37 °C and 10 % CO_2 with 5 µM Indo-1-AM. The Ca^{2+} measurements were performed on a BD FACS Celesta™. In brief, 10^6 cells per sample in 0.5 ml of FACS buffer were pre-warmed for 10 min at 37 °C. Next, the baseline intracellular, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured for 2 min before addition of 1 µg/ml anti-chicken IgM. After an additional 4 min, 2 µM ionomycin (I0634, Sigma) was added to the samples to obtain the maximal $[Ca^{2+}]_i$. Cells were kept at 37 °C prior and during their injection from the sample tube into the flow cytometer instrument. The fluorescence ratio of Indo-1 violet (emission wavelength about 400 nm, Ca^{2+}-bound) to Indo-1 blue (emission wavelength about 475 nm; Ca^{2+}-free) following excitation at about 355 nm was measured by using FACSDiva™ software (BD Biosciences).

For data evaluation, the experimental results recorded in the .fcs files generated by the FACSDiva[TM] software (BD Biosciences) were imported into the FlowPy Python tool (http://flowpy.wikidot.com) and then transferred to Excel spread sheets. In experiments analyzing two cell populations at the same time, DT40 cells expressing WT BTK (unstained), and DT40 cells expressing BTKC481S (stained), the matrix was fractionated into two parts, M1 and M2, according to the fluorescence intensities determined for intracellular CFSE. The individual points shown in Fig. 2c correspond to the times in full seconds *vs.* the medians of all fluorescence ratios representing $[Ca^{2+}]_i$, following their correction for basal fluorescence intensity in the absence of anti-IgM and normalization according to the maximal values reached after addition of 2 μM ionomycin. Between serial measurements, the latter values were normalized to 100 % of the two maxima obtained in the two untreated control cell preparations. Nonlinear least squares curve fitting of selected portions of the data to equations of either a first-order invasion and first-order elimination process (Bateman; response to anti-IgM) of a simple hyperbolic saturation function (response to ionomycin), in both cases allowing for different lag periods after the addition of the stimuli during the curve-fitting processes, was done using GraphPad Prism[®], versions 8.4.3 or 4.03, to determine the values corresponding to the maximal $[Ca^{2+}]_i$ concentrations for normalization to allow for quantitative comparison between experiments.

Flow cytometric analysis of BCR cell surface expression:

The cells (1×10^7 cells) were washed once with 2.2 ml PBS and then incubated for 30 min at 4 °C at a density of 2×10^7 cells/ml with increasing concentrations (0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 μg/ml) of FITC-conjugated mouse anti-chicken IgM or FITC-conjugated mouse IgG_{2b} isotype control in PBS (Southern Biotech), as recommended by the manufacturer. Fluorescence intensity of 5×10^3 cells was measured on BD FACS Celesta[TM] using FACSDiva[TM] software (BD Biosciences). The medians of fluorescence intensities were calculated using the FlowJo software V. 9.3.2 and normalized to the median of the isotype control. The data was fit by nonlinear least-squares curve fitting to a three-parameter sigmoidal-dose response using GraphPad Prism[®], version 4.03 The global curve fitting procedure contained in Prism was used to decide between a three- and a four-parameter sigmoidal dose-response model and

to determine whether the best fit values of selected parameters differed between data sets. The simpler model was selected unless the extra sum of squares F-test had a p value of less than 0.05.

BH3 profiling

Mitochondrial BH3 profiling was carried out as described earlier (Seyfried, CDDis 2019, Montero Cancer Cell 2015). For baseline BH3 profiling, tumor cells were obtained from the Eµ-TCL1 model from spleens of mice treated with vehicle, vecabrutinib or ibrutinib. For dynamic BH3 profiling, primary patient CLL samples were cultured ex vivo for 16 hours in RPMI 1640 supplemented with 20% FCS and exposed to DMSO control, 1 µM vecabrutinib or 1 µM ibrutinib.

Cells were stained with the fixable viability dye Zombie Violet (423113, Biolegend) for 15 minutes (1:1000) in PBS at room temperature. The cells were then permeabilized with digitonin (0.002%) and exposed for 60 minutes to the BH3-peptides (murine samples: 1 µM, 0.3 µM and 0.1 µM of BIM peptide, 10 µM and 3 µM of BAD, HRK and MS1 peptide; patient samples: 0.1 µM, 0.3 µM and 1 µM of BAD), a DMSO negative control and 25 µM alamethicin (BML-A150-0005, Enzo) serving as positive control for released cytochrome c. After peptide-exposure, cells were fixed in paraformaldehyde for 10 minutes and then incubated in N2 buffer for 10 minutes. Cytochrome c staining (612308, Biolegend) was performed overnight at 4°C. Samples were measured on a CytoFLEX XS flow cytometer (Beckman Coulter) and the data was analyzed using FlowJo 10.7.1 (Becton Dickinson). For the dynamic BH3 profiling, delta priming to vecabrutinib and ibrutinib was calculated as the change in the cytochrome c release of treated compared with control-treated cells.

Stimulation of splenocytes with soluble anti-CD3 antibody:

TCR activation and analysis was performed as reported earlier[4]. Briefly, single cell suspensions of the splenocytes were prepared and RBCs were lysed and removed using the RBC lysis buffer (Invitrogen). 2×10^5 cells were seeded in triplicates into U bottom 96 well plates and pretreated for 1 h at 37 °C with ibrutinib, vecabrutinib, or tirabrutinib. TCR stimulation was performed by adding 1 µg/ml anti-CD3ε monoclonal

antibody (eBioscience) and incubating for 3 h at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2. The cells were washed by centrifuging the 96 well plate and resuspending the pellets in 100 µl of ice-cold PBS. The cells from the 3 wells were pooled into a single well of a 96 well plate and stained for 30 minutes at 4 °C with TCRβ antibody to detect TCR internalization following CD3 stimulation along other antibodies for cell surface markers and eBioscience™ Fixable Viability Dye eFluor® (ThermoFisher Scientific). After washing, the cells were fixed and permeabilized using Foxp3 / Transcription Factor Staining Buffer Set (eBioscience™) and stained for Foxp3+ regulatory T-cells (Tregs) and Nur77, the immediate early TCR signaling component, to detect TCR activation. The cells were analysed using a 96 well plate format Cytoflex flow cytometer.

Analysis of T-cell function:

The impact of vecabrutinib and ibrutinib treatment on T-cell functions was analysed by assessing cytokine release and granzyme B production of CD8+ T-cells as previously described[4]. Cells were suspended in DMEM supplemented with 10 % FSC, 10 mM HEPES/NaOH, pH 7.4, 1 mM sodium pyruvate, and 0.1 % (v/v) β-mercaptoethanol, and seeded at a density of 2×10^6 cells/well into a 96 well U bottom plate. The cells were incubated for 6 h at 37 °C and 5 % CO_2 in Cell Stimulation Cocktail (eBioscience), containing PMA and ionomycin as stimuli and the protein transport inhibitors brefeldin A and monensin. The cells were then washed and incubated with fluorescent labelled antibodies for surface proteins along with eBioscience™ Fixable Viability Dye eFluor® (ThermoFisher Scientific) for 30 minutes at 4°C. Fluorescently labelled antibodies were used for the different cytokines and granzyme B after fixing and permeabilizing the cells using eBioscience™ Intracellular Fixation Buffer (ThermoFisher Scientific) for 30 minutes at room temperature. The cells were then analyzed using flow cytometry.

Analysis of drug impact on T-cells from CLL patients:

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll (Biochrom) density gradient centrifugation. PBMCs were viably frozen and when needed, frozen PBMCs were thawed. 3 × 106 PBMCs were seeded into U bottom 96 well plates. TCR

stimulation was performed by 1 µg/ml anti-CD3 or a combination of 1 µg/ml anti-CD3 (clone UCHT1, Biolegend) and 2µg/ml anti-CD28 (clone 28.2, Biolegend) antibodies in the presence of DMSO as vehicle control or ibrutinib, vecabrutinib, or tirabrutinib at a concentration of 1µM for 48h at 37°C and 5 % CO2. For flow cytometric analyses, cells were washed with PBS and stained for 30 minutes at 4 °C with eBioscience^{TM} Fixable Viability Dye eFluor® (ThermoFisher Scientific) as well as fluorescently labelled antibodies against surface markers. Cells were fixed using eBioscience^{TM} Intracellular Fixation Buffer (ThermoFisher Scientific) for 30min at room temperature, washed and stored at 4°C in the dark until data acquisition.

Effect of BTK inhibitors on isolated T-cells:

T-cells were isolated from NR4A1-GFP mouse splenocytes after Ficoll using EasySep^{TM} Mouse T cell isolation kit (Stemcell) according to the manufacturer's instructions. Human T-cells were isolated from PBMCs of healthy individuals using EasySep^{TM} Human T cell isolation kit (Stemcell). The isolated T-cells were treated with DMSO or 1µM and / or 5µM of ibrutinib, vecabrutinib or tirabrutinib. The isolated murine and human T-cells were activated using Dynabeads™ Mouse T-Activator CD3/CD28 and Dynabeads™ Human T-Activator CD3/CD28 beads, respectively. The cells were incubated for 6 h at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2, following which the cells were stained for 30 minutes at 4 °C with CD25, CD69 along with T-cell markers CD3, CD4 and CD8. The cells were fixed using IC fixation buffer (eBioscience) and analysed using flow cytometry. For analysis of proliferation, the human T-cells were stained using the cell proliferation dye eFluor^{TM} 670, followed by treatment with the inhibitors as mentioned above and stimulation with Dynabeads™ Human T-Activator CD3/CD28 for 72 hours at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2. The cells were then stained for T-cell markers CD3, CD4 and CD8 for 30 minutes at 4 °C, fixed using IC fixation buffer (eBioscience) and analysed using flow cytometry.

<u>DiOC$_6$/PI staining to determine cell viability:</u>

Loss of mitochondrial transmembrane potential following drug treatment was assessed using DiOC$_6$/PI staining and FACS. Splenocytes isolated from leukemic recipient mice were purified using Ficoll density gradient centrifugation (Biochrom and Sigma). The isolated mononuclear cells were cultured in RPMI 1640 containing 10 % FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at a density of 10^5 cells per well in a U bottom 96 well plate and treated with different concentrations of ibrutinib, vecabrutinib or venetoclax as single agents or in combination with 1 µM venetoclax. For analysing drug impact on human CLL cell viability assay, cells purified by Ficoll density gradient centrifugation (Biochrom and Sigma) were cultured in 96 well plates in 50% of HS5 conditioned medium and 50% of RPMI 1640 containing 20% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin and treated with the inhibitors or DMSO control and incubated at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2. After drug treatment for 4 days, the cells were stained for 20 minutes at 37 °C with 45 nM DiOC$_6$ (Sigma Aldrich) in RPMI 1640 with 0.5 % (w/v) BSA. The cells were then centrifuged and resuspended in FACS staining buffer (PBS with 2 % (v/v) FBS) containing 2 µg/ml PI and then analysed by with FACS immediately. Percentage viability was calculated by normalizing the DiOC$_6^+$ PI$^-$ populations in the inhibitor-treated samples to those treated with the vehicle containing DMSO.

<u>Statistical tests and quantifications:</u>

Statistical analyses were performed using GraphPad Prism®, version 6.0. For non-normally distributed data sets, the non-parametric Mann-Whitney U test was used. Paired variables were compared using the paired Student's T-test. Survival curves were analysed using the log-rank-test (Mantel-Cox). Statistical significance was defined as $P < 0.05$. In figures, *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.0001$; ns, $P > 0.05$.

Materials:

For western blotting, rabbit polyclonal antibody reactive against human and chicken BTK (F52632) was obtained from NSJ Bioreagents. The rabbit polyclonal antibody reactive against human and chicken PLCγ2 (#3872) was purchased from Cell Signaling. Mouse monoclonal antibody reactive against human and chicken β-Actin (#A5441) was from Sigma-Aldrich. Antibodies used for western blot on murine CLL cells isolated from vecabrutinib, ibrutinib or vehicle treated mice for apoptotic proteins include, BCL-xl (sc-8392), BCL2 (sc-492), BAX (sc-23959), ß-actin (sc-47778) and Lamin b (sc-6216) purchased from Santa Cruz Biotechnology, phospho-BAD (9296s), BAD (9292s) and BIM (2933s) from Cell Signaling Technology, MCL1 (AP 1312a) from Abgent and α-tubulin from Abcam. Antibodies used for flow cytometry are listed in Suppl. Table. 1. q-RT PCR was performed as described before[5]. The sequences of primers used for the qPCR are given in Suppl. Table. 2. CFSE (eBioscience[TM], 65-0850-84) and Indo-1- AM (I1223) were from Thermo Fisher Scientific. The BTK inhibitors ibrutinib (PCI-32765) and vecabrutinib (SNS-062) were purchased from Selleckchem and kindly provided by Sunesis, respectively.

References:

1.   Döhner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N. Engl. J. Med.* 2000;343(26):1910–6.

2.   Stilgenbauer S, Schnaiter A, Paschka P, et al. Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. *Blood*. 2014;123(21):3247–54.

3.   Fu G, Gascoigne NRJ. Multiplexed labeling of samples with cell tracking dyes facilitates rapid and accurate internally controlled calcium flux measurement by flow cytometry. *J. Immunol. Methods*. 2009;350(1–2):194–199.

4.   Hanna BS, Roessner PM, Scheffold A, et al. PI3Kδ inhibition modulates regulatory and effector T-cell differentiation and function in chronic lymphocytic leukemia. *Leukemia*. 2019;33(6):1427–1438.

5.   Scheffold A, Jebaraj BMC, Tausch E, et al. IGF1R as druggable target

mediating PI3K-δ inhibitor resistance in a murine model of chronic lymphocytic leukemia. *Blood*. 2019;blood.2018881029.

Suppl. Table 1 List of antibodies used for flow cytometry

Antibodies	Clone	Supplier
anti-CD19	ID3	eBioscience
anti-CD5	35-7.3	BD Biosciences
anti-CD3	17A2	Biolegend
anti-CD4	RM4-5	BD Biosciences
anti-CD8a	53-6.7	BD Biosciences
anti-CD44	IM7	BD Biosciences
anti-CD127	SB/199	BD Biosciences
anti-CD69	H1.2F3	Biolegend
anti-Ki67	B56	BD Biosciences
anti-CD25	PC61	BD Biosciences
anti-CD103	2E7	Biolegend
anti-CD357	DTA-1	Biolegend
anti-CD152	UC10-4B9	Biolegend
anti-FOXP3	PCH101	eBioscience
anti-FOXP3	FJK-16S	eBioscience
anti-IL2	JES6-SH4	Biolegend
anti-Granzyme B	NGZB	eBioscience
anti-IFNg	XMG1.2	eBioscience
anti- phospho ERK	MILAN8R	eBioscience
anti- Nur77	REA704	Milteny Biotech
anti-TCRbeta	H57-597	BD Biosciences
anti-CD3e	145-2C11	eBioscience
anti-chicken IgM	M-4	Southern Biotech
anti-chicken IgM	M-1	Southern Biotech
anti-mouse IgG_{2b} iso.	A-1	Southern Biotech

Suppl. Table 1 (continued) List of antibodies used for flow cytometry

Antibodies	Clone	Supplier
anti-CD4	RPA-T4	BD Biosciences
anti-CD5	UCHT2	BD Biosciences
anti-CD8	SK1	BD Biosciences
anti-CD4	SK3	BD Biosciences
anti-CD19	H1B19	BD Biosciences
anti-CD69	FN50	Biolegend
anti-CD25	M-A251	BD Biosciences

Suppl. Table 2: List of primers used for RT-qPCR based gene expression analysis

Gene	Forward primer	Reverse primer
Bim	5´GGAGACCAGTTCAACGAAACTT	5´AACAGTTGTAAGATAACCATTTGAGG
Bid	5´GCGTCTGCGTGGTGATT	5´TCCCAGTAAGCTTGCACAGG
Bad	5´AACAGTCATCATGGAGGCGC	5´CCCGCTGGGTACGAACTG
Bax	5´GTGAGCGGCTGCTTGTCT	5´GGTCCCGAAGTAGGAGAGGA
Bcl2	5´GTACCTGAACCGGCATCTG	5´GGGGCATATAGTTCCACAA
Mcl1	5´AAAGGCGGCTGCATAAGTC	5´TCCTCCGGAGACACGATG
Bcl-xl	5´TTTGGTGGGACAAGTGCAGGA	5´CTGTGAACTCCGCCCAGC

Suppl. Table 3: Patient characteristics of CLL cohort used for study of drug impact on viability and apoptotic priming (BH3 profiling)

ID	Age	Sex	VH	FISH	BTK	Viability assay	BH3 profiling
#1	74	M	Unmutated	Del17p13	C481S	Yes	Yes
#2	49	M	Unmutated	Normal	C481S	Yes	Yes
#3	57	M	n.a.	n.a.	C481S	Yes	No
#4	76	F	Unmutated	Del13q14	C481S	Yes	No
#5	63	F	n.a.	Normal	WT	Yes	Yes
#6	55	M	Unmutated	Normal	WT	Yes	Yes
#7	64	M	n.a.	Del13q14	WT	Yes	No
#8	71	M	Mutated	Normal	WT	Yes	No
#9	53	F	Mutated	Del13q14	WT	Yes	No
#10	60	M	n.a.	Del13q14	WT	Yes	Yes
#11	53	M	Mutated	Del13q14	WT	Yes	Yes
#12	65	M	Mutated	Del13q14	WT	Yes	No
#13	55	M	Unmutated	Normal	WT	No	Yes

Suppl. Table 4: Patient characteristics of CLL cohort used for analysis of drug impact on T-cells

ID	Age	VH	FISH	Stage
#14	84	unmutiert	13q del	n.a.
#15	61	unmutiert	Trisomy 12	Binet C
#16	54	unmutiert	Normal	Binet A
#17	44	unmutiert	Del13q14	Binet A
#18	82	mutiert	Del13q14	Binet A
#19	64	mutiert	Normal	Binet A
#20	69	mutiert	Trisomy 12	Binet A
#21	60	mutiert	Del13q	Binet A
#22	82	mutiert	Del13q	Binet A

Suppl. Table 5: IC50 values calculated from direct kinase assay (Fig. 1c-1e)

IC$_{50}$ (nM), [ATP]= 50 µM	WT BTK	C481S BTK	Fold Change
Vecabrutinib	4.6	1.1	0.2
Ibrutinib	0.1	6.6	66
Acalabrutinib	4.2	707	168

Suppl. Table 6: Kinase inhibition profile of vecabrutinib

Kinase	IC50 (nM)
BTK*	3
LCK†	8
LCK	13
ITK*	14
TEC†*	14
BLK*	23
cSRC	84
NEK11	90
PKG1α	184
AURKB	191
BMX*	224
LYN	259
HCK	276
ERBB4*	317
PKG1β	327
YES	402
TXK*	474
FYN	651
CHK2	823
EGFR*	6644
ABL	> 1000
FLT3	> 1000
JAK3*	> 1000
BRK	>1000
CSK	>1000
RET	>1000
FGR	500 (Kd)

* Cysteine residue aligning
with BTK C481
† Activated

Suppl. Fig. 1
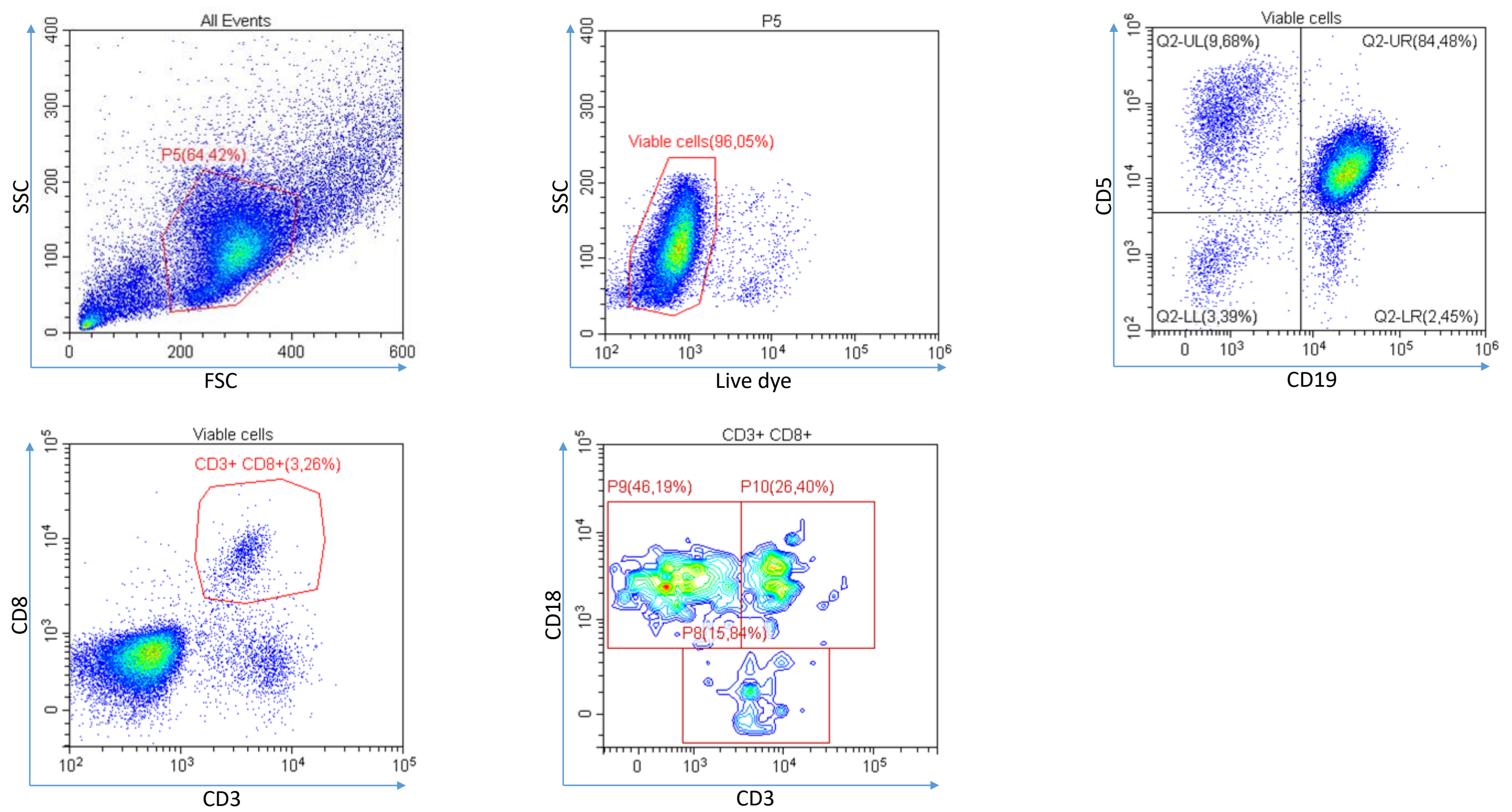
Suppl. Fig. 1: Gating strategy for analysis of CD8+ T cell subsets using FACS

Suppl. Fig. 2
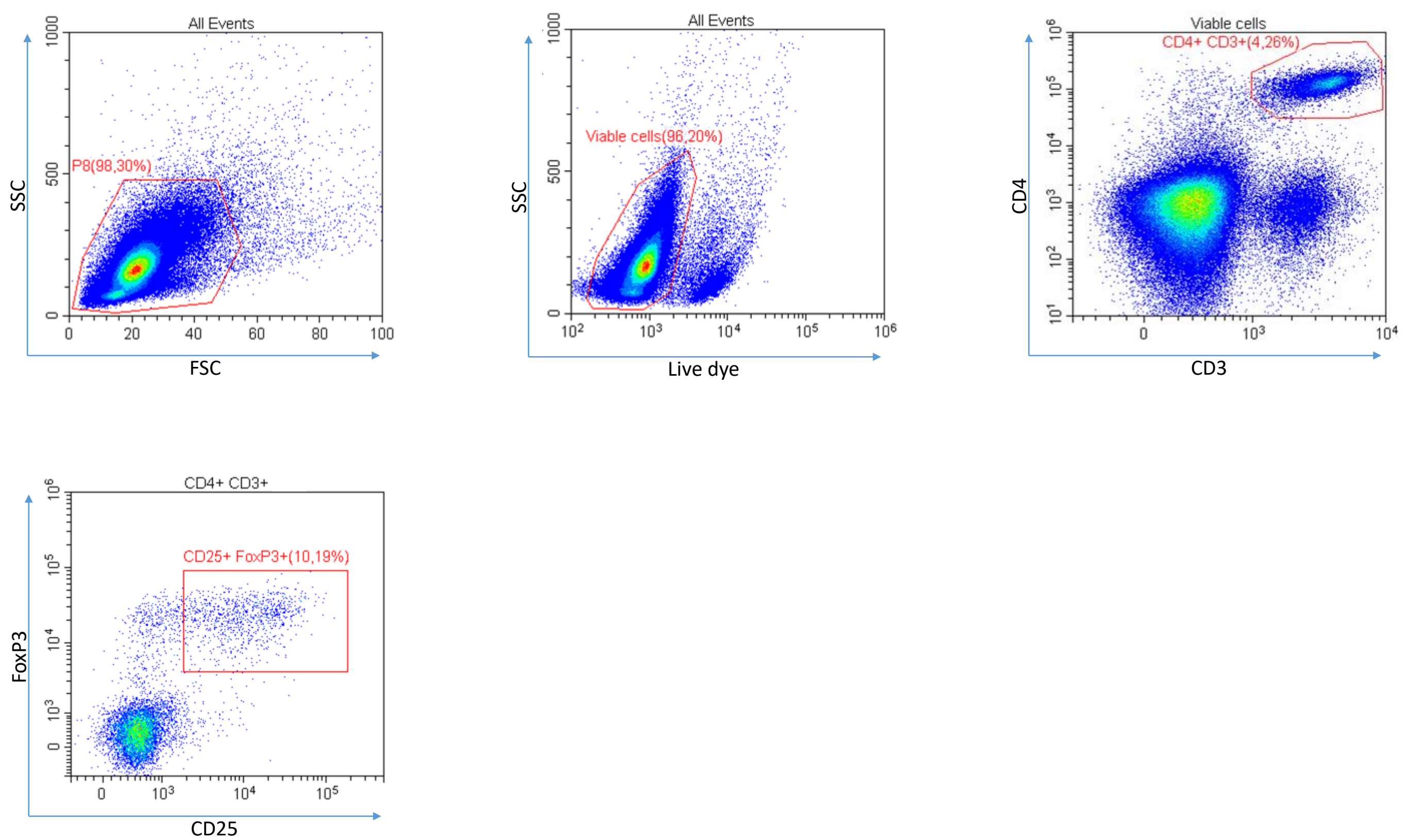
Suppl. Fig. 2: Gating strategy for analysis of CD4 CD15+ FoxP3+ Treg cells using FACS
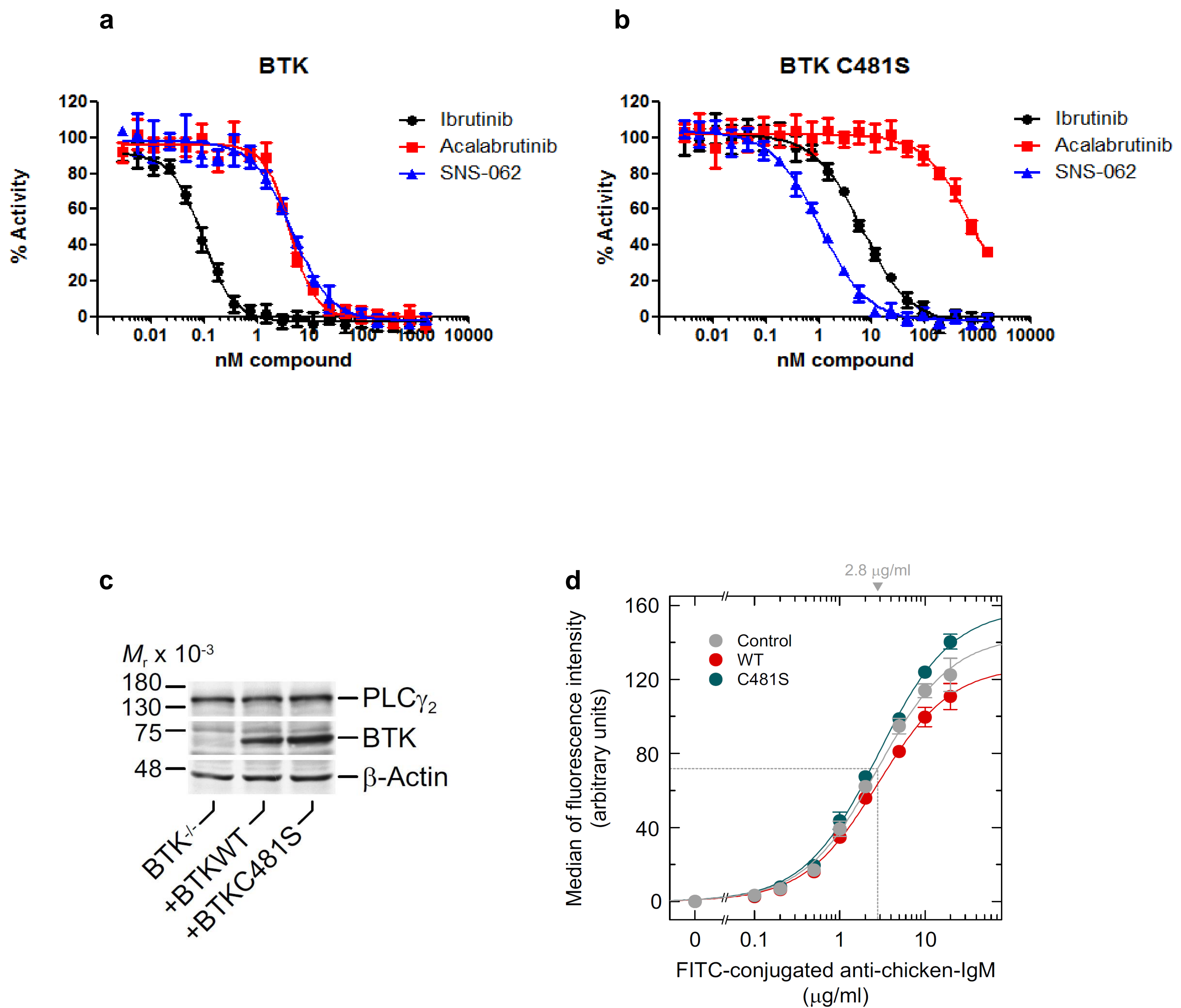
Suppl. Fig. 3 Inhibition of kinase activity of (a) Wt and (b) C481S mutant BTK by ibrutinib, vecabrutinib and acalabrutinib, as measured by direct kinase assay. Error bars indicate standard deviation from biological replicates. (c) Representative western blot of BTK protein expression in BTK^{-/-} DT40 cells prior to and following reconstitution with Wt or C481S mutant BTK. (d) Flow-cytometric analysis of BCR surface expression in BTK^{-/-} DT40 cells and upon reconstitution with Wt or C481S mutant BTK. Fig c and d are representative figures of 3 biological replicates
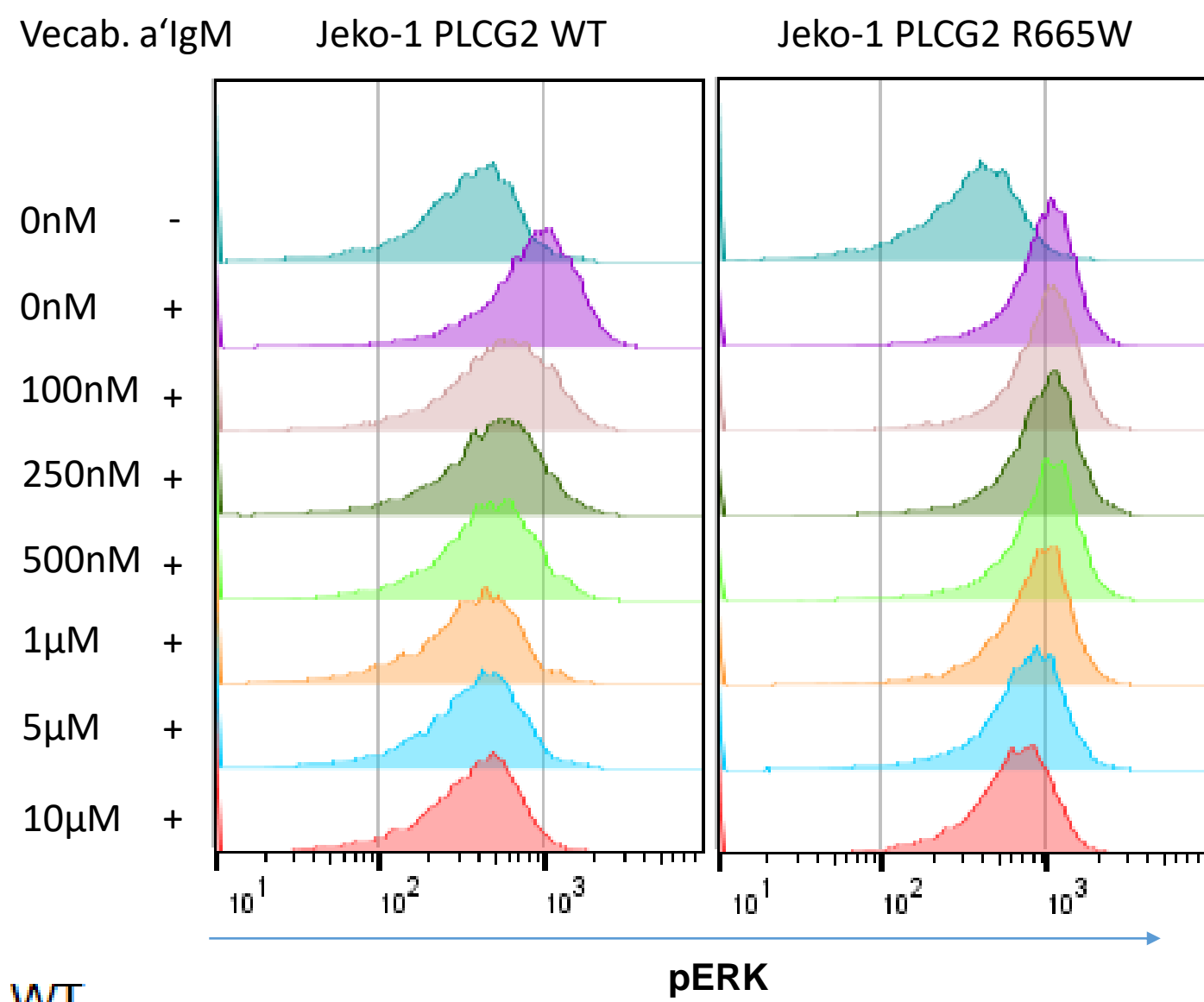
Suppl. Fig. 4

a

$M_r \times 10^{-3}$

130 —

75 — —BTK

48 — —β-Actin

−	−	−	−	PLCγ₂
−	+	−	−	BTKE41K
−	−	+	−	BTKE41K|K430R
−	−	−	+	BTKE41K|C481S

b

$M_r \times 10^{-3}$

130 — —PLCγ₂

75 — —BTK

48 — —β-Actin

+	+	+	+	+	+	+	+	+	+	PLCγ₂
−	−	−	+	+	+	−	−	−	−	BTKE41K
−	−	−	−	−	−	+	−	−	−	BTKE41K|K430R
−	−	−	−	−	−	−	+	+	+	BTKE41K|C481S
−	+	−	−	+	−	−	−	+	−	Acalabrutinib
−	−	+	−	−	+	−	−	−	+	Vecabrutinib
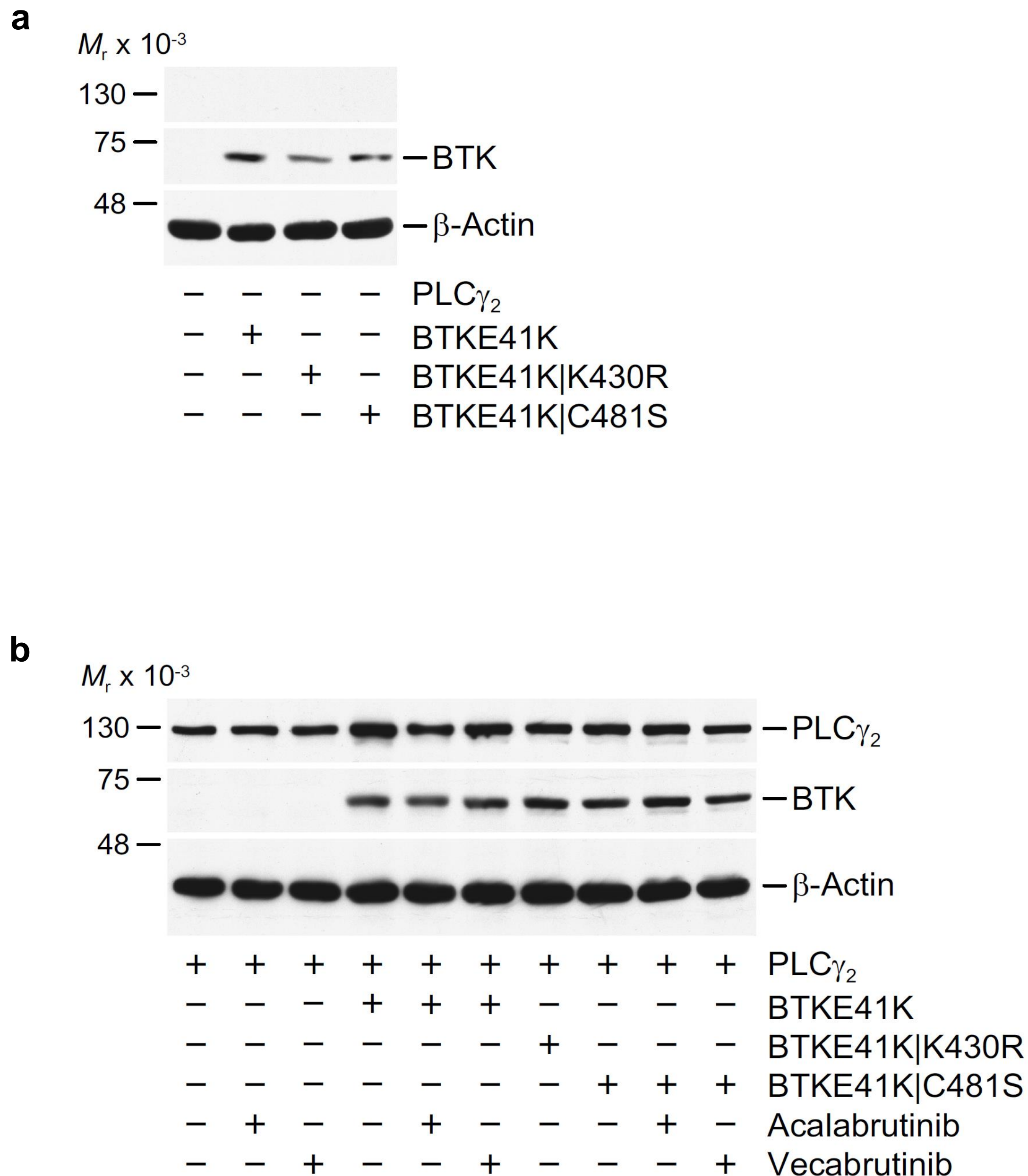
Suppl. Fig. 4: (a) Overexpression of the constitutively active E41K BTK mutant, alone or together with the K430R or C481S BTK mutants in COS-7 cells. (b) changes in BTK and PLCγ2 expression upon acalabrutinib or vecabrutinib treatment of COS-7 cells overexpressing E41K BTK mutant, alone or in combination with the K430R or C481S BTK mutants. The western blots are representatives of 3 biological replicates.
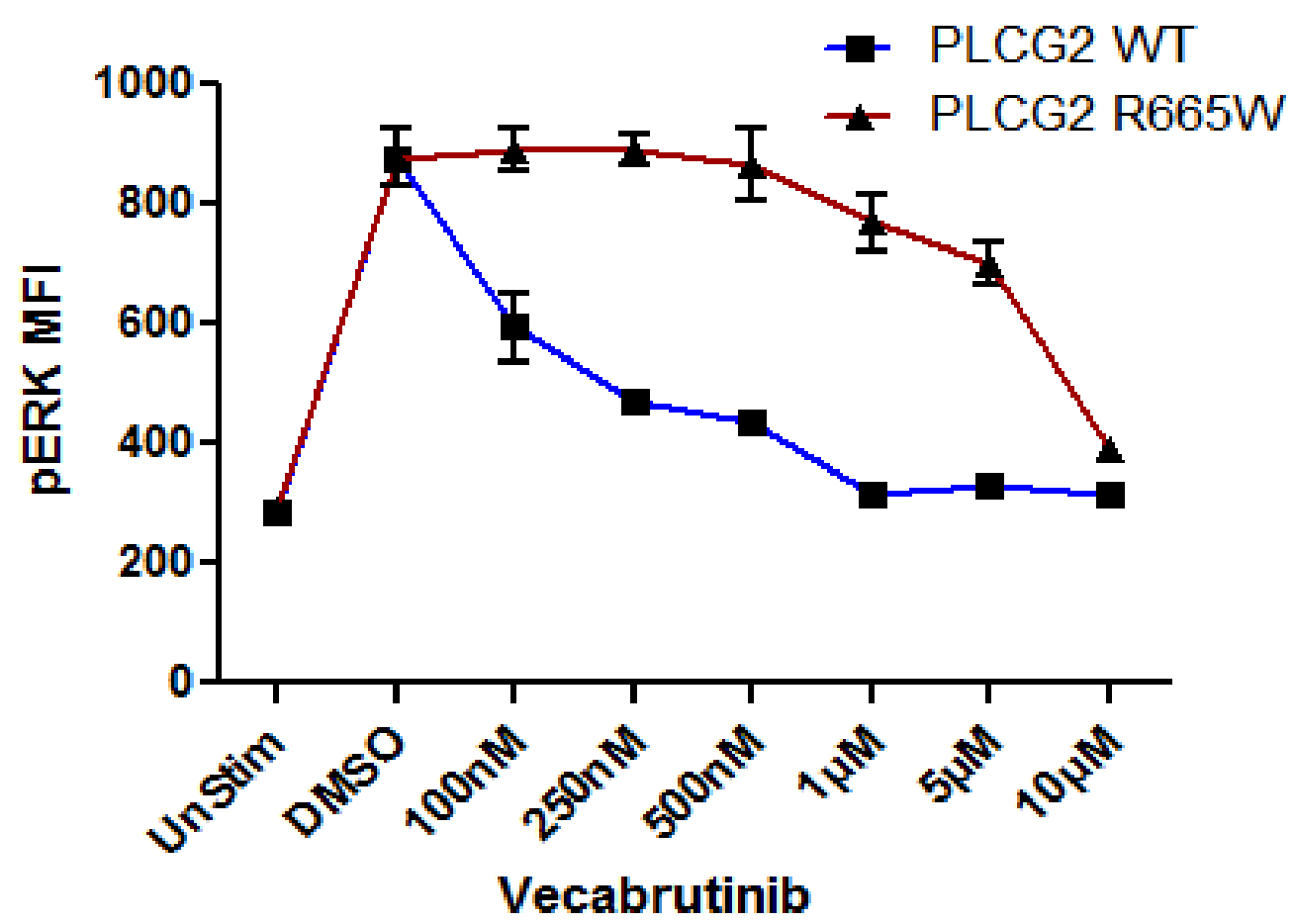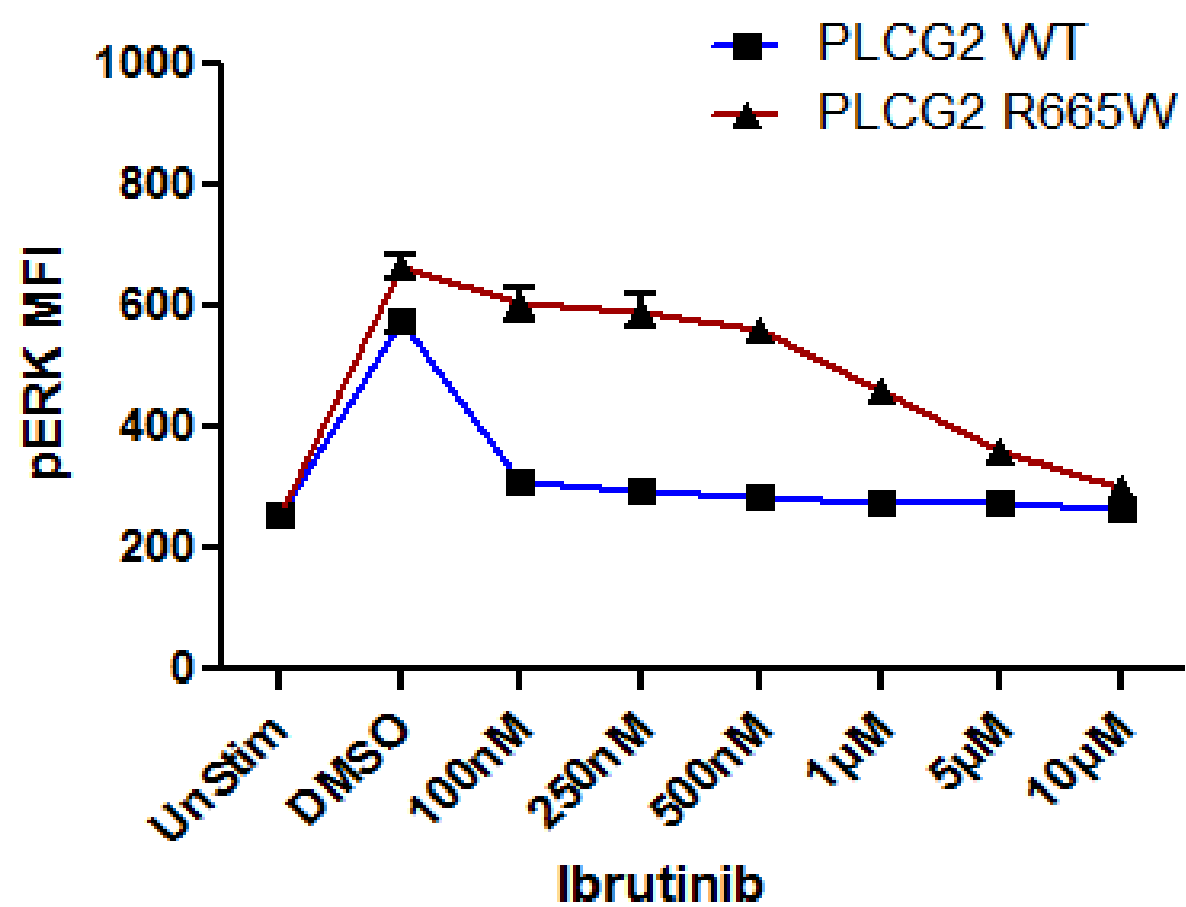
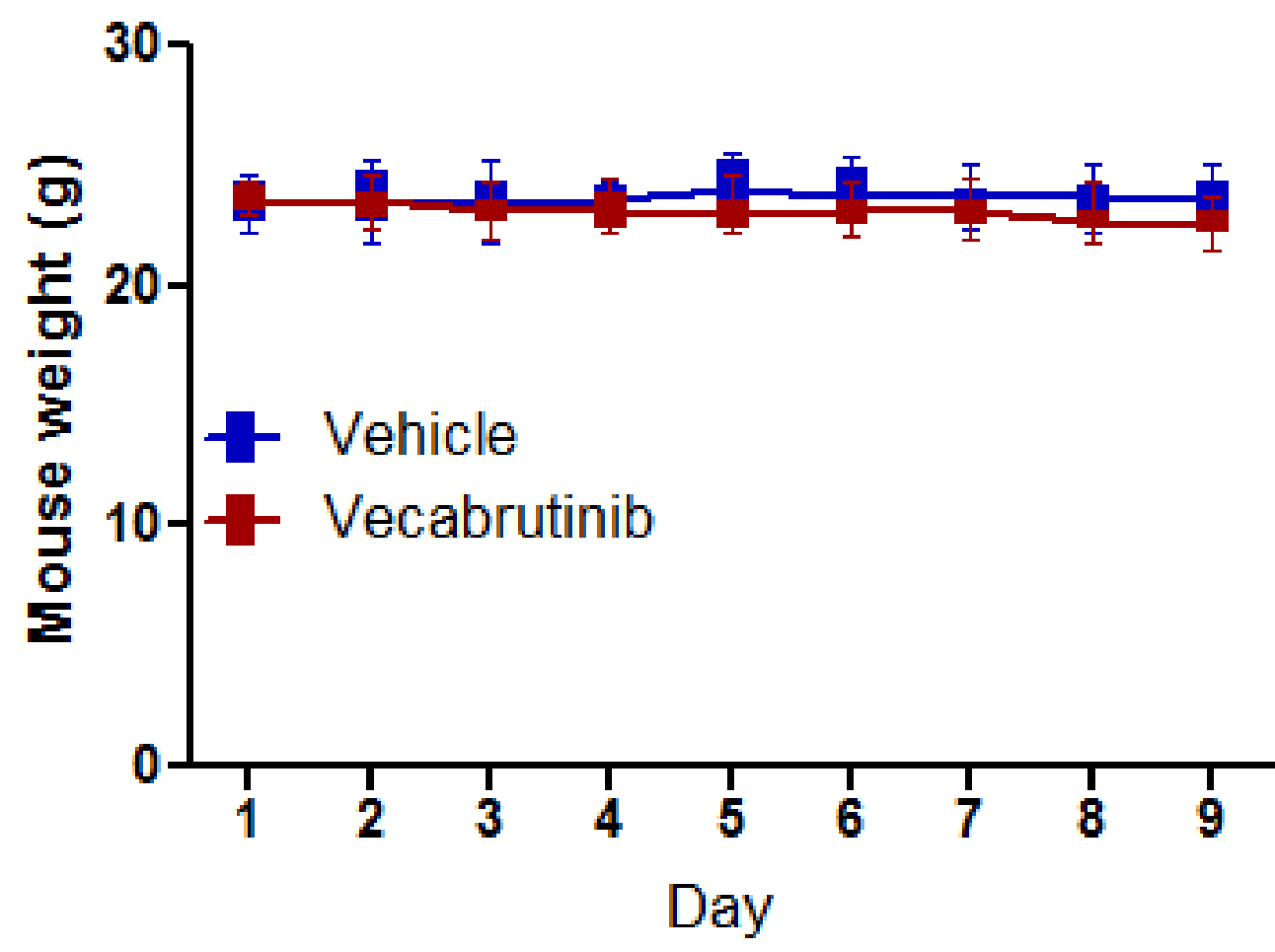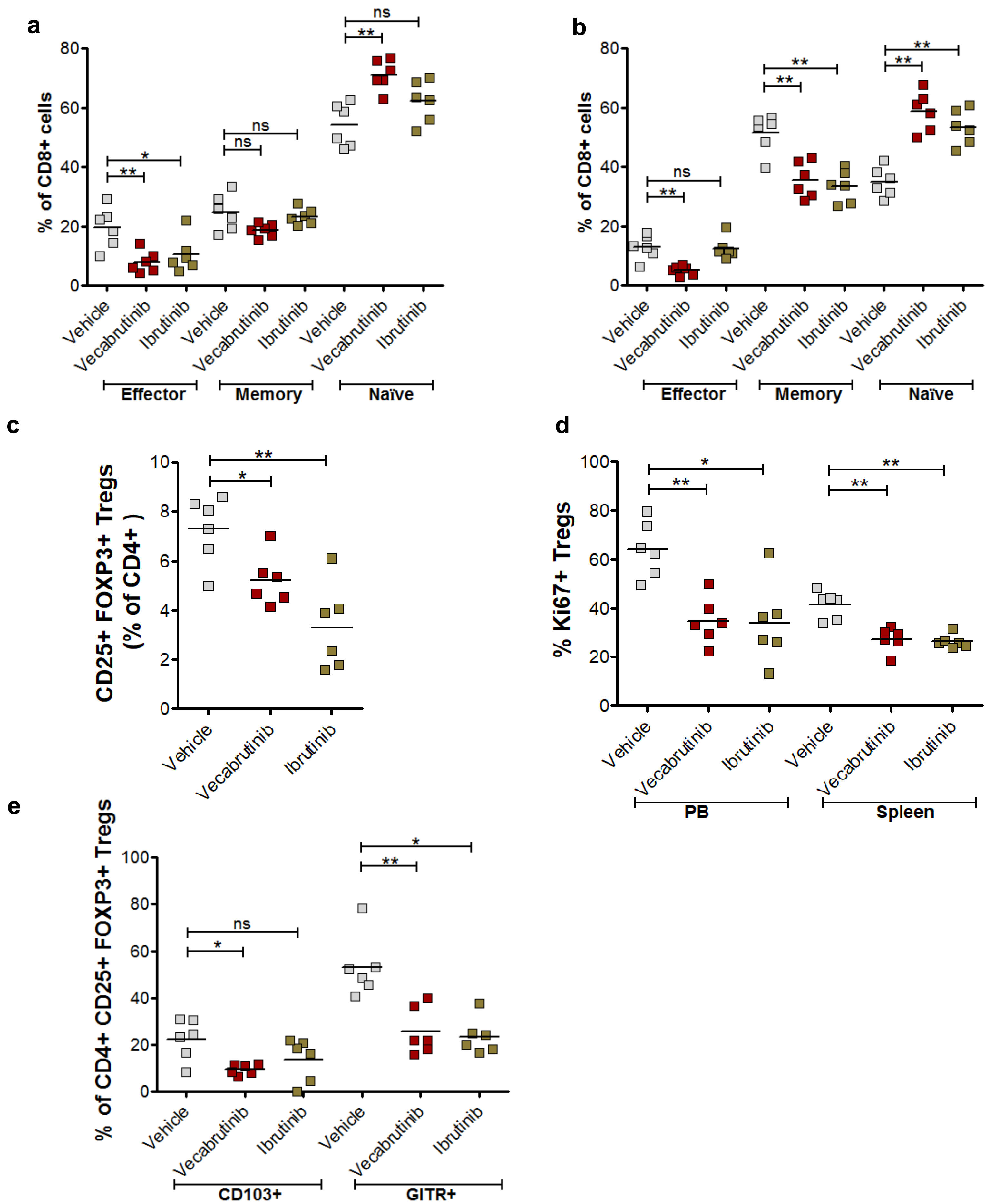**Suppl. Fig. 5**

a
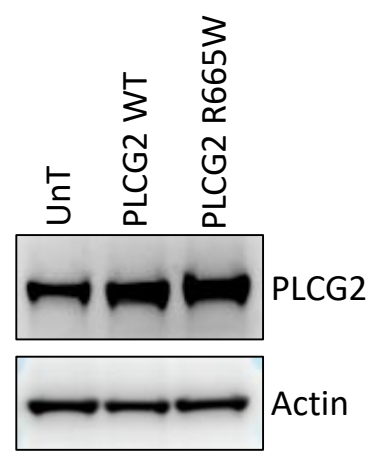
b

c

d

Suppl. Fig. 5 (a) Western blot showing overexpression of Wt and R665W mutant PLCγ2 in Jeko-1 cells. (b) Representative FACS plots showing changes in pERK levels upon stimulation with anti-IgM in cells pretreated with vecabrutinib. (c) Similar to ibrutinib, vecabrutinib treatment led to a decrease in pERK only at high concentrations in R665W PLCγ2 mutant expressing cells. All data presented here are representative of more than 3 biological replicates.
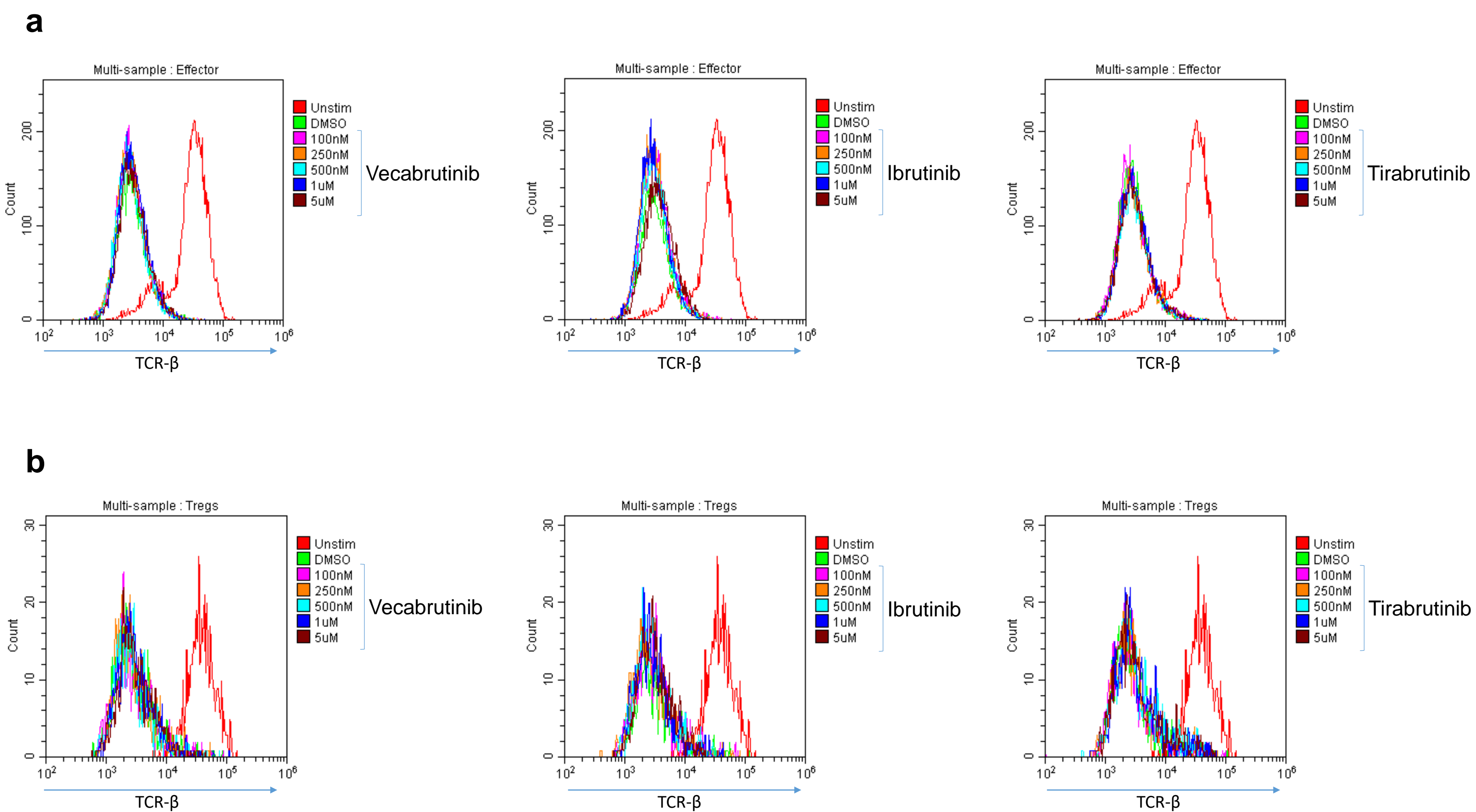
Suppl. Fig. 6

Suppl. Fig. 6: Weights of mice treated with vecabrutinib vs. vehicle
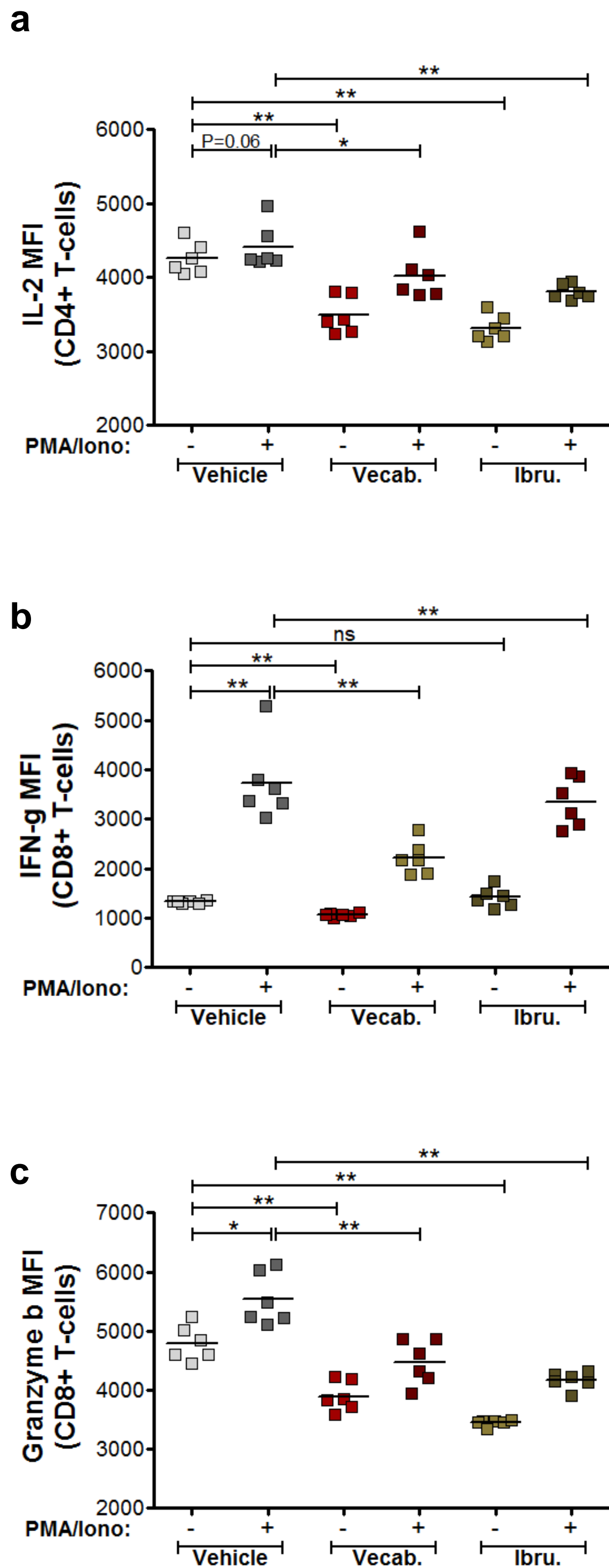
Suppl. Fig. 7

Suppl. Fig. 7: Changes in effector (CD127^{low} CD44^{int-hi}) and memory (CD44+ CD127+) and naïve (CD44- CD127+) T-cell populations in (a) peripheral blood and (b) bone marrow; and (c) changes in CD4+ CD25+ FoxP3+ regulatory T cells (Tregs) in peripheral blood upon treatment with vecabrutinib, ibrutinib or vehicle. (d) Proliferation (% Ki67+) of Tregs in peripheral blood and spleen upon treatment with the inhibitors compared to vehicle. (e) Expression of CD103 and GITR by Tregs in peripheral blood upon vecabrutinib, ibrutinib or vehicle treatments.
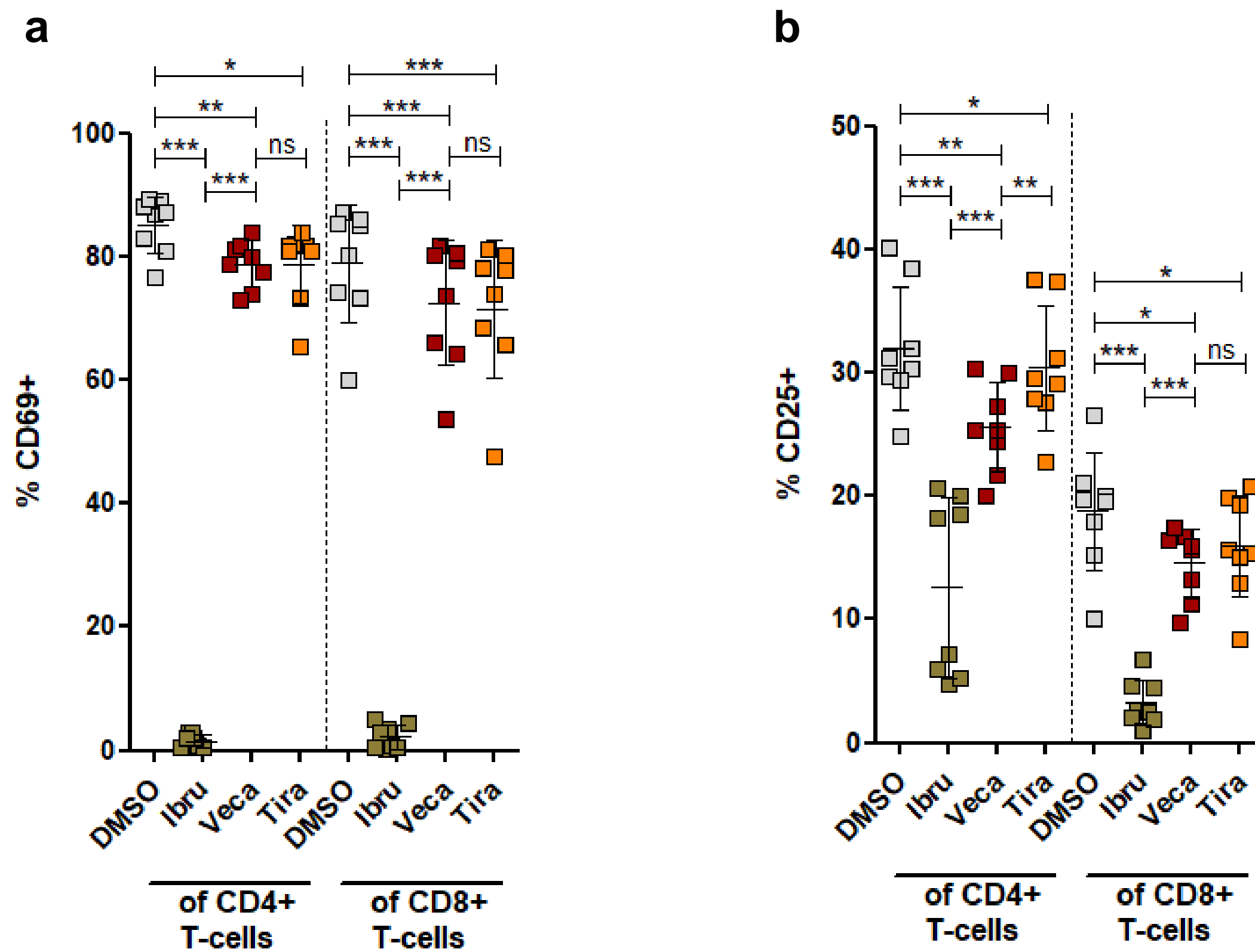
Suppl. Fig. 8

Suppl. Fig. 8: Representative FACS plot showing changes in TCR-beta expression in (a) CD8+ effector (CD127^{low} CD44^{int-hi}) and (b) CD4+ CD25+ FoxP3+ regulatory T cells (Tregs) in splenocytes following pre-treatment with different doses of vecabrutinib, ibrutinib and tirabrutinib, followed by stimulation of TCR using anti-CD3 antibody.
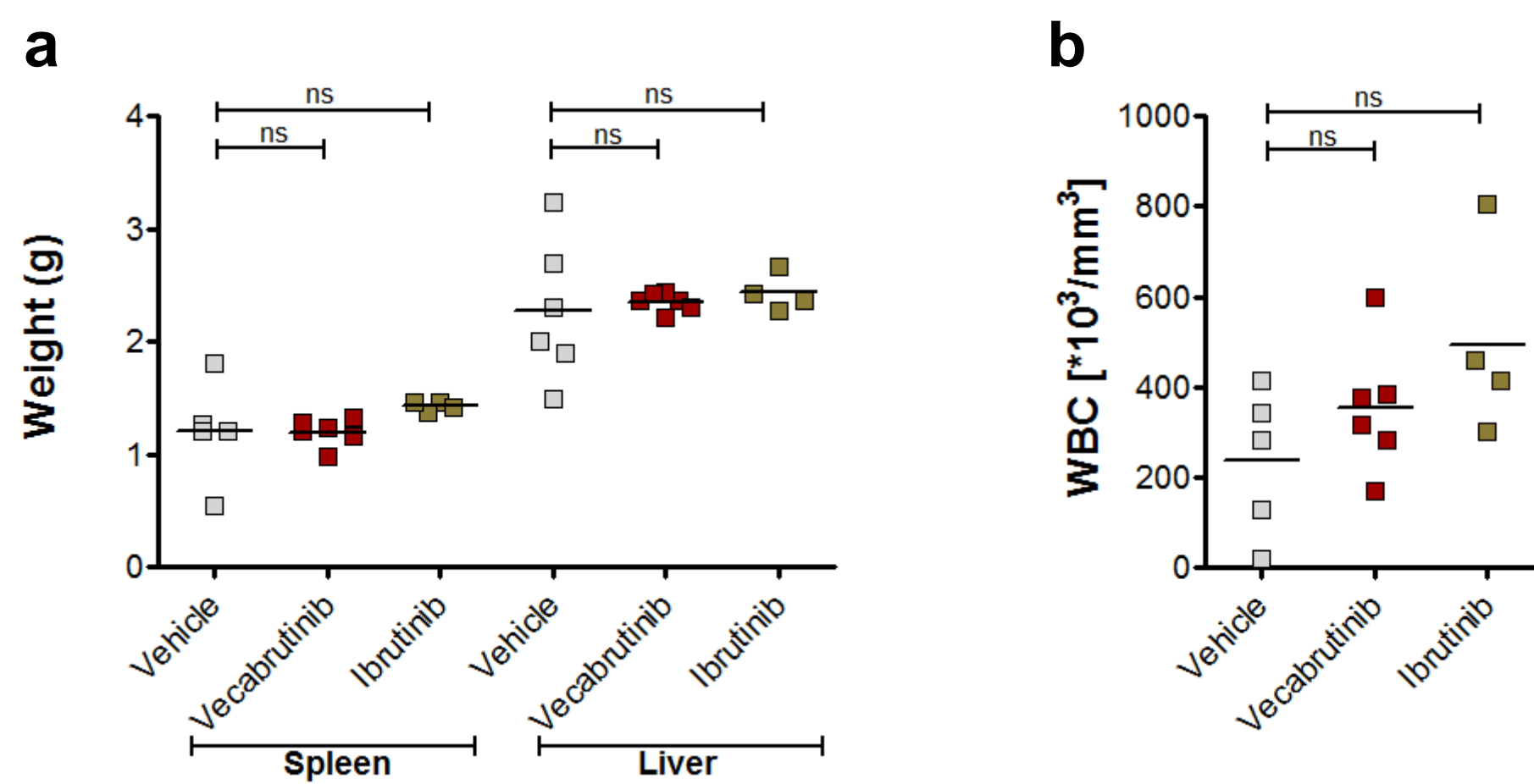
Suppl. Fig. 9

a

b

c

Suppl. Fig. 9 Functional assessment of splenic CD8+ T-cells isolated following 10 days of treatment with vehicle, vecabrutinib or ibrutinib treated mice and stimulated with PMA/Ionomycin measured by production of: (a) IL-2 by CD4+ T-cells (b) IFN-g and (c) Granzyme b by CD8+ T-cells.
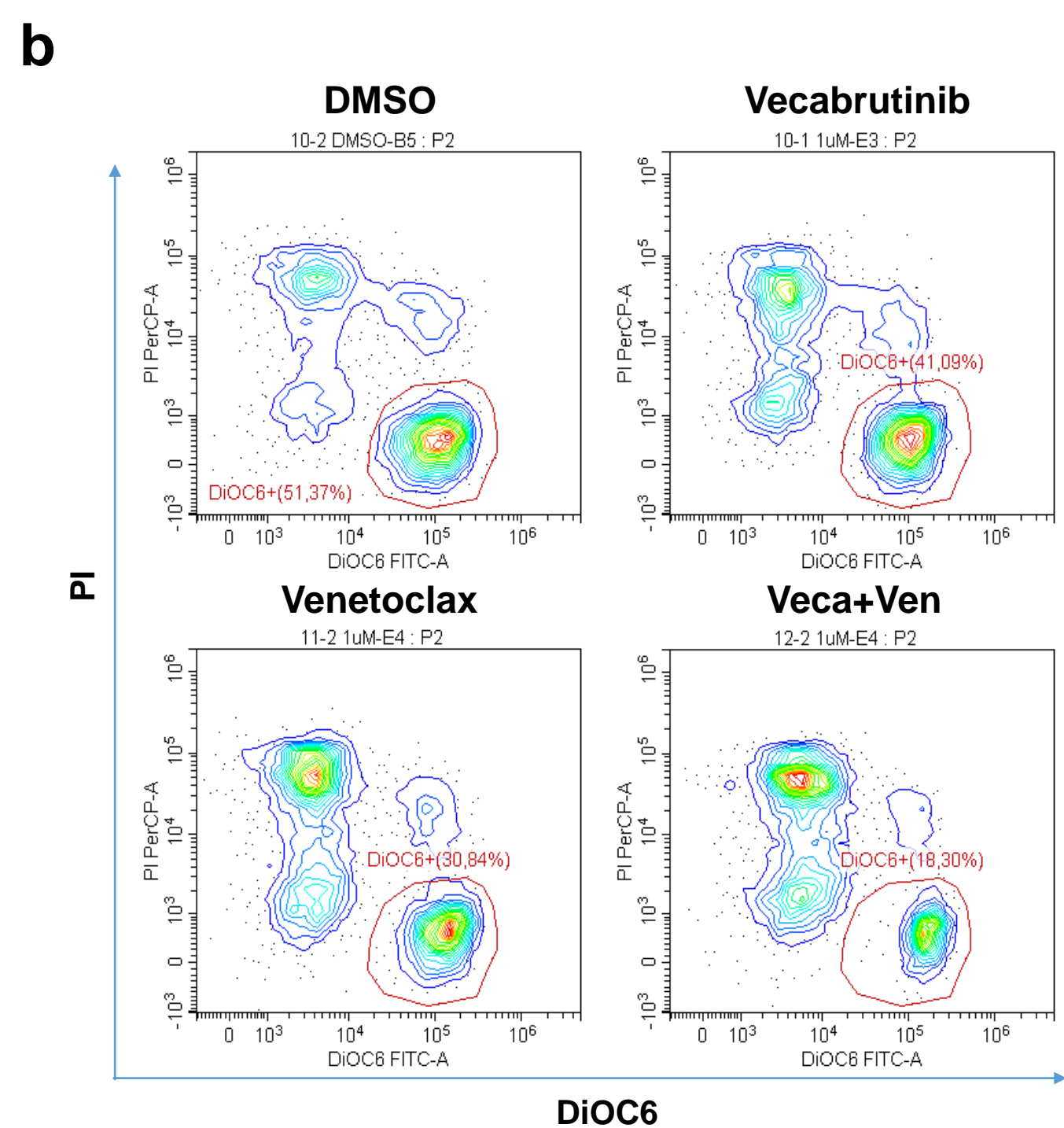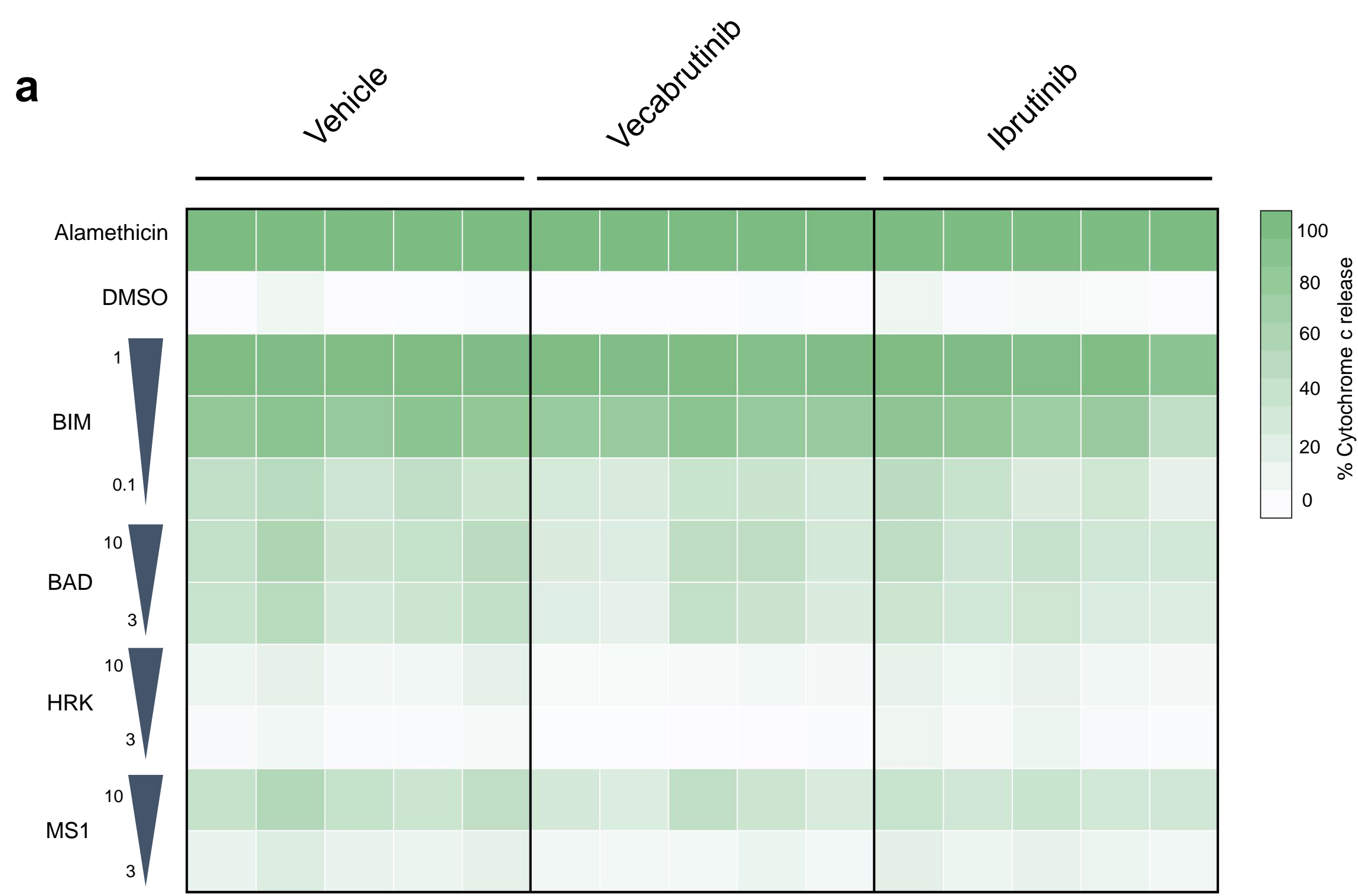
Suppl. Fig. 10

Suppl. Fig. 10: T-cell activation measured by (a) relative expression of CD69 and (b) relative expression of CD25 on human T-cells isolated from healthy individuals, treated ex vivo with DMSO or 5μM of ibrutinib, vecabrutinib or tirabrutinib, and stimulation with anti-CD3/CD28 beads for 6 hours.

Suppl. Fig. 11:

Suppl. Fig. 11: Analysis of (a) spleen, liver weights and (b) WBC count of mice from the survival analysis (Fig. 5a)

Suppl. Fig. 12:

a

b

Suppl. Fig. 12: (a) q-RT-PCR analysis of tumors that progressed under BTK inhibitor treatment for the expression of the pro-apoptotic (*Bim*, *Bid, Bad, Bax*) and anti-apoptotic (*Bcl-xl*, *Mcl1* and *Bcl2*) genes. (b) Western blotting analysis for comparison of protein expression of phospho-BAD, BAD, BCL-xl, BIM, BAX, BCL2 and MCL1 in splenic tumors from vehicle, vecabrutinib and ibrutinib treated mice.

Suppl. Fig. 13:

a

b

Suppl. Fig. 13: (a) Heatmap of baseline mitochondrial BH3 profiling in the murine Eµ-TCL1 model after treatment with vehicle control, vecabrutinib or ibrutinib. (b) Representative FACS plots showing *ex vivo* drug sensitivity measurement using DiOC6/PI FACS staining.