Differential effects of BTK inhibitors ibrutinib and zanubrutinib on NK-cell effector function in patients with mantle cell lymphoma

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Figure S1 (staining panels)

NK cell panel 1

Receptor	Alt. name	Clone	Colour
CD56	NCAM 1	NCAM16.2	BUV563
CD3	+ fix aqua	OKT3	BV510
CD14		M5E2	BV510
CD19		HIB19	BV510
CD16		3G8	APC-H7

NK cell panel 2

Receptor	Alt. name	Clone	Colour
KIR3DL1		DX9	BUV395
CD56		NCAM16.2	BUV563
CD69	CLEC2C	FN50	BUV737
KIR3DL2		539304	AF 405
CD3/14/19	+fix aqua		BV510
DNAM-1	CD226	DX11	BV650
			BV786
FceRy		FCABS400F	FITC
NKG2C	CD159C	#134591	PERCP
KIR2DL1	CD158a	MM0439-8J29	PE
KIR2DS4	CD158i	179315	AF 594
KIR2DL1/S	CD158a/h	EB6B	Pe-Cy7
NKG2A	CD159C	Z199	APC
CD16		3G8	APC-H7

NK cell panel 3

Receptor	Alt. name	Clone	Colour
CD56		NCAM16.2	BUV563
CD57		HNK-1	BUV805
CD62L		DREG-56	Pac Blue
CD3/14/19	+fix aqua		BV510
NKp46	CD335	9E2	BV650
NKp30	CD337	p30-15	BV786
KIR2DL3	CD158b2	180701	AF488
			PERCP
NKp44		FAB22491p	PE
KIR2DL2/L3/S2	CD158b	DX27	PeC7
NKG2D	CD314	#149810	APC
CD16		3G8	APC-H7

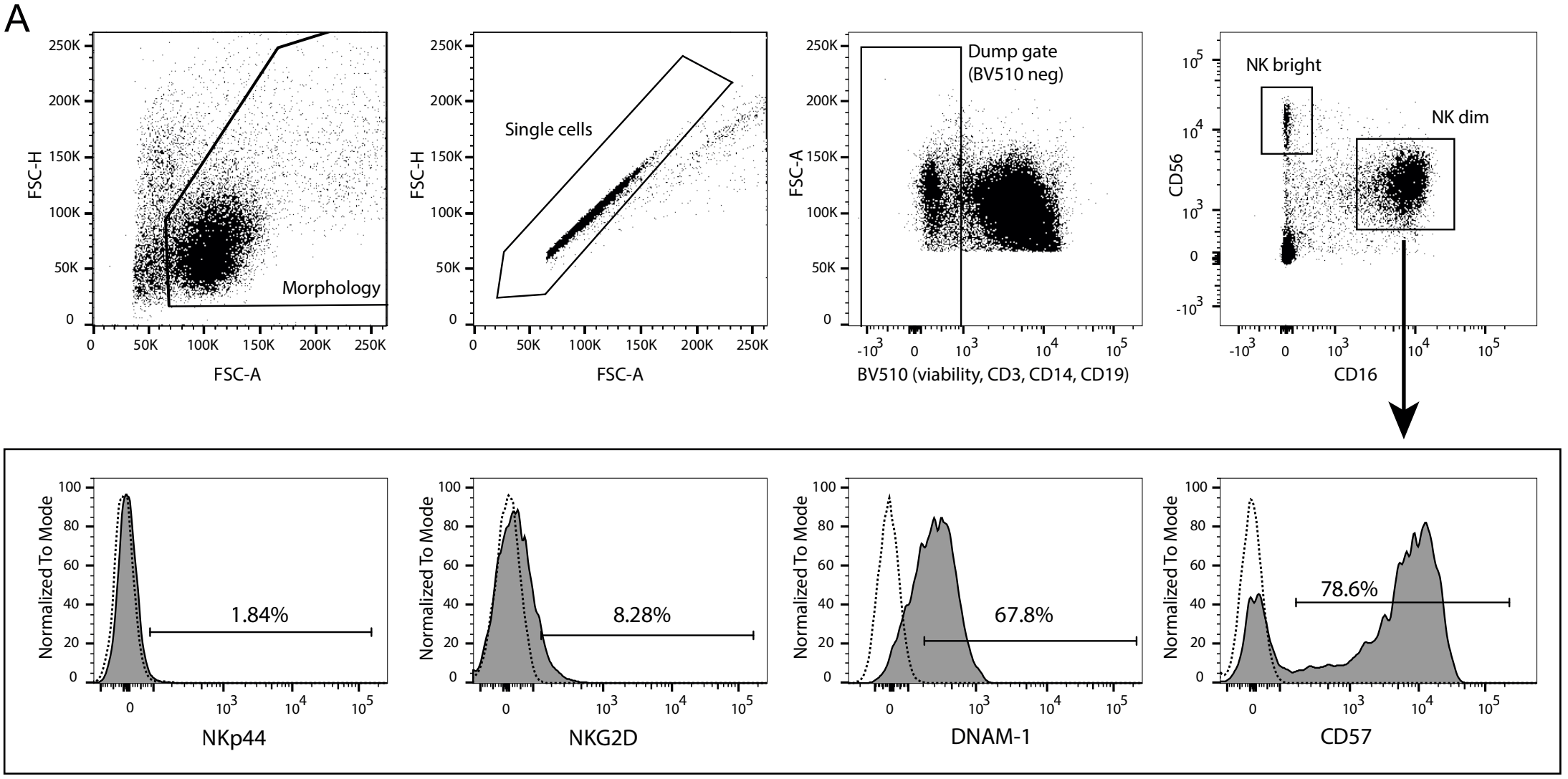
B cell panel 1

Receptor	Alt. name	Clone	Colour
CD3	+ fix aqua	OKT3	BV510
CD14		M5E2	BV510
CD5		L17F12	BV605
CD19		SJ25C1	APC-H7

B cell panel 2

Receptor	Alt. name	Clone	Colour
HLA-ABC		G46-2.6	Horizon-V450
CD3/CD14	+ fix aqua		BV510
CD5		L17F12	BV605
CD155		TX24	BV650
CD23		M-L233	BV711
PD-L1	CD274	29E.2A3	BV785
HLA-G		87G	AF488
ULBP2,5,6		#165903	PERCP
CD112	Nectin-2	#610603	PE
MICA/B		159207	AF 594
CD20		2H7	Pe-Cy7
HLA-E		3D12	APC
ULBP-1		FAB1380N	AF700
CD19		SJ25C1	APC-H7

Figure S2 (gating strategy)



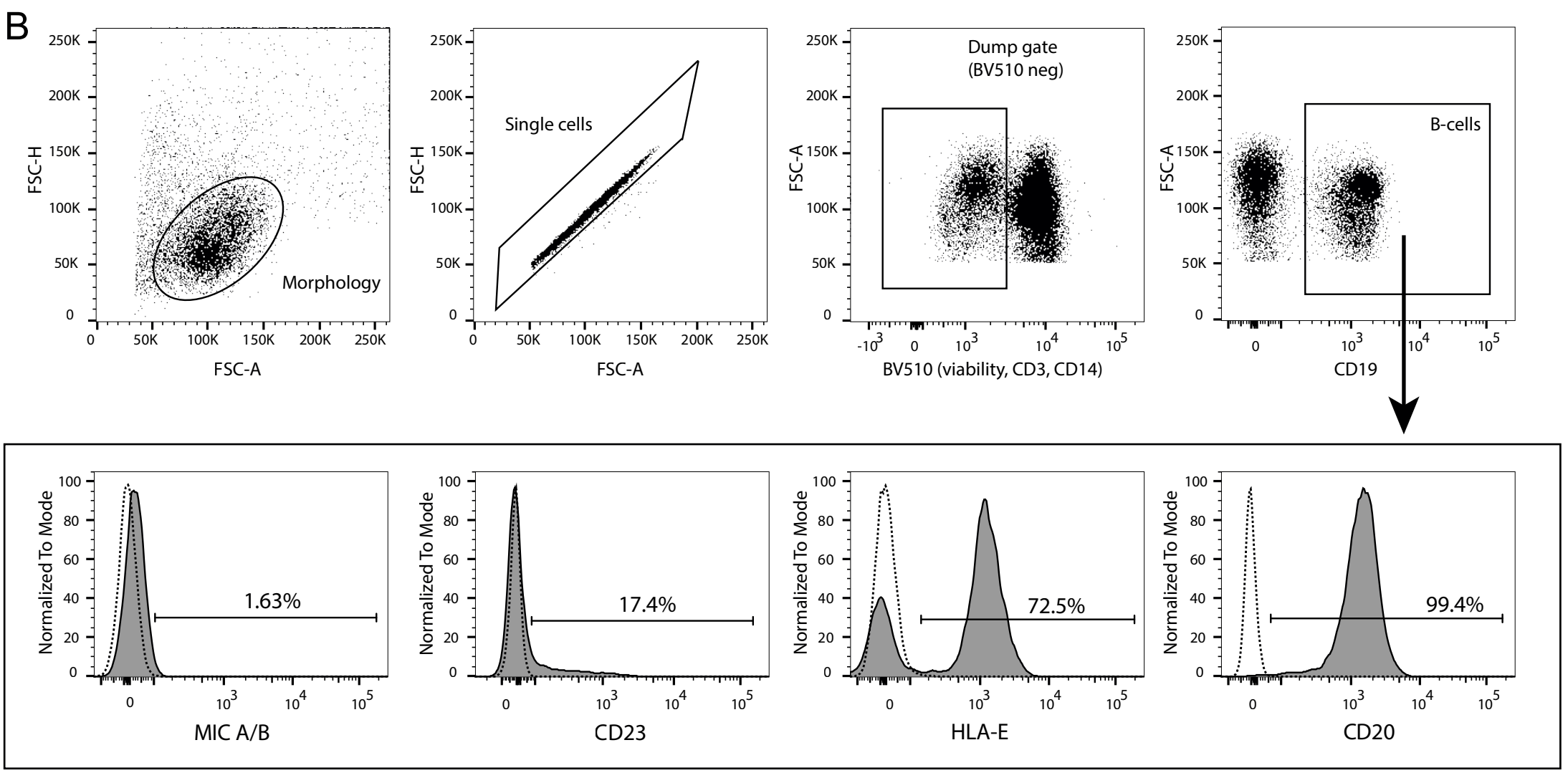
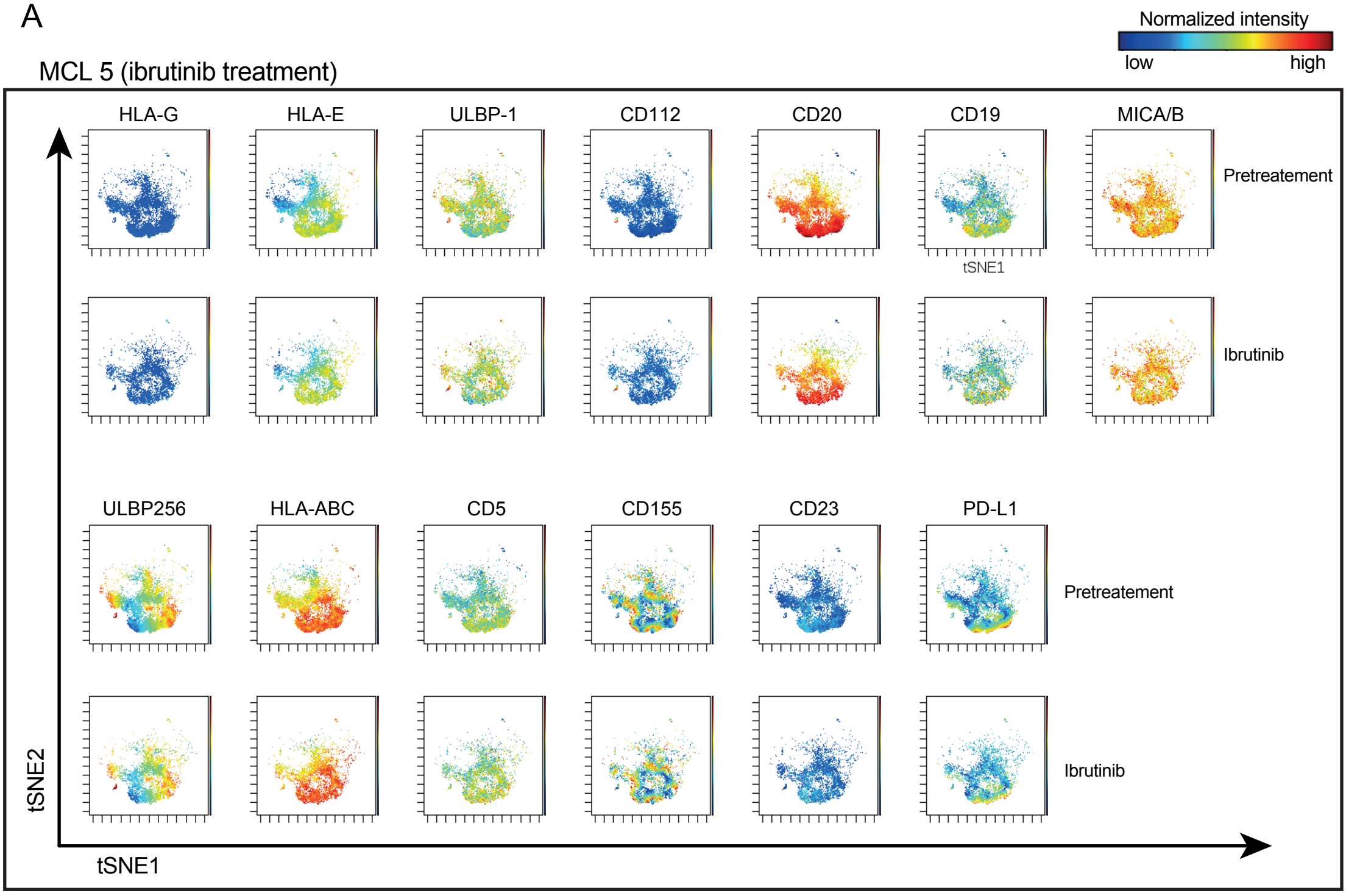


Figure S3

A


Normalized intensity
low
high
MCL 5 (ibrutinib treatment)
HLA-G
HLA-E
ULBP-1
CD112
CD20
CD19
MICA/B
Pretreatement
tSNE1
Ibrutinib
ULBP256
HLA-ABC
CD5
CD155
CD23
PD-L1
Pretreatement
Ibrutinib
tSNE2
tSNE1

B

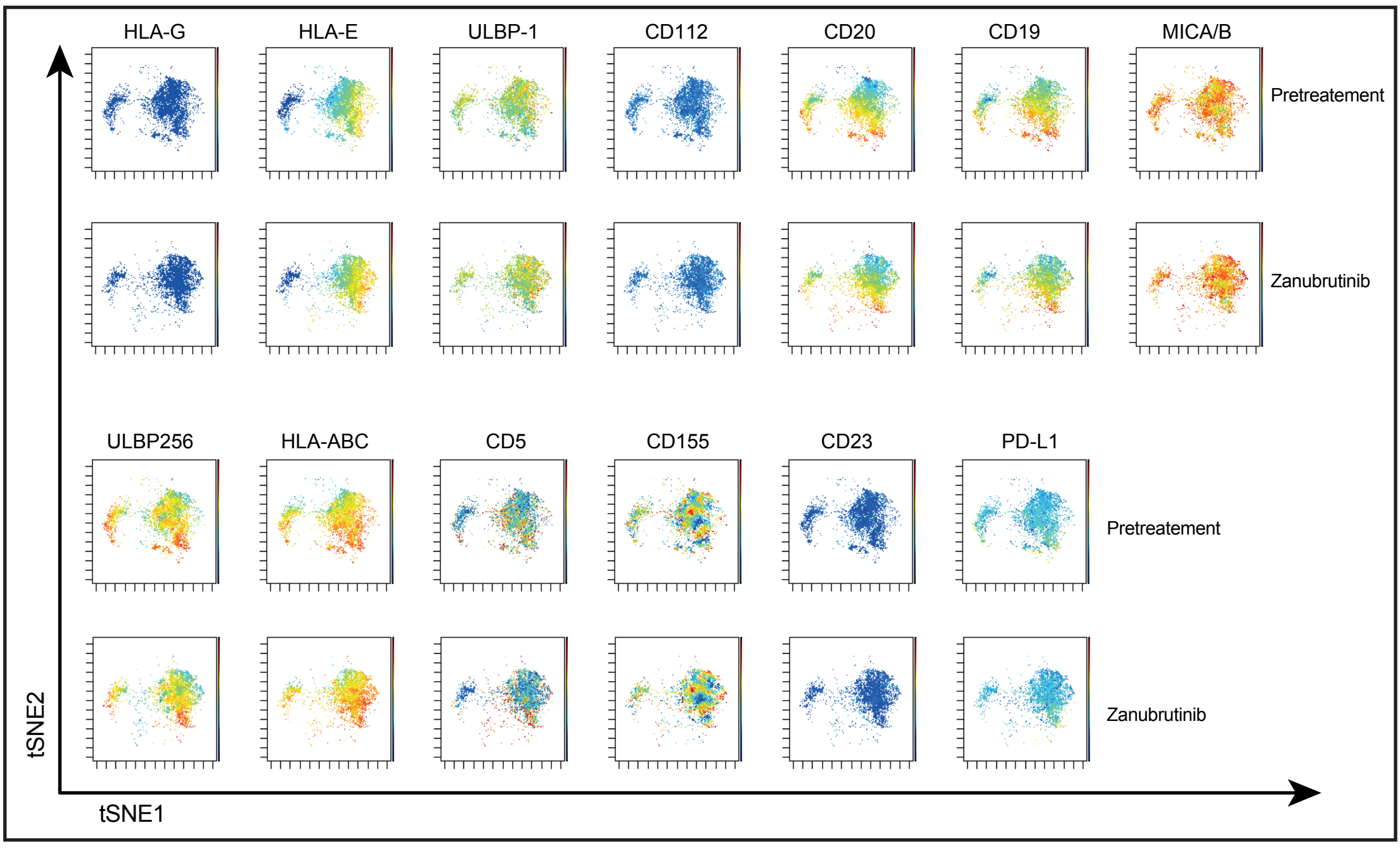
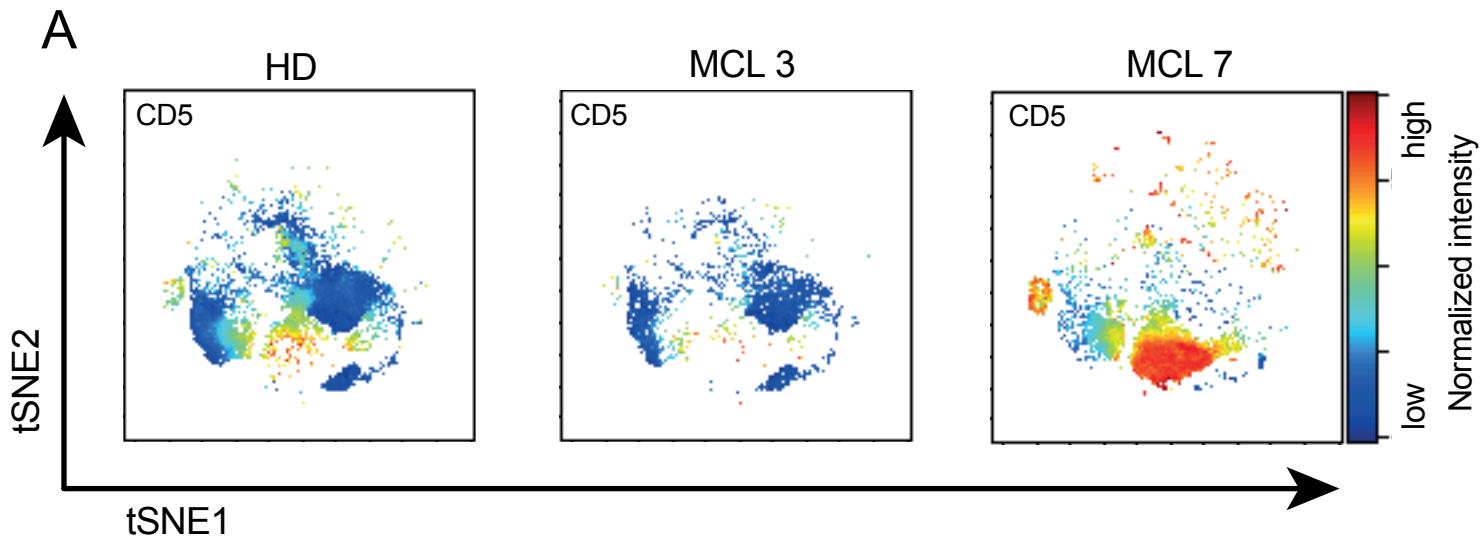
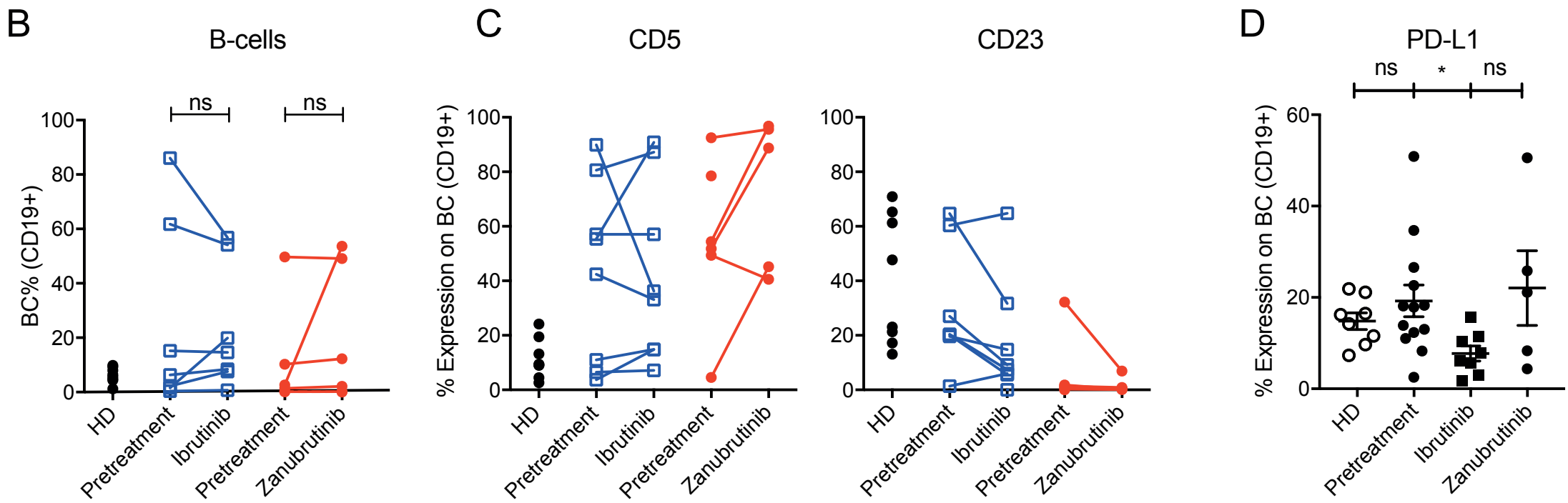
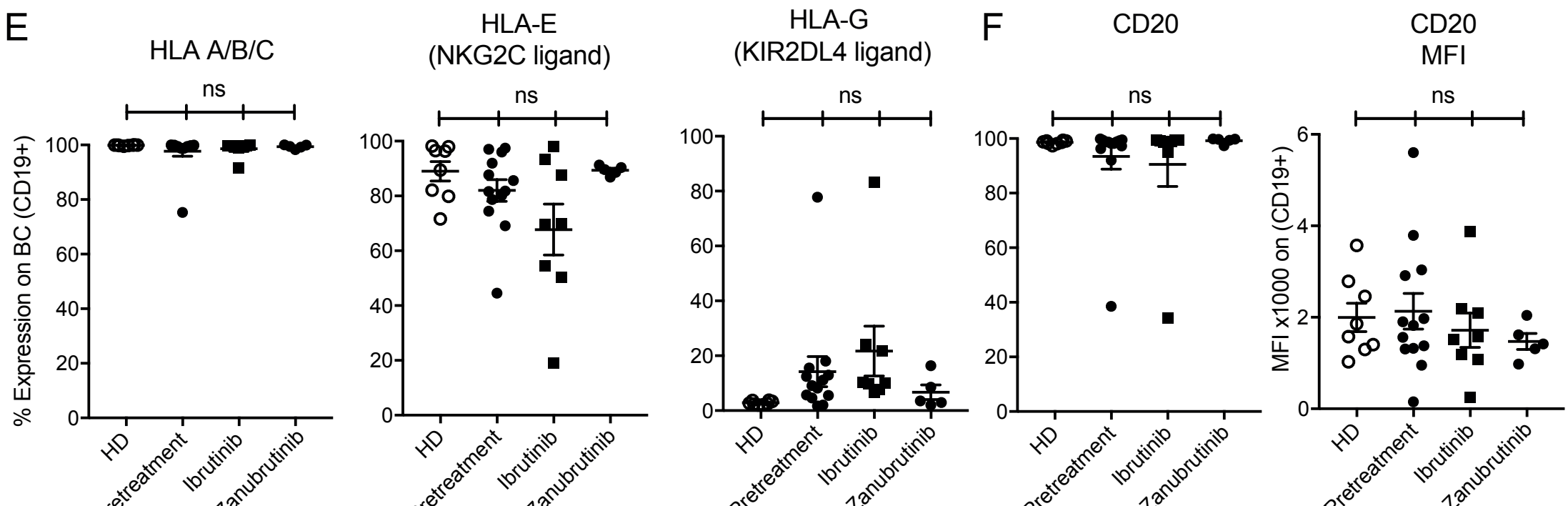

MCL 8 (zanubrutinib treatment)
HLA-G
HLA-E
ULBP-1
CD112
CD20
CD19
MICA/B
Pretreatement
Zanubrutinib
ULBP256
HLA-ABC
CD5
CD155
CD23
PD-L1
Pretreatement
Zanubrutinib
tSNE2
tSNE1

Figure S4


A
HD
MCL 3
MCL 7
CD5
CD5
CD5
high
low
Normalized intensity
tSNE2
tSNE1


B
B-cells
BC% (CD19+)
ns
ns
C
CD5
CD23
% Expression on BC (CD19+)
D
PD-L1
ns
*
ns
HD
Pretreatment
Ibrutinib
Pretreatment
Zanubrutinib


E
HLA A/B/C
HLA-E
(NKG2C ligand)
HLA-G
(KIR2DL4 ligand)
F
CD20
CD20
MFI
% Expression on BC (CD19+)
MFI x1000 on (CD19+)
ns
HD
Pretreatment
Ibrutinib
Zanubrutinib

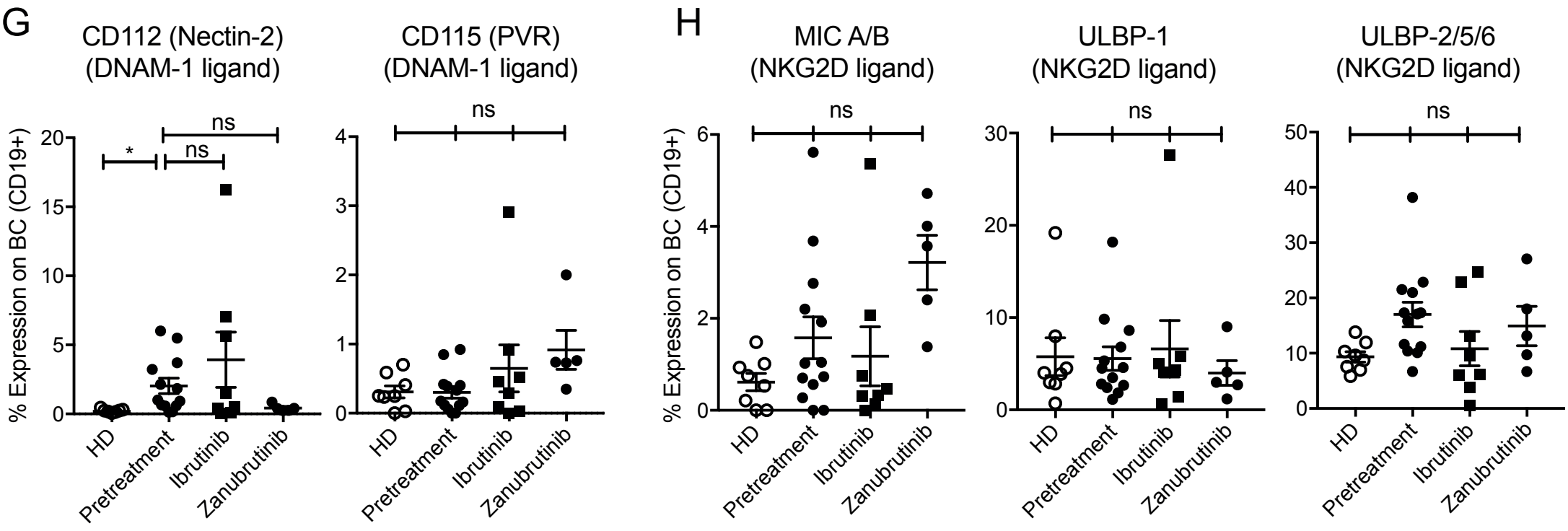

G
CD112 (Nectin-2)
(DNAM-1 ligand)
CD115 (PVR)
(DNAM-1 ligand)
H
MIC A/B
(NKG2D ligand)
ULBP-1
(NKG2D ligand)
ULBP-2/5/6
(NKG2D ligand)
% Expression on BC (CD19+)
*
ns
HD
Pretreatment
Ibrutinib
Zanubrutinib

Figure S5

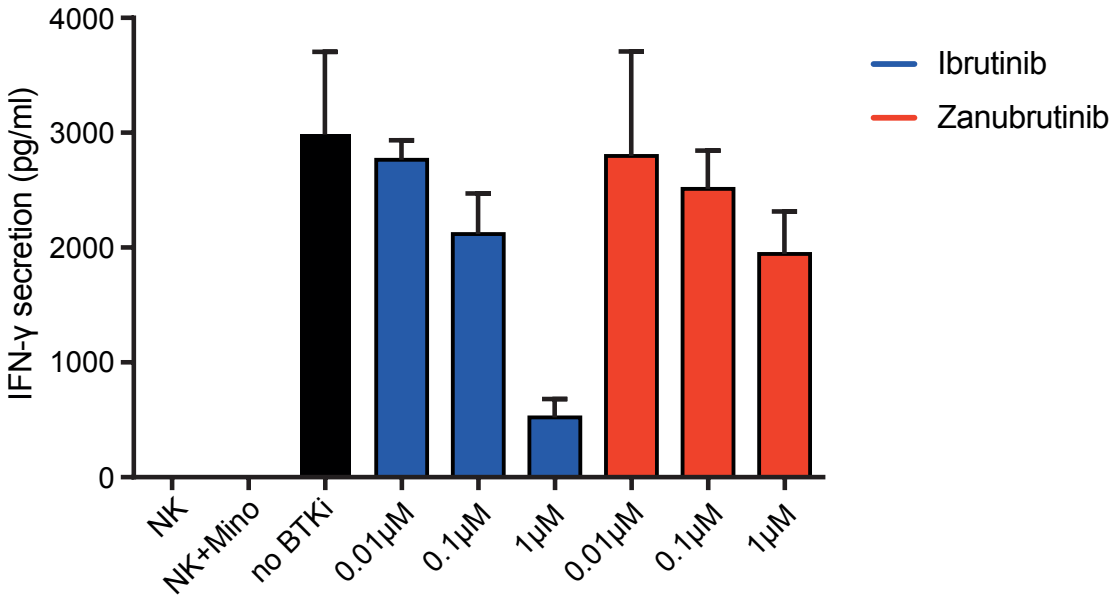
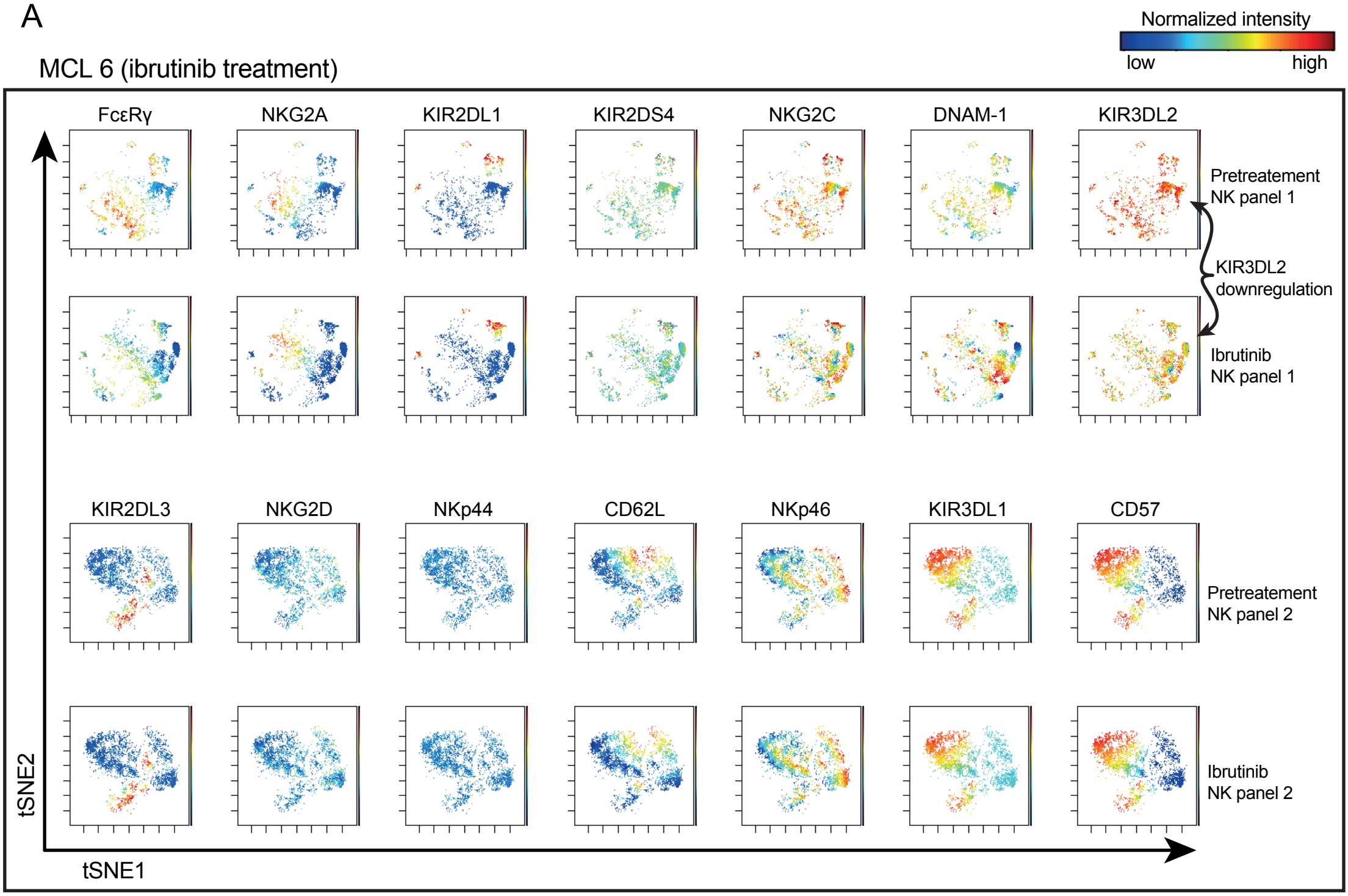

IFN-γ secretion (pg/ml)
4000
3000
2000
1000
0
NK
NK+Mino
no BTKi
0.01μM
0.1μM
1μM
0.01μM
0.1μM
1μM
Ibrutinib
Zanubrutinib

Figure S6

A


Normalized intensity
low
high
MCL 6 (ibrutinib treatment)
FcεRγ
NKG2A
KIR2DL1
KIR2DS4
NKG2C
DNAM-1
KIR3DL2
Pretreatement NK panel 1
KIR3DL2 downregulation
Ibrutinib NK panel 1
KIR2DL3
NKG2D
NKp44
CD62L
NKp46
KIR3DL1
CD57
Pretreatement NK panel 2
Ibrutinib NK panel 2
tSNE2
tSNE1

B

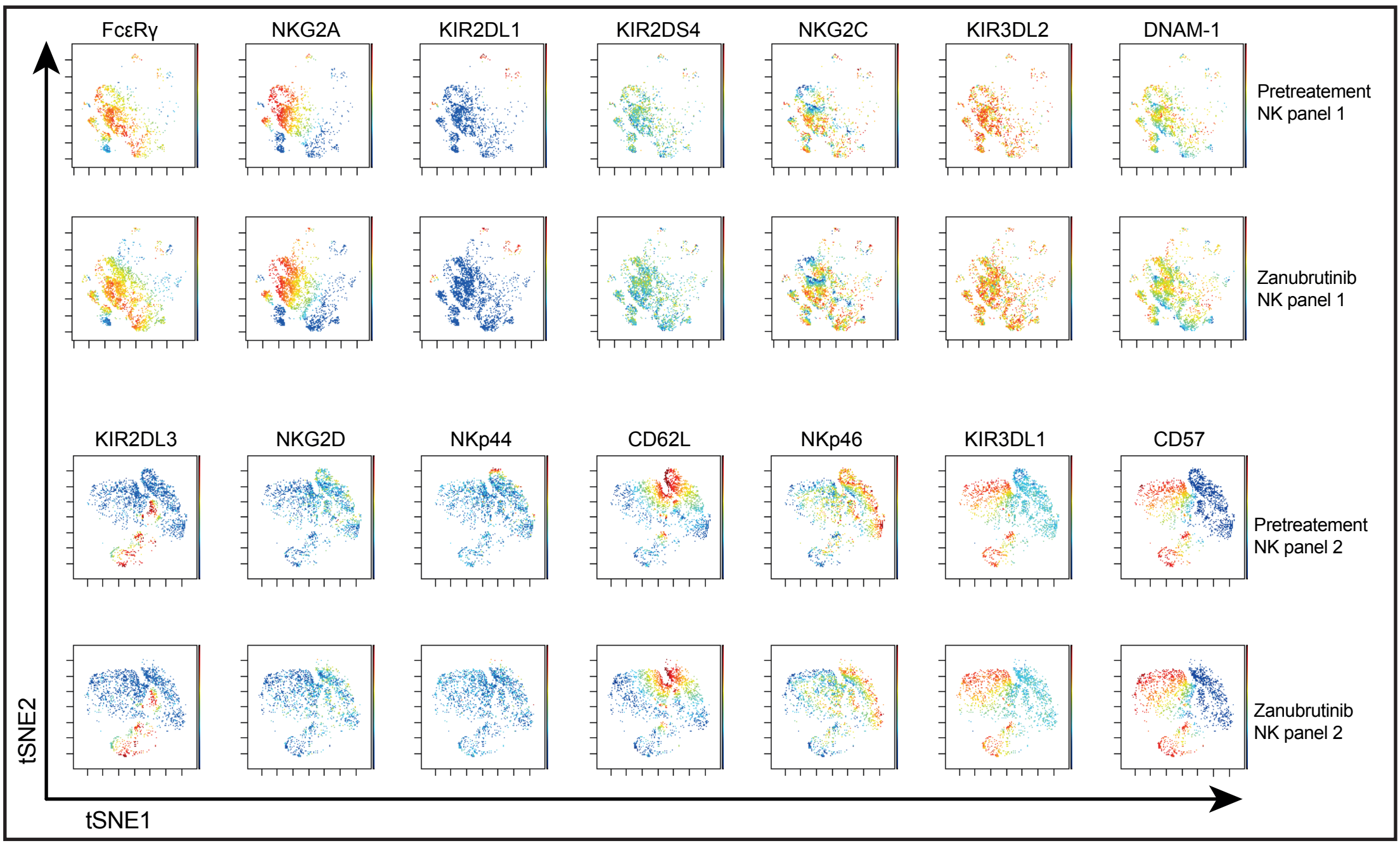
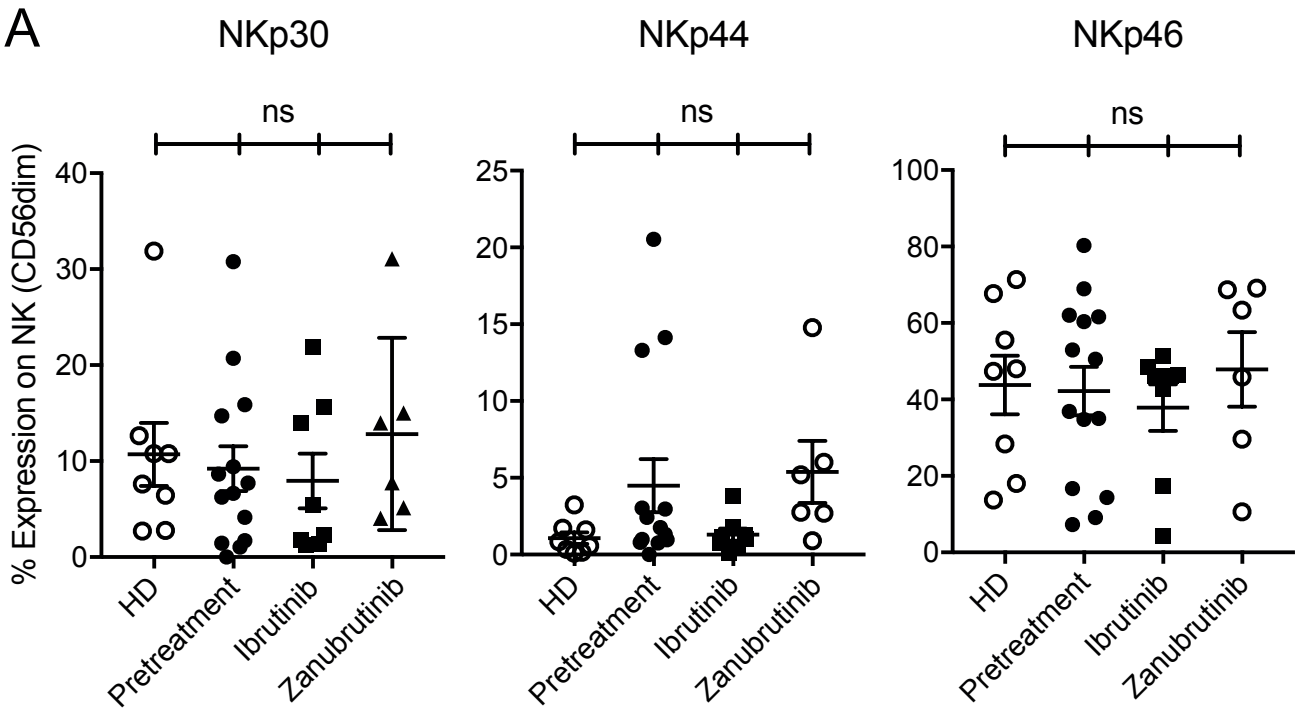
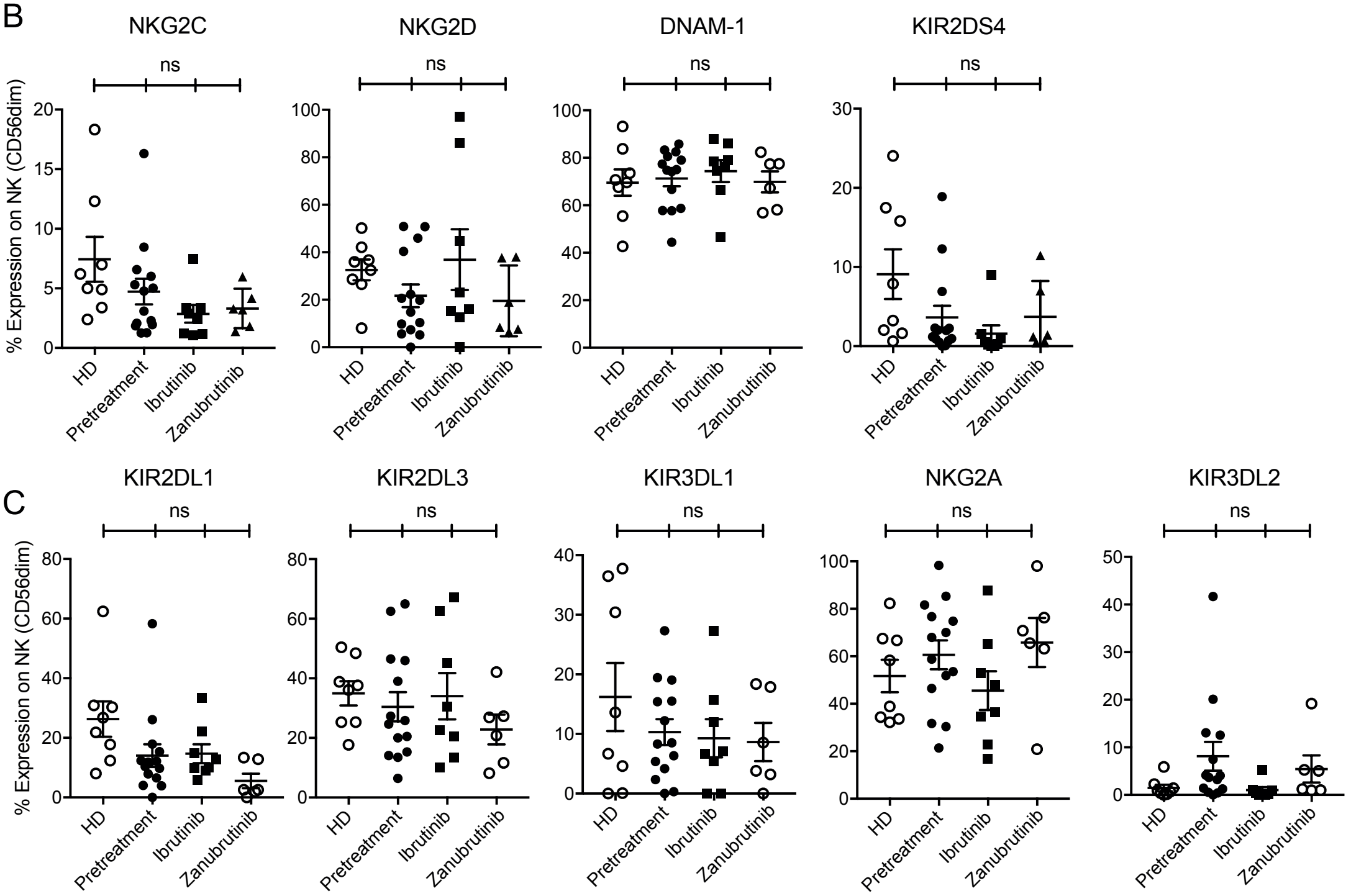

MCL 9 (zanubrutinib treatment)
FcεRγ
NKG2A
KIR2DL1
KIR2DS4
NKG2C
KIR3DL2
DNAM-1
Pretreatement NK panel 1
Zanubrutinib NK panel 1
KIR2DL3
NKG2D
NKp44
CD62L
NKp46
KIR3DL1
CD57
Pretreatement NK panel 2
Zanubrutinib NK panel 2
tSNE2
tSNE1

Figure S7


FcεRγ
NKG2A
KIR2DL1
KIR2DL1/S1
CD16
KIR2DS4
NKG2C
KIR3DL2
DNAM-1
KIR3DL1
CD56
Healthy donors
tSNE2
tSNE1
MCL patients
(ibrutinib cohort)
tSNE2
tSNE1
MCL patients
(zanubrutinib cohort)
tSNE2
tSNE1

Figure S8


A
NKp30
NKp44
NKp46
ns
% Expression on NK (CD56dim)
HD
Pretreatment
Ibrutinib
Zanubrutinib


B
NKG2C
NKG2D
DNAM-1
KIR2DS4
C
KIR2DL1
KIR2DL3
KIR3DL1
NKG2A
KIR3DL2
ns
% Expression on NK (CD56dim)
HD
Pretreatment
Ibrutinib
Zanubrutinib

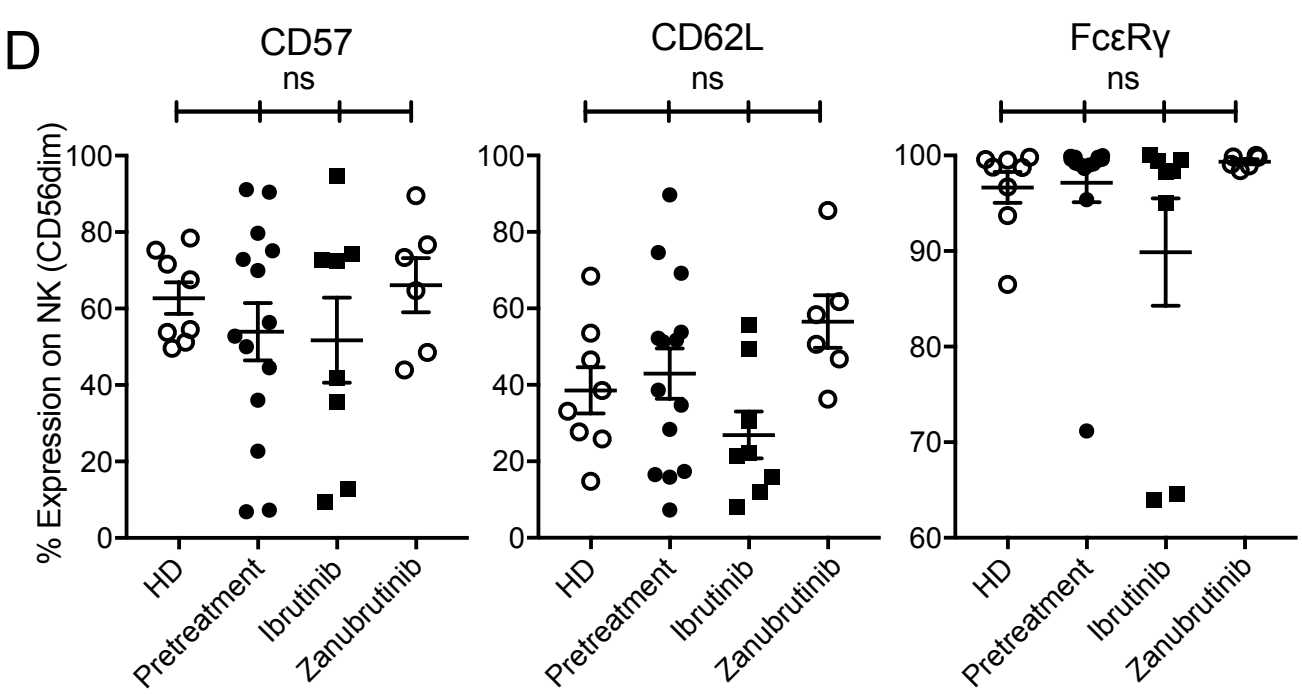
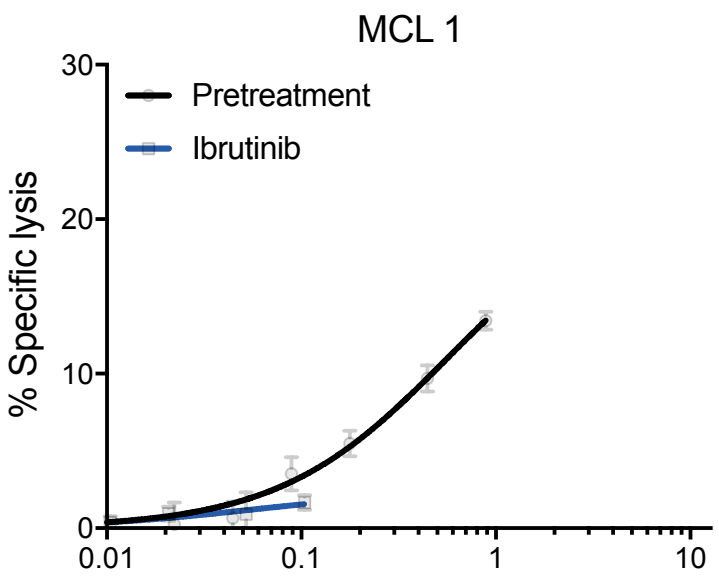
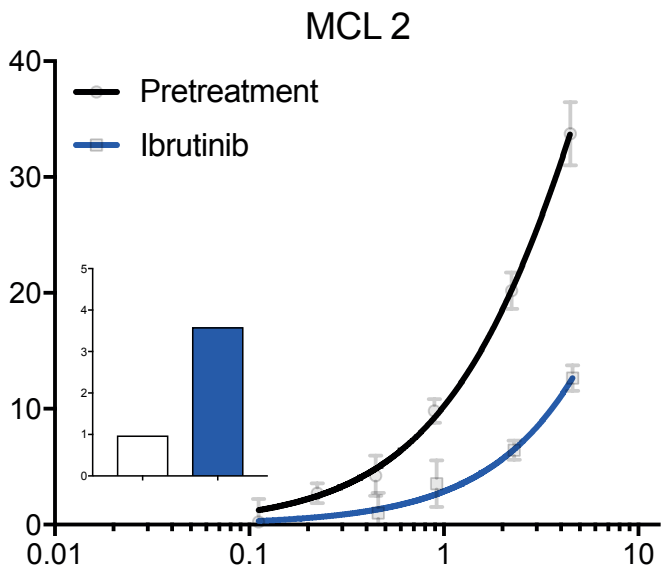
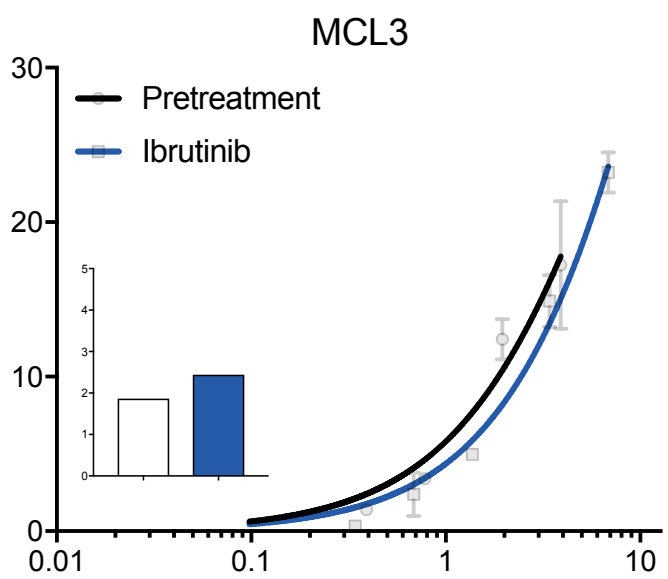

D
CD57
CD62L
FcεRγ
ns
% Expression on NK (CD56dim)
HD
Pretreatment
Ibrutinib
Zanubrutinib

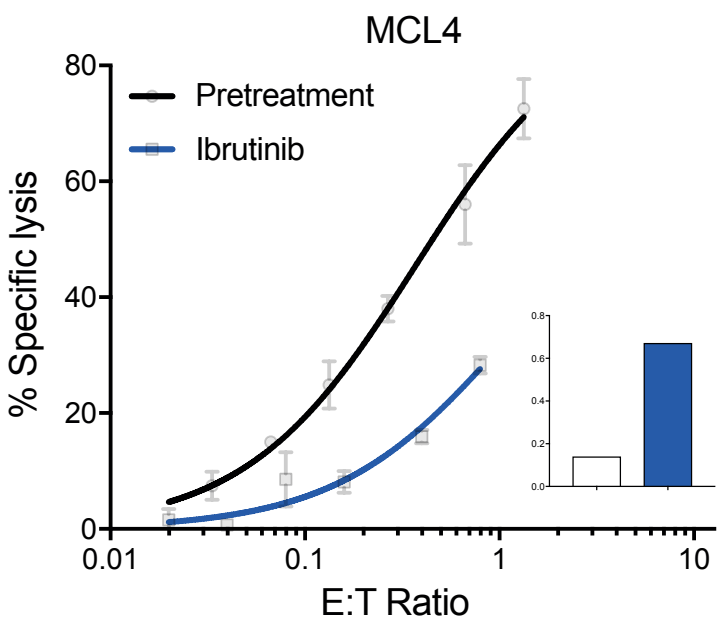
Figure S9

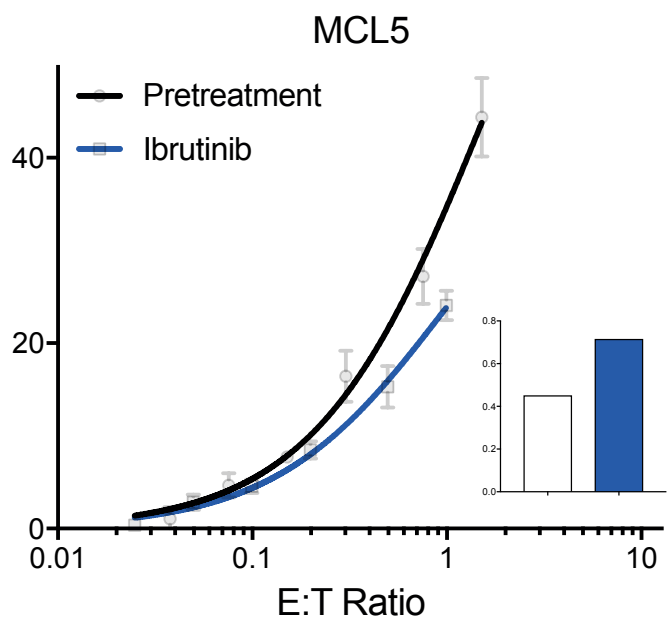
A

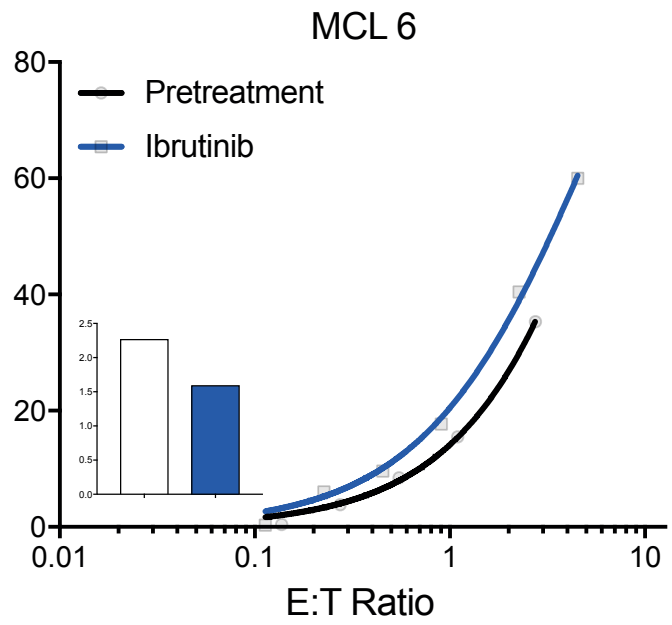

MCL 1
Pretreatment
Ibrutinib
% Specific lysis
0
10
20
30
0.01
0.1
1
10


MCL 2
Pretreatment
Ibrutinib
0
10
20
30
40
0.01
0.1
1
10

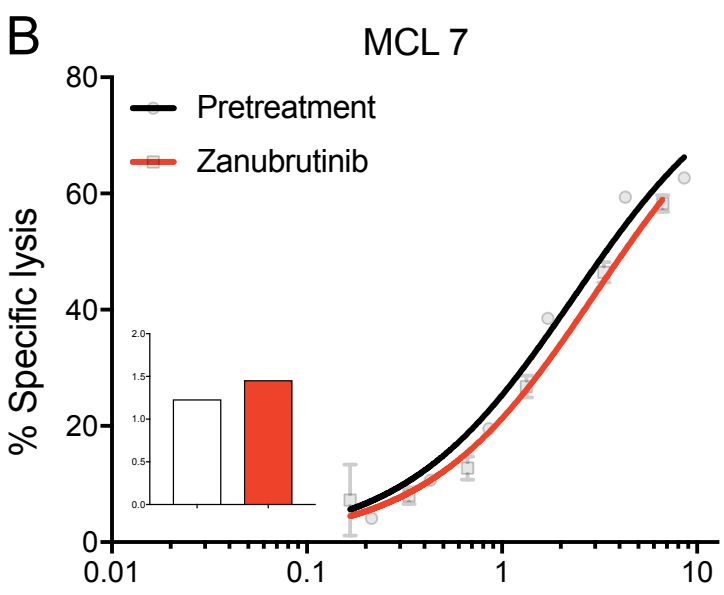

MCL3
Pretreatment
Ibrutinib
0
10
20
30
0.01
0.1
1
10

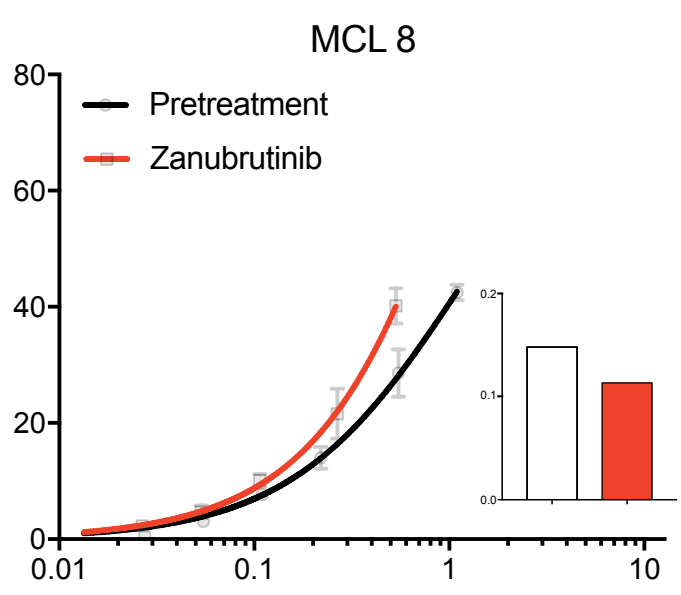

MCL4
Pretreatment
Ibrutinib
% Specific lysis
0
20
40
60
80
0.01
0.1
1
10
E:T Ratio

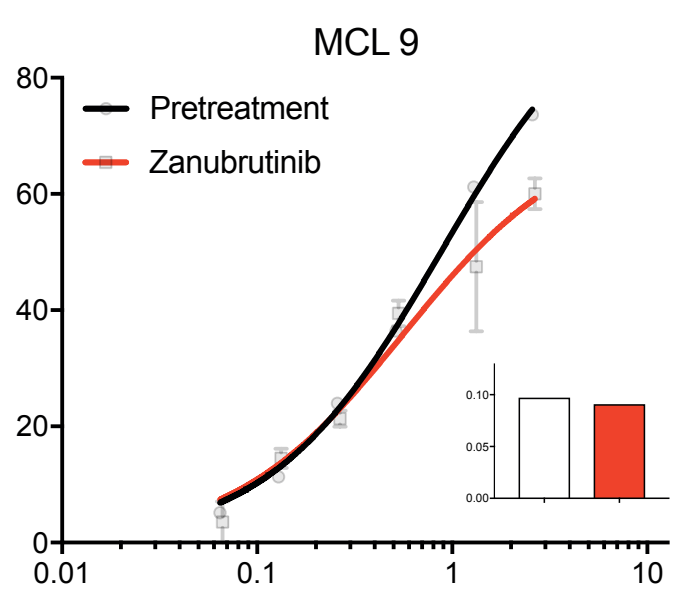

MCL5
Pretreatment
Ibrutinib
0
20
40
0.01
0.1
1
10
E:T Ratio

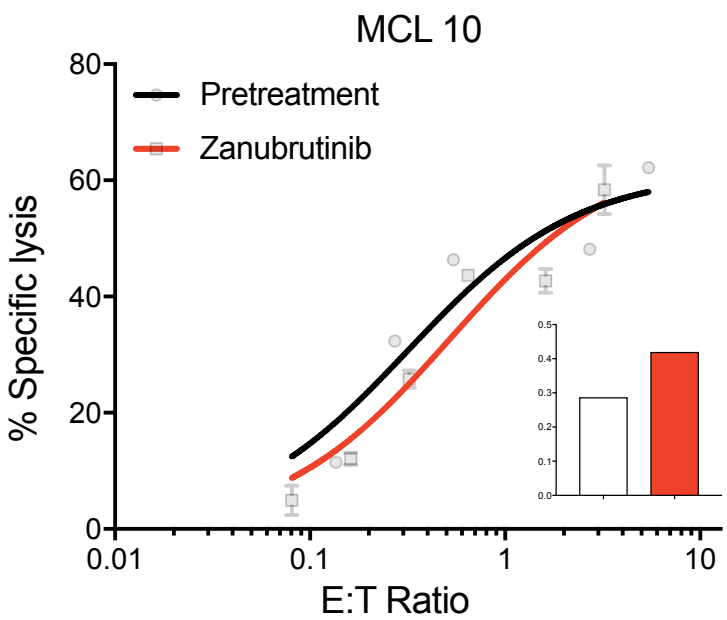

MCL 6
Pretreatment
Ibrutinib
0
20
40
60
80
0.01
0.1
1
10
E:T Ratio

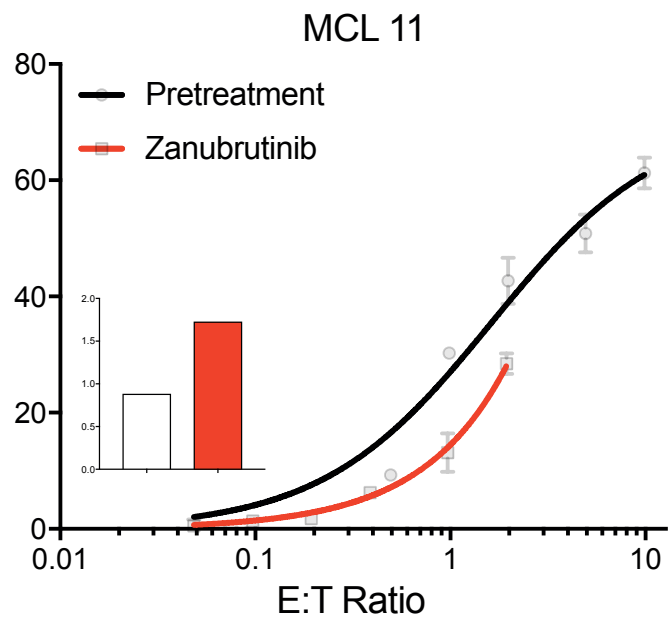
B


MCL 7
Pretreatment
Zanubrutinib
% Specific lysis
0
20
40
60
80
0.01
0.1
1
10


MCL 8
Pretreatment
Zanubrutinib
0
20
40
60
80
0.01
0.1
1
10


MCL 9
Pretreatment
Zanubrutinib
0
20
40
60
80
0.01
0.1
1
10


MCL 10
Pretreatment
Zanubrutinib
% Specific lysis
0
20
40
60
80
0.01
0.1
1
10
E:T Ratio


MCL 11
Pretreatment
Zanubrutinib
0
20
40
60
80
0.01
0.1
1
10
E:T Ratio

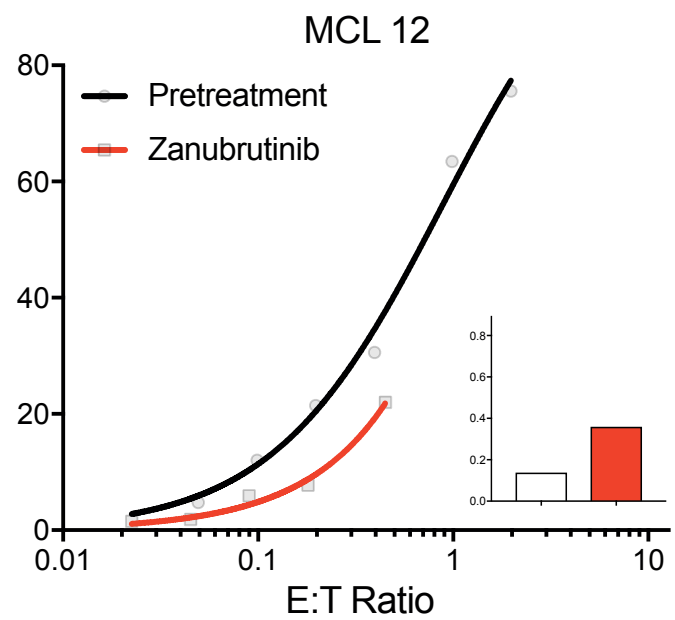
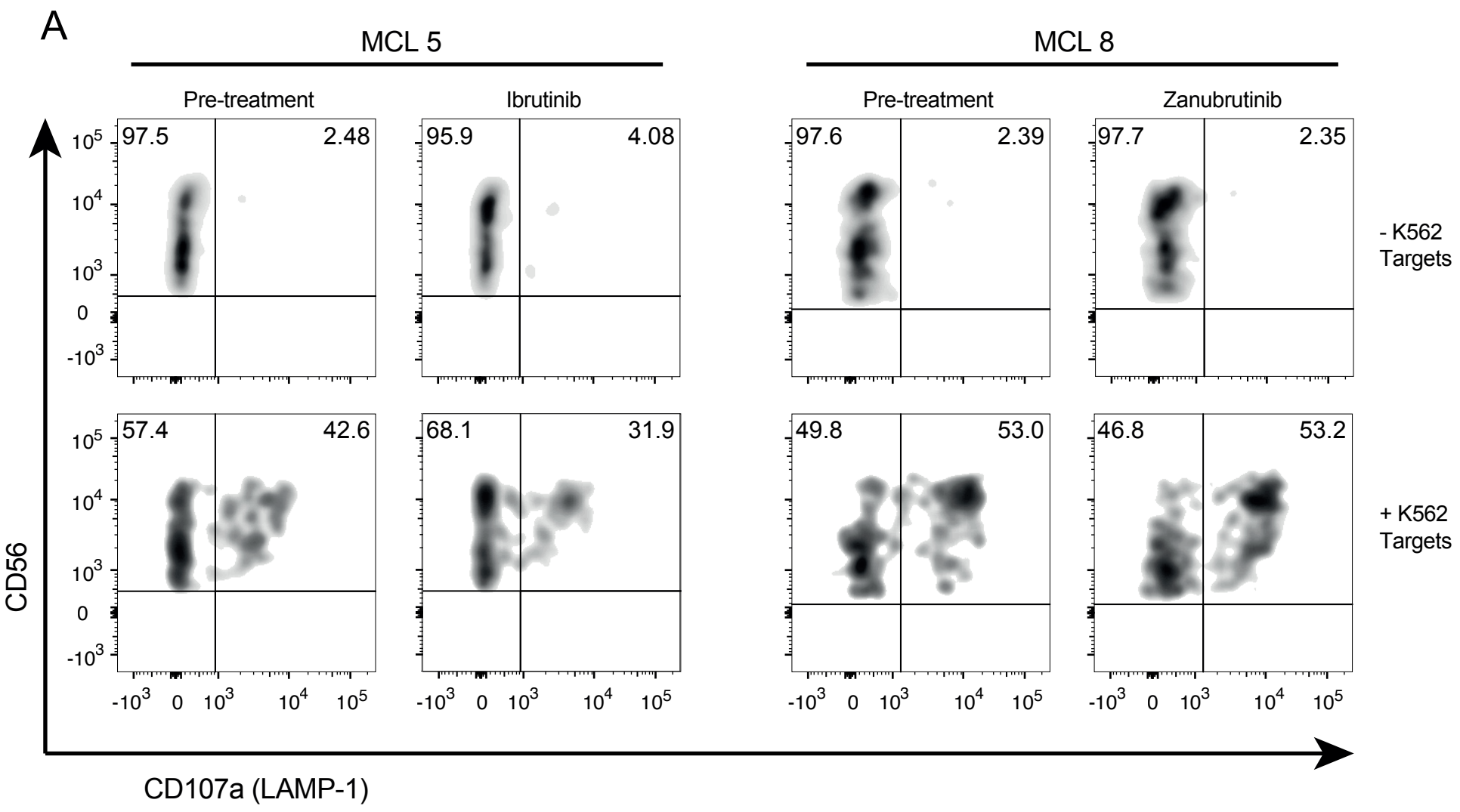

MCL 12
Pretreatment
Zanubrutinib
0
20
40
60
80
0.01
0.1
1
10
E:T Ratio

Figure S10


A
MCL 5
MCL 8
Pre-treatment
Ibrutinib
Pre-treatment
Zanubrutinib
97.5
2.48
95.9
4.08
97.6
2.39
97.7
2.35
- K562 Targets
57.4
42.6
68.1
31.9
49.8
53.0
46.8
53.2
+ K562 Targets
CD56
CD107a (LAMP-1)

Supplementary table

Patient	Treatment	Gender	Age	Time since Dx	Previous therapies
MCL 1	Ibrutinib	M	77	59 months	R-CHOP, R-DHAP, Temsirolimus
MCL 2	Ibrutinib	M	50	42 months	R-hyperCVAD then autograft
MCL 3	Ibrutinib	M	53	71 months	R-hyperCVAD then autograft
MCL 4	Ibrutinib	M	55	11 months	Nordic protocol then autograft
MCL 5	Ibrutinib	M	69	40 months	R-CHOP alt + Ara-C then autograft
MCL 6	Ibrutinib	F	81	11 months	Mini-CHOP
MCL 7	Zanubrutinib	M	80	6 months	R-CHOP, Chlorambucil
MCL 8	Zanubrutinib	M	64	39 months	R-CHOP then autograft
MCL 9	Zanubrutinib	M	74	69 months	R-CHOP, FCR, R-CEP, ABT-199, radiation
MCL 10	Zanubrutinib	F	68	60 months	R-CHOP/DHAP then autograft, FCR, Rituximab
MCL 11	Zanubrutinib	M	68	167 months	Chlorambucil, rituximab, Nordic then autograft
MCL 12	Zanubrutinib	F	68	9 months	Chlorambucil, rituximab, Nordic then autograft
MCL 13	Ibrutinib	M	72	25 months	R-CHEP
MCL 14	Ibrutinib	M	62	26 months	Nordic protocol (but not autografted)

Supplementary figure legends

Figure S1: FACS panels used for staining of receptor expression on MCL patients NK cells and B cells

Table shows the 5 panels used for phenotyping.

Figure S2: Representative gating strategy

PBMCs from MCL patients were isolated and stained with surface markers, permeabilized and stained for intracellular markers according to the panels in figure S1. Cells were gated for morphology, single cells, lineage negative and NK cells (based on CD16 and CD56) or B cells ($CD19^+$). Shown are 4 examples of surface receptors with different expression patterns (background in dotted line, stained cells in solid grey).

Figure S3: t-SNE analysis of B cell receptor repertoire in MCL patients before and after treatment with BTK inhibitors

(A,B) t-distributed stochastic neighbour embedding (t-SNE) map of $CD19^+$ B cells from two MCL patients. t-SNE analysis was performed on all MCL patients to explore the heterogeneity of $CD19^+$ B cells. Shown are representatives of 2 MCL patients treated with ibrutinib (A) or zanubrutinib (B).

Figure S4: B cell receptor repertoire in MCL patients before and after treatment with BTK inhibitors

PBMCs from MCL patients or HDs were stained for surface markers (staining panels in Figure S1, representative gating in Figure S2B). Graphs show expression or mean fluorescence intensity (MFI) of HDs, MCL patients before treatment or after ibrutinib or zanubrutinib treatment.

(A-C) Characterisation of B cells. t-SNE analysis was performed on all MCL patients to explore the heterogeneity of $CD19^+$ B-cells. t-SNE map of two representative MCL patients (A), percentage of $CD19^+$ B-cells (B) and CD5 and CD23 expression of all MCL patients (C).

(D-G) Surface expression of PD-L1 (D) HLA-A/B/C (E, left), HLA-E (E, middle), HLA-G (E, right), CD20 (F), DNAM-I ligands (G) and NKG2D ligands (H) on B cells. * $p<0.05$ (paired t-test).

Figure S5: Zanubrutinib is an equally potent, but more selective, inhibitor of BTK than ibrutinib *in vitro*

Mino cells and NK92MI cells were co-seeded and treated with vehicle or various concentrations of BTK inhibitors in the presence of GA-101 and IFN-γ levels in the conditioned medium were measured.

Figure S6: Therapy with BTK inhibitors does not affect NK cell receptor repertoire

(A,B) t-distributed stochastic neighbour embedding (t-SNE) map of $CD3^-/CD56^{dim}/CD16^+$ NK cells from two MCL patients. t-SNE analysis was performed on all MCL patients to explore the heterogeneity of NK cells. Shown are representatives of 2 MCL patients treated with ibrutinib (A) or zanubrutinib (B).

Figure S7: NK cell receptor repertoire in MCL patients is similar to that of healthy controls

t-distributed stochastic neighbour embedding (t-SNE) map of NK cell panel 2 on $CD3^-/CD56^{dim}/CD16^+$ NK cells from all MCL patients and healthy donors. Rows represent individuals. Columns show the relative expression of each receptor. All t-SNE results for NK cell panel 3, B- cell panel 2 as well as comparison pre- and posttreatment available on request.

Figure S8: NK cell receptor repertoire in MCL patients before and after treatment with BTK inhibitors

(A-D) PBMCs from MCL patients or HDs were stained for surface and intracellular markers (staining panels as in Figure S1, representative gating is shown in Figure S2A). Graphs show expression or mean fluorescence intensity (MFI) of activating receptors (A,B), inhibitory receptors (C) and CD57, CD62L, FcεRγ (D) of HD and MCL patients before treatment or after ibrutinib or zanubrutinib treatment.

Figure S9: Individual curves of NK cell cytotoxicity

PBMCs taken prior to or after therapy with ibrutinib (A) or zanubrutinib (B) were incubated with ^{51}Cr-labeled K562 target cells for 4 h at the indicated effector to target cell ratios (normalized for the percentage of NK cells). Shown are individual datapoints that we extrapolated using a Michaelis-Menten equation. Inserted graphs show the effector:target ratio required to kill the number of cells at the linear part of the curve.

Figure S10: Representative degranulation assay

(A) PBMCs were incubated for 3 h in the absence (−K562) or presence (+ K652) of K562 target cells. NK cell degranulation was assessed by measurement of CD107a surface labelling in the $CD3^-/CD56^+$ cell populations.

Supplementary table; patient information

Table containing MCL patient information including age, gender, prior and current treatment. Patient 13 and 14 are separated because we could not analyse NK cell function due to low NK cell numbers (no killing seen even at highest achievable E:T ratio). These patients were included in the phenotypical analysis.

Abbreviations:

R-: rituximab; CHOP: cyclophosphamide, doxorubicin (**h**ydroxydaunomycin), vincristine (**O**ncovin®), prednisolone; DHAP: dexamethasone, high-dose cytarabine (**A**ra-C), cisplatin (**P**latinum); hyperCVAD: cyclophosphamide, vincristine, doxorubicin, dexamethasone in multiple fractions per day (hyperfractionated); Nordic protocol: CHOP with high doses [maxi-CHOP] in 21-day intervals, alternating with rituximab + high-dose cytarabine; Mini-CHOP: low dose CHOP; FCR: fludarabine, cyclophosphamide and rituximab; CEP: cyclophosphamide, etoposide and prednisone; CHEP: cyclophosphamide, doxorubicin (**h**ydroxydaunomycin), etoposide and prednisone; ABT-199: venetoclax.

Details of these protocols in:

Kluin-Nelemans HC, Hoster E, Hermine O, et al. Treatment of older patients with mantle-cell lymphoma. N. Engl. J. Med. 2012;367(6):520–31.

Vose JM. Mantle cell lymphoma: 2017 update on diagnosis, risk-stratification, and clinical management. Am. J. Hematol. 2017;92(8):806–813.

Supplementary Materials and Methods

ITK inhibition

Day 1 Activated NK cells were incubated with various concentrations of the ITK inhibitor 5-Aminomethylbenzimdazole or DMSO as control for 1 hour. Then, NK cell cytotoxicity assays and degranulation assays were performed as described below in the presence of the ITK inhibitor. Phosphorylation of ITK was checked after 4 hours incubation of isolated NK cells with 5-Aminomethylbenzimdazole and 1000 IU/ml IL-2 using a purified mouse anti-Btk (pY551)/Itk (pY511) antibody (clone 24a/BTK(Y551), BD Pharmingen).

Biochemical IC50 determination of BTK and ITK

Zanubrutinib and Ibrutinib were tested for inhibition of BTK and ITK in assays based on the time-resolved fluorescence-resonance energy transfer (TR-FRET) methodology. Briefly, the assays were carried out in 384-well low volume black plates in a reaction mixture containing BTK kinase, 5μM ATP, 2μM peptide substrate and 0-10 μM compound in buffer containing 50 mM Tris pH7.4, 10 mM MgCl2, 2 mM MnCl2, 0.1 mM EDTA, 1 mM DTT, 0.005% Tween-20, 20 nM SEB and 0.01% BSA. The kinase was incubated with the compound for 60 minutes at room temperature and the reaction was initiated by the addition of ATP and peptide substrate. After reaction at room temperature for 60 minutes, an equal volume of stop/detection solution was added. Plates were sealed and incubated at room temperature for 1 hour, and the TR-FRET signals were recorded on a PHERAstar FS plate reader (BMG Labtech). The protocol of ITK assay is similar to BTK assay except for the following modification: 3μM ATP and 2μM ITK substrate were used in the kinase reaction.

BTK occupation assay

Z-138 cells were treated with increasing concentration of zanubrutinib or ibrutinib for 2 hours. The cell lysates were loaded to ELISA plate pre-immobilized with detection probe. After overnight

incubation, plates were washed with PBS-T 3 times and probe conjugated BTK protein was detected by a BTK antibody. The potency of compounds was calculated basing on the inhibition of ratio between signal intensity at OD450 nm. IC50 values were calculated with GraphPad Prism software using the sigmoidal dose-response function.

Inhibition of Rec-1 Tumor Cell Proliferation

The growth-inhibitory activity of compounds in Rec-1 was determined using a CellTiter-Glo luminescent cell viability assay (Promega). Briefly, Rec-1 cells (4000 cells/well) were seeded in a 96-well plate then treated with a 10-point dilution series of compounds for 6 days. Following the compound treatment, 30µl of CellTiter-Glo reagent was mixed with 100µl of cell culture on an orbital shaker for 5 minutes to allow cell lysis, followed by 5 minutes incubation at room temperature to allow development and stabilization of luminescent signal, which corresponds to the quantity of ATP thus the quantity of metabolically active cells. Luminescent signal was measured using PHERAstar FS reader (BMG Labtech).

Inhibition of Cellular BTK Activation

The BTK pY223 cellular assay was an HTRF-based assay (Cisbio), conducted in Ramos cells to evaluate the inhibition of cellular BTK activation by compounds. It is designed to quantitatively determine the endogenous levels of phosphorylation at BTK Tyr-223, which is necessary for full activation of BTK. Briefly, Ramos cells were serum starved in 0.5% FBS-containing RPMI-1640 overnight. Following starvation, 8×10^5 cells per well were seeded in a 96-well plate and incubated with compounds at various concentrations in a CO_2 incubator for 3 hours. After incubation, cells were stimulated with 1mM pervanadate (PV) or Na_3VO_4 (OV) for 20 min, then lysed at RT for 10 min. 2µl/well of 1 x antibody mixture prepared by diluting anti-BTK-d_2 and anti-pBTK-K in detection buffer was dispensed into the OptiPlate-384 assay plate (Perkin Elmer), then mixed with 18µl of cell lysate. After mixing gently and spinning briefly, the plate was sealed up and kept in dark at RT for

18 hours. The fluorescence emission was measured at two different wavelengths (665 nm and 620 nm) on a compatible HTRF reader (PHERAstar FS, BMG). The potency of compounds was calculated based on the inhibition ratio between signal intensities at 665nm and 620nm.

ITK occupation assay

HEK293-FlagITK cells were treated with increasing concentration of zanubrutinib and ibrutinib for 4 hours. The cell lysates were loaded to an ELISA plate pre-immobilized with a detection probe. After overnight incubation, plates were washed with PBS-T 3 times and probe conjugated ITK protein was detected by an ITK antibody. The potency of compounds was calculated basing on the inhibition of ratio between signal intensity at OD450 nm. IC50 values were calculated with GraphPad Prism software using the sigmoidal dose-response function.

p-PLCγ1 Cellular Assay

Once activated by T cell receptor (TCR) aggregation, ITK directly phosphorylates PLCγ1 residue Tyr783 and subsequently actives the NF-κB pathway, leading to increased production of Interleukin 2 (IL-2). Jurkat cells were treated with indicated concentration of ibrutinib or zanubrutinib for 2 hrs. After treatment, cells were exposed to 10 mM of hydrogen peroxide for 10 min. PLCγ1, p-PLCγ1 (Y783) were detected by western blot analysis. IC50 is calculated using Quantity One and Prism 5 software.

IL-2 production assay

HuT87 cells and HEK293/OS8V cells were pre-incubated with increasing concentrations of BTK inhibitors for 2 hours. After incubation, 30,000 HuT87 cells, together with BTK inhibitors, were transferred to 30,000 HEK293/OS8V cells to activate TCR on HuT87. After co-culturing for 20 hours, the media were harvested and IL-2 level was measured with a commercial IL-2 ELISA kit (R&D systems DY202E) following manufacture's protocol. The potency of compounds was

calculated basing on the inhibition of ratio between signal intensity at OD450nm. IC50 values were calculated with GraphPad Prism software using the sigmoidal dose-response function.

NK cell IFN-γ inhibition by BTK inhibitors

Mino cells and NK92MI cells were co-seeded and treated with vehicle or various concentrations of BTK inhibitors for one hour, followed by co-treatment with vehicle or rituximab (2 ug/well) for an additional 24 hrs. IFN-γ levels in the conditioned medium were measured using Human IFN-γ ELISA Ready-SET-Go kit and presented as mean of IFN-γ ± standard deviation (SD).

NK cell mediated killing assay of Mino cells

Mino cells and NK92MI cells were co-seeded and treated with vehicle or various concentrations of BTK inhibitors for one hour, followed by co-treatment with with vehicle or rituximab (2 ug/well) for additional 5 hrs. Cytotoxicity was determined by LDH release into the culture medium.

Patient material and ethics

Patient material collection and ethics for MCL patients treated with ibrutinib are described in [8] (study protocol: NCT02343120). Samples of zanubrutinib treated MCL patients were obtained from the first-in-human phase I study of zanubrutinib in patients with advanced lymphoid malignancies and included patients with MCL both in the dose escalation and dose expansion cohorts (majority on 320mg daily), study protocol: NCT0471391.

Cell culture

Peripheral blood mononuclear cells (PBMCs) from patients diagnosed with mantle cell lymphoma (MCL) and healthy donors were separated from peripheral blood by ficoll isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB), washed and cryopreserved in FCS containing 10% DMSO.

Cytotoxicity assay

NK cell cytotoxicity was measured in 4h chromium (^{51}Cr) release assays as described previously (Sutton VR, Waterhouse NJ, Baran K, et al. Measuring cell death mediated by cytotoxic lymphocytes or their granule effector molecules. *Methods* 2008;44(3):241-49). The number of NK cells in PBMC was calculated for each sample according to the percentage of $CD3^-$ /$CD56^{dim}$/$CD16^+$ cells, and plotted as effector:target (E:T) ratio.

NK cell degranulation assays

CD107a/Lamp-1 externalization was measured to quantify NK cell degranulation. NK cells were harvested as described above and incubated with K562 targets at 1:2 E:T ratio for 3h at 37°C/5% CO2. CD107a externalization was assessed in $CD3^-$ $CD56^{dim}$ cells; spontaneous externalization of CD107a was assessed over 3 h in the absence of target cells.

Phenotyping

Cells were washed twice in PBS containing 2% FCS and 0.1% sodium azide (NaN3, Sigma-Aldrich), incubated with mouse anti–human Ab (see figure S1). Cells were then washed twice in PBS/2% FCS/0.1% NaN_3), fixed, permeabilized, and stained for FcεRγ. Cell events were acquired on FACS symphony and analysed using FACS Diva (BD Bioscience) or FlowJo Version 10.2 software. For t-distributed stochastic neighbour embedding (t-SNE) analysis, raw FCS files were processed and analyzed using Cytobank https://www.cytobank.org/).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 software.