Supplementary Figures and Methods:

Pre-clinical characterization of ISB 1342, a CD38xCD3 T-cell engager for relapsed/refractory multiple myeloma

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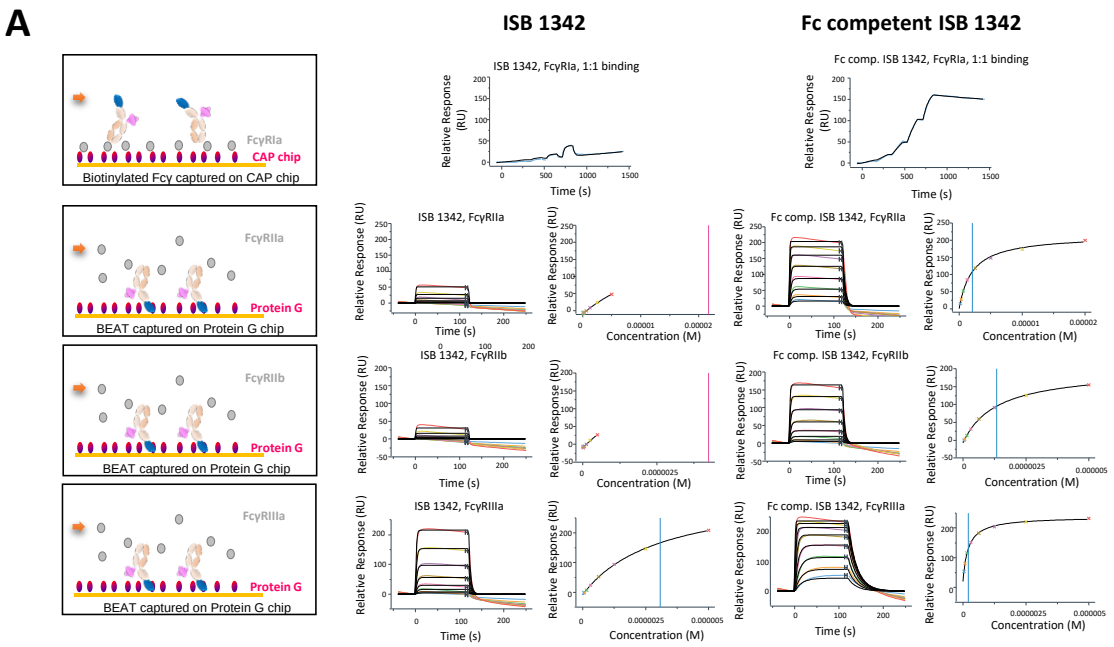
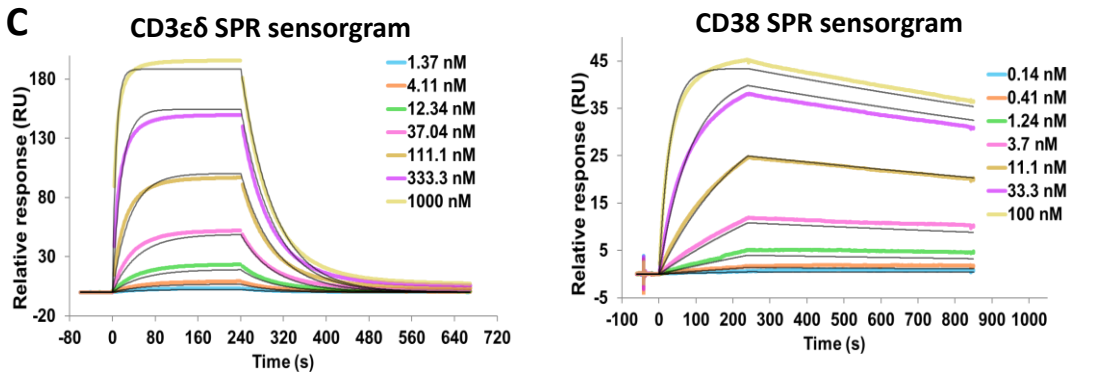
Supplementary Figures



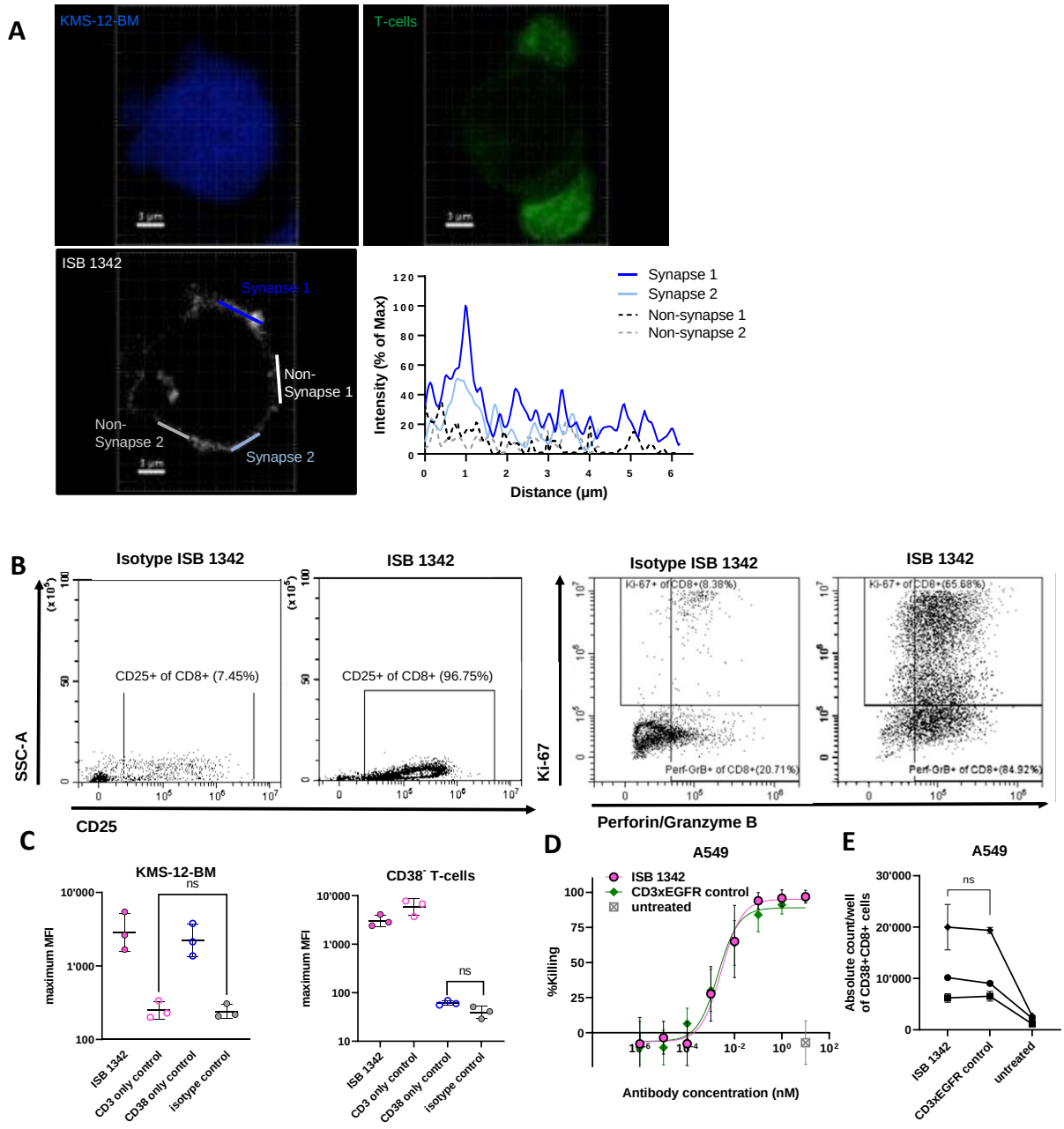
B

Molecule	K_D to FcγRIa (nM)	K_D to FcγRIIa (µM)	K_D to FcγRIIb (µM)	K_D to FcγRIIIa (µM)
ISB 1342	1330 ± 900	Weak binding	Weak binding	2.56 ± 0.46
Fc competent ISB 1342	2.11 ± 0.11	0.59 ± 0.07	1.29 ± 0.06	0.2 ± 0.02
Fold reduction for ISB 1342 over Fc competent counterpart	630	ND	ND	13



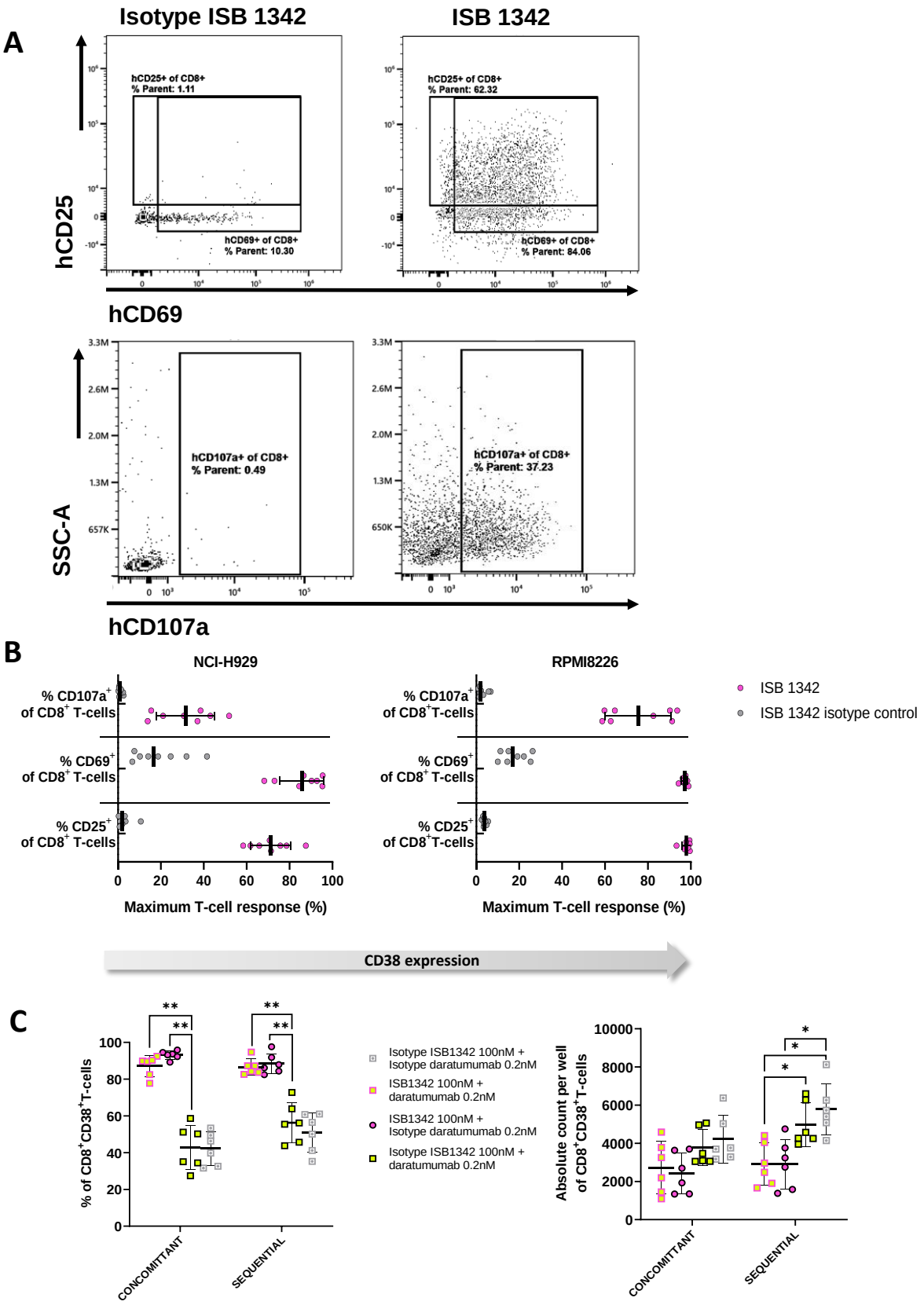
Supplementary Figure 1. Additional characterization of ISB 1342 biophysical properties. (A) Binding to the FcγR is silenced in ISB 1342. The binding of ISB 1342 to Fcγ receptors Ia, IIa, IIb and IIIa was measured by SPR and compared to a structurally identical version of ISB 1342 that did not include the LALA mutations (Fc competent ISB 1342). For each receptor and molecule tested, the fitted sensorgrams of a representative measurement are shown. Data are expressed as number of resonance units (abbreviated RU; y axis) vs. time (seconds; x axis). Colored lines represent experimental data and black lines represent the fitted data using the 1:1 kinetics model. For FcγRIIa, IIb and IIIa, the data were fit using a steady state affinity model. For FcγRIa, IIa and IIb, the binding to Fc receptors was too weak to infer a K_D

value, while the K_D to FcγRIIIa was decreased by a factor 13, as summarized in the table in B. (B) Summary table of dissociation constants (K_D). Data are reported as the average of at least 4 independent measurements ± standard deviation. (C) Fitted sensorgrams of independent measurements show the binding of ISB 1342 to human CD3εδ (left panel) and human CD38 (right panel) by SPR. Data are expressed as number of resonance units (abbreviated RU; y axis) vs. time (seconds; x axis). Curves represent single concentration injections from 0.1 µM for CD38 binding and 1 µM for CD3εδ binding with serial dilutions of 1/3. Colored lines represent experimental data and black lines represent the fitted data using the 1:1 kinetics model.



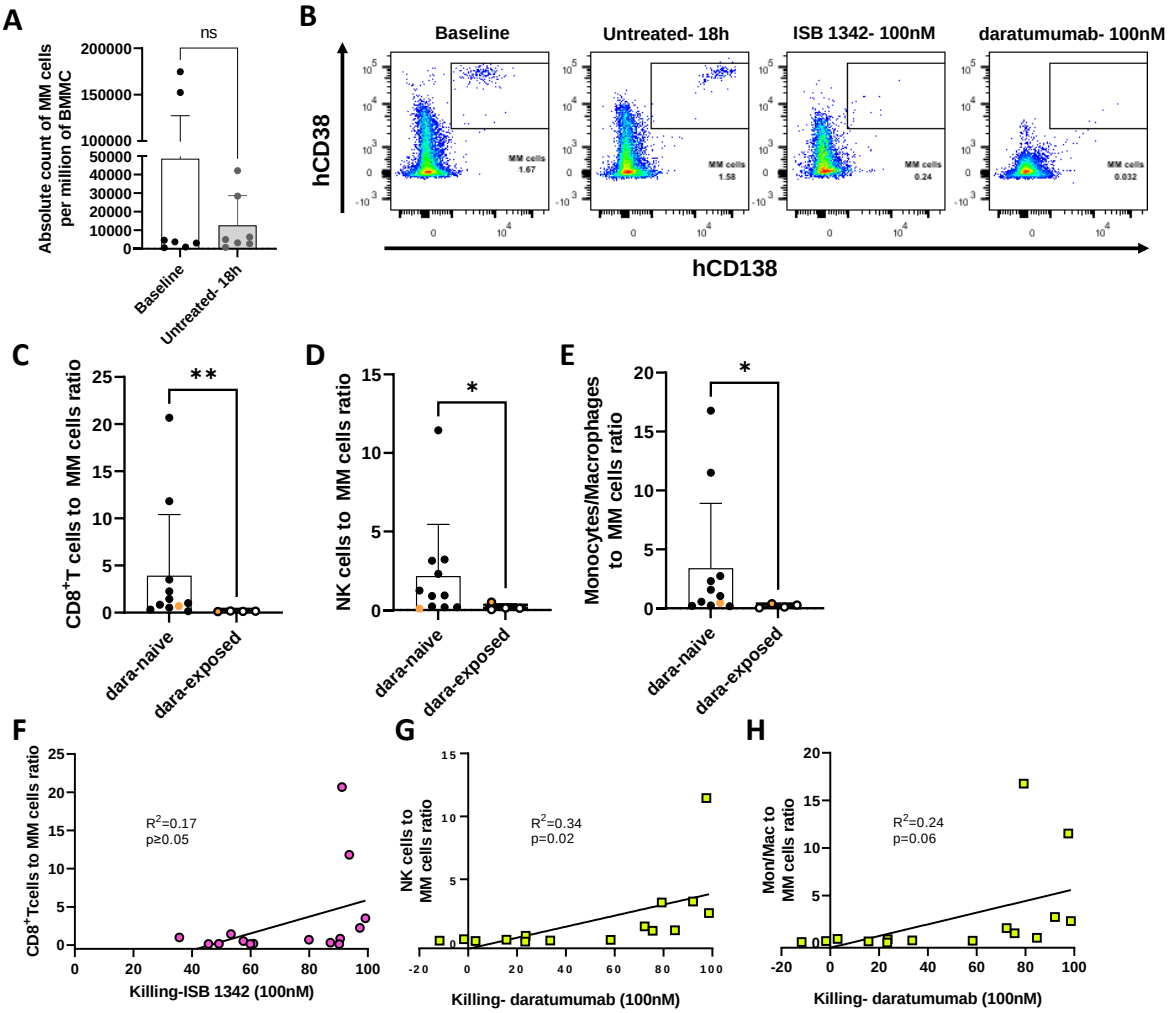
Supplementary Figure 2. ISB 1342 binding and activity on MM cells and T-cells. (A) Confocal images of KMS-12-BM cell line (blue), T-cells (green) and ISB 1342 (white) acquired with Zeiss LSM 800 inverted confocal microscope and processed with Imaris software. Magnification x40. Intensity of ISB1342 staining was measured at the synapse (blue) and non-synapse (grey/ black/white) locations using Image J Plot profile tool and normalised to maximum intensity. **(B)** Representative dot plots showing activation (CD25), proliferation (Ki-67) and degranulation profile (perforin/granzyme B) on human $CD8^+$T-cells from hPBMCs co-cultured with KMS-12-BM cell line upon treatment with maximum dose of ISB 1342 or its isotype control for 72h in a RDL assay. **(C)** Binding of ISB 1342 or control molecules on KMS-12-BM cells and on CD38 negative healthy T-cells. Data represent mean ± SD from 3 measurements on KMS-12-BM or 3 T-cells donors in 3 independent experiments, analyzed using one-way ANOVA and Tukey's post-hoc test, ns: not significant. **(D-E)** Cytotoxicity towards A549 cell line expressing both CD38 and EGFR (D) and absolute count per well of $CD8^+$T-cells expressing CD38 (E) after treatment with ISB 1342 (10nM) or CD3xEGFR (10nM) control molecule in presence of healthy PBMC (E:T 10:1) for 48

hours. Data represent mean ± SD from 4 PBMC donors using non-linear regression analysis (D) or mean ± SEM from replicates with treatment at 10nM for each PBMC donor (E, one line/symbol=1donor) performed in 2 independent experiments in duplicates. Data was analysed using a two-way ANOVA and a Tukey's post hoc test.

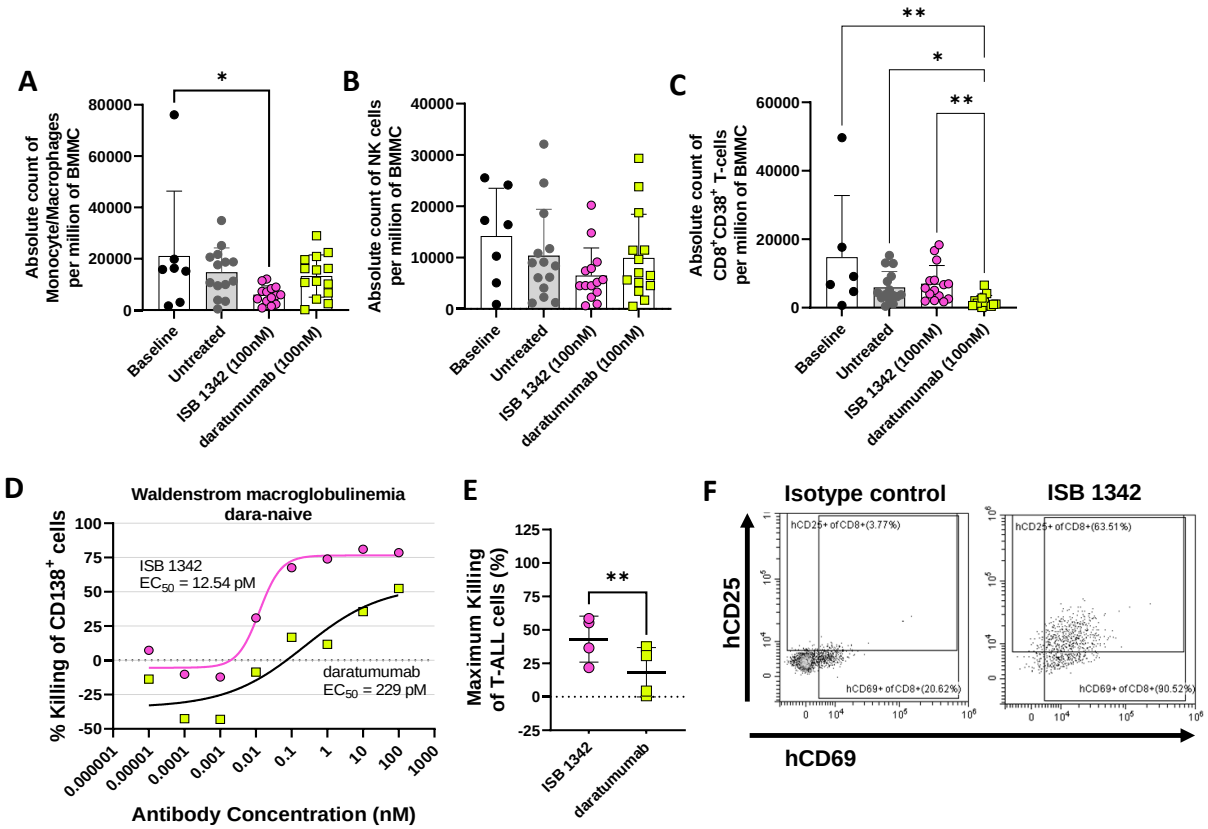


Supplementary Figure 3. T-cell profiling in MMoAK. (A) Representative dot plots showing activation (CD25, CD69) and degranulation profile (CD107a) on human $CD8^+$ T-cells from hPBMCs co-cultured with NCI-H929 cell line upon treatment with maximum dose of ISB 1342 or its isotype control for 48h in a MMoAK assay. **(B)** Maximum T-cell response in a MMoAK

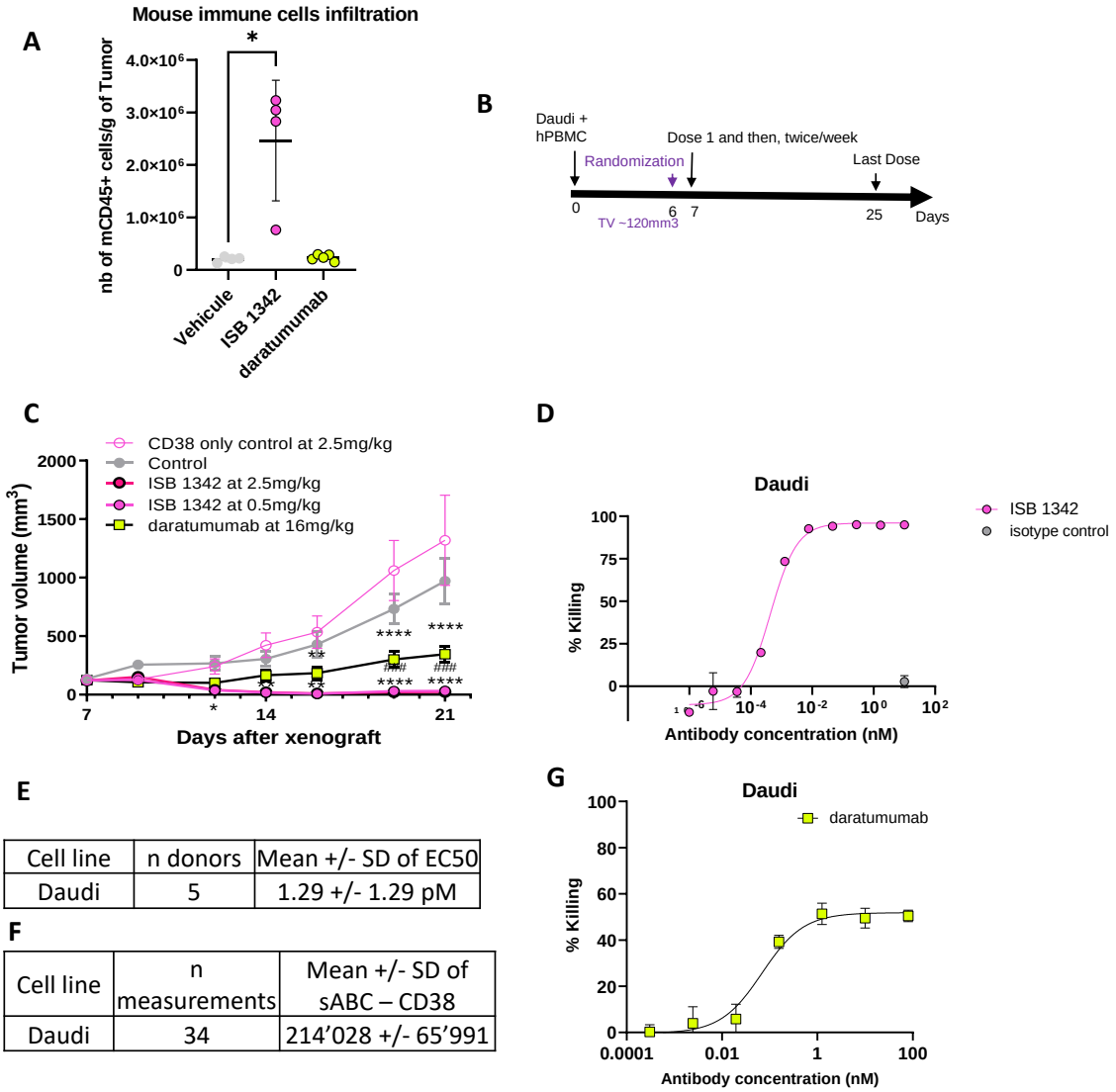
assay with MM cell lines in presence of ISB 1342 (pink symbols) or its isotype control (grey symbols), healthy PBMC (E:T 5:1), normal human serum and rhIL-2 for 48 hours. Dots represent the maximum response for each PBMC donor and bars represent mean ± SD of the maximum response from up to 8 PBMC donors per treatment and cell line from 5 independent experiments. **(C)** Percentage (left) and absolute count per well (right) of $CD38^{+}CD8^{+}$T-cells in a MMoAK assay with NCI-H929 cell line in presence of a single dose of daratumumab or its isotype control, treated concomitantly or sequentially with a dose-range of ISB 1342 or its isotype control. Dots represent the mean of experimental replicates for each PBMC donor and bars represent mean ± SD from 6 PBMC donors from 1 experiment.



Supplementary Figure 4: Quantification of effector cells in sample from patients with MM. (A) Absolute count of $CD38^{+}CD138^{+}$ tumor cells per million of BMMC in MM patient samples at baseline versus 17-24h in culture in untreated conditions. Dots represent individual samples and data are mean ± SD compared using a Wilcoxon paired t-test. (**B**) Representative dots plots showing $CD38^{+}CD138^{+}$ tumor cell population at baseline, after 17-24h in culture, untreated versus ISB 1342 and daratumumab at 100nM from MM patient sample 4. (**C-E**) Ratio of $CD8^{+}$T-cells (C), NK cells (D) and Monocytes/Macrophages (E) to $CD38^{+}CD138^{+}$ MM tumor cells in patient samples at baseline. PCL patients 1 and 12 highlighted in orange are blood samples. Dots represent individual samples and data are mean ± SD compared using a Mann-Whitney test. (**F-H**) Correlation between cytotoxicity induced by ISB 1342 (100nM) and $CD8^{+}$T-cells ratio to MM cells in patient samples (F), and between cytotoxicity induced by daratumumab (100nM) and NK cells (G) as well as Monocytes/Macrophages (H) (Mon/Mac). Dots represent individual samples and data are analysed using linear regression.



Supplementary Figure 5: ISB 1342 does not deplete other CD38$^+$ cells and induces cytotoxicity towards Waldenstrom macroglobulinemia and T-cell acute lymphoblastic leukemia (T-ALL). (A-C) Absolute count of monocytes/macrophages (A), NK cells (B) and CD8$^+$CD38$^+$T-cells (C) per million of BMMC in MM patient samples at baseline, and after 17-24h culture in untreated, ISB 1342 (100nM) and daratumumab (100nM) treated conditions. Dots represent individual samples and data are mean ± SD compared using a one-way ANOVA followed by a Tukey's (A) or Kruskal-Wallis (B, C) post-hoc analysis. (**D**) Representative cytotoxicity curves of CD138$^+$ MM cells by ISB 1342 and daratumumab at 24h in samples from a patient with Waldenstrom macroglobulinemia. Data is mean ± SEM of replicates analysed using non-linear regression analysis. **(E)** Maximal cytotoxicity of T-ALL with ISB 1342 or daratumumab in PBMC or BMMC samples from patients with T-ALL. Dots represent individual samples and data is mean ± SD compared using a paired t-test. (**F**) Representative dot plots showing activation profile (CD25 and CD69 expression) of human CD8$^+$T-cells from BMMC of one MM patient upon treatment with ISB 1342 or its isotype control for 48h in an *ex vivo* killing assay (patient sample 2).



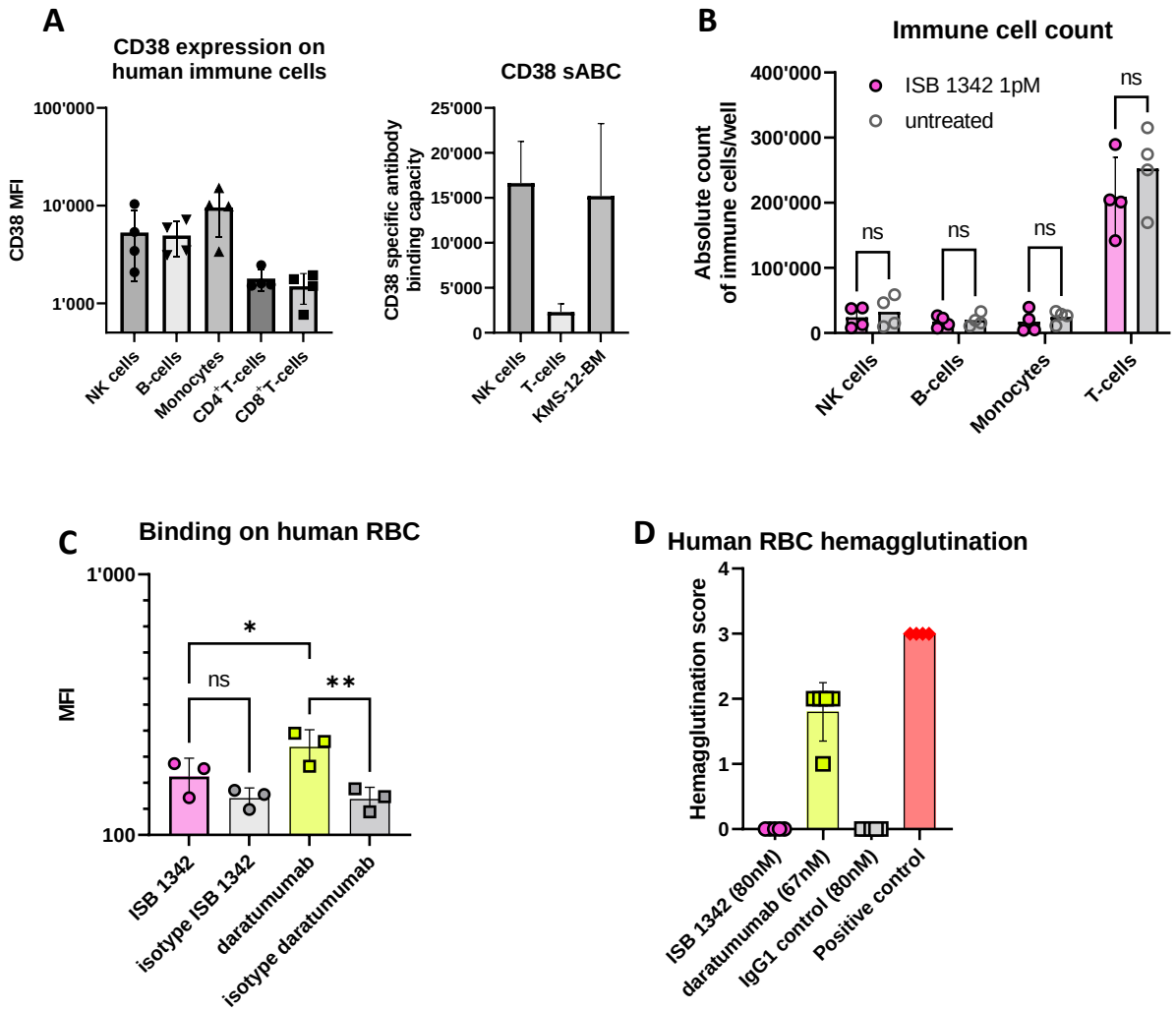
E

Cell line	n donors	Mean +/- SD of EC50
Daudi	5	1.29 +/- 1.29 pM

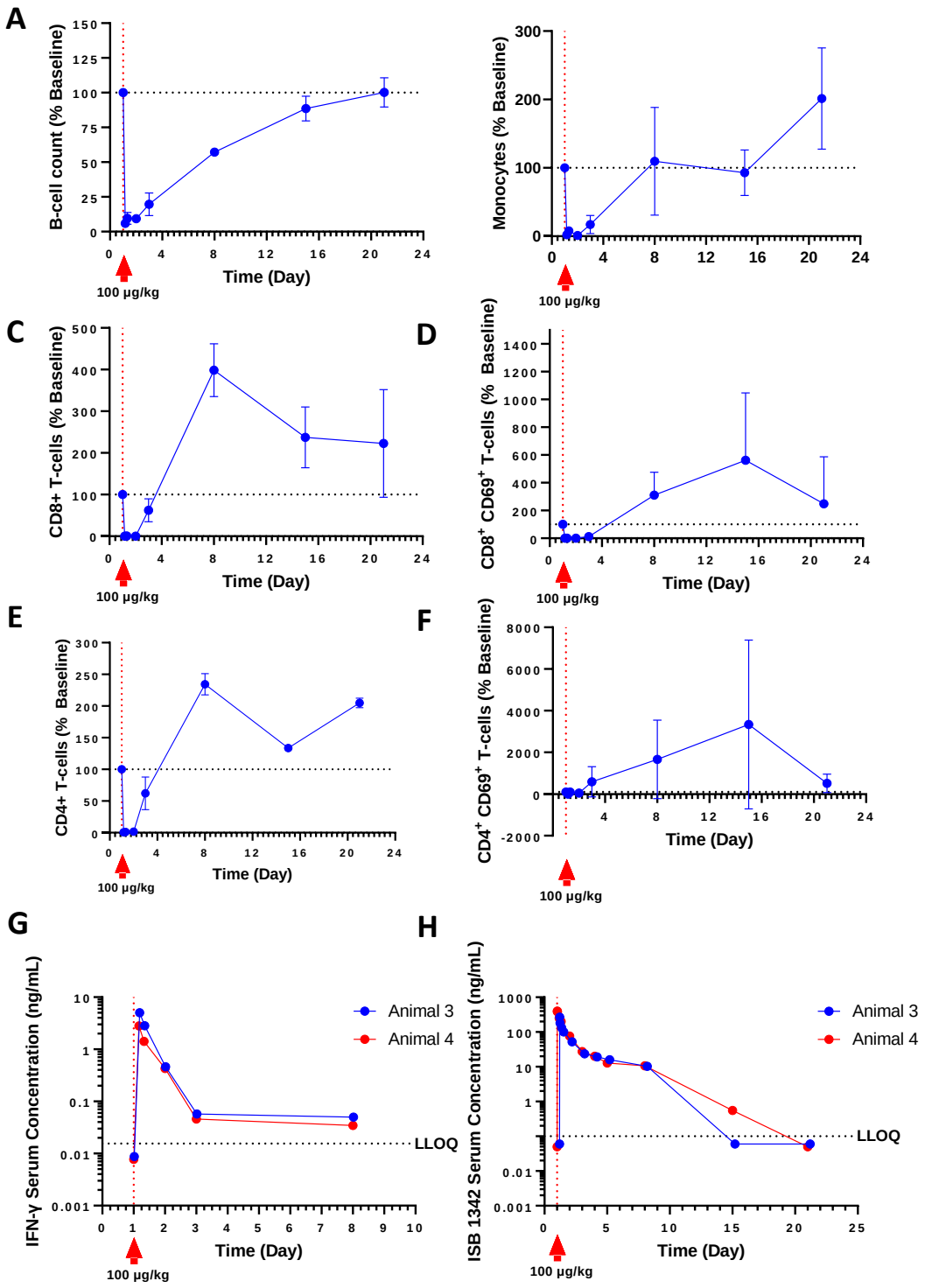
F

Cell line	n measurements	Mean +/- SD of sABC – CD38
Daudi	34	214'028 +/- 65'991

Supplementary Figure 6. ISB 1342 activity *in vivo*. (A) Infiltration of murine (m) CD45+ cells (defined as live hCD45$^-$mCD45$^+$) in tumors of KMS-12-BM xenografted mice after vehicle, ISB 1342 or daratumumab treatments. Data are mean ± SD for 5 mice compared using one-way ANOVA followed by Dunnet's post-hoc test to vehicle. *p<0.05. **(B-C)** Experimental design (B) and measurement of tumor growth (C) in the Daudi s.c. xenograft human PBMC (hPBMC)-transferred NOD-SCID mouse model (B). *In vivo* activity was followed for ISB 1342 at 0.5 or 2.5 mg/Kg and daratumumab at 16 mg/Kg injected i.v. twice per for three weeks with 4-5 mice per group. Data are mean mm^3 ± standard error of the mean (SEM) determined by caliper measurements. Data were compared for both models using two-way ANOVA followed by Tukey's post-hoc comparison. * is showing significant differences between ISB1342 and vehicle control; # is showing differences between daratumumab and ISB 1342. **(D)** Cytotoxicity of Daudi cell line after treatment with ISB 1342 or control molecules in presence of healthy PBMC (E:T 5:1) for 72 hours. Data represent mean ± SD from duplicates for one representative PBMC donor. **(E)** Mean +/- SD of EC50 of Daudi cell line cytotoxicity induced by ISB 1342 in presence of 5 healthy PBMC donors in 3 independent experiments. **(F)** Absolute number of specific Antibody Bound per Cell (sABC) for CD38 indicating the relative target density on Daudi cell line. **(G)** Cytotoxicity of Daudi cell line in a Multiple Mode of Action Killing in vitro assay (MMoAK) in presence of daratumumab, healthy PBMC (E:T 5:1), normal human serum and rhIL-2 for 48 hours. Data represent mean ± SD of duplicates from one representative donor.



Supplementary Figure 7. ISB 1342 safety profile on human immune cells and Red Blood cells (RBC). (A) CD38 expression (left) on NK cells ($CD56^+$), B-cells ($CD20^+$), monocytes ($CD14^+$) and $CD4^+$ or $CD8^+$T-cells in PBMC from human healthy donors. Each dot represents the log of the MFI for one donor and the bars the log of the mean ± SD of 4 donors from 2 independent experiments. Absolute number (right) of specific Antibody Bound per Cell (sABC) indicating the relative CD38 density on MM cell lines. Data represent mean ± SD of 10 to 63 measurements per cell type. **(B)** Absolute count of NK cells ($CD56^+$), B-cells ($CD20^+$), monocytes ($CD14^+$) and T-cells ($CD3^+$) in an *in vitro* assay where PBMC from healthy donors were incubated with ISB 1342 or its isotype control for 48 hours. The absolute count per well was measured by flow-cytometry. Each dot represents one PBMC donor and the bars mean ± SD of 4 donors from 2 independent experiments compared using a two-way ANOVA followed by Sidak's post-hoc analysis. **(C)** Binding on human RBC of ISB 1342 and daratumumab or their isotype controls. Each dot represents the log of the MFI for one RBC donor and the bars the log of the mean ± SD of 3 donors from 1 experiment that were compared using a one-way ANOVA followed by a Tukey post-hoc comparison. **(D)** Human RBC agglutination by indirect Coombs agglutination assay. Each symbol represents the hemagglutination score for one RBC donor, bars represent mean ± SD of 5 donors.



Supplementary Figure 8: Impact of ISB 1342 on circulating leukocytes and systemic soluble factors in cynomolgus monkeys. (A-F) Cynomolgus monkeys were injected with one single dose of ISB 1342 i.v. (100 µg/kg) at day 1. Levels of peripheral B-cells (A), monocytes (B), $CD8^+$ T-cells (C), activated $CD8^+$ $CD69^+$ T-cells (D), $CD4^+$ T-cells (E) and activated $CD4^+$ $CD69^+$ T-cells (F) were measured using flow cytometry. Data represent mean ± SD of 10^3 counts/µl normalized to baseline counts for two animals (one male, one female). (G, H) Levels of circulating IFN-γ (G) and ISB 1342 (H) were measured using ELISA. Data represent levels per animal and LLOQ is the lower limit of quantification for the assay.

Supplementary Tables

Supplementary Table 1: List of antibodies and labelling reagents.

Antibody	Clone	Supplier	Assay
CFSE	NA	Sigma-Aldrich	RDL
eFluor 670	NA	ThermoFisher	RDL, MMoAK
Anti-human CD25	BC96	ThermoFisher	RDL, MMoAK
Antu- human Ki-67	SolA15	ThermoFisher	RDL
Anti-human granzyme B	MA523639	ThermoFisher	RDL
Anti-human perforin	353320	Biolegend	RDL
Anti-human CD107a	eBioH4A3	ThermoFisher	MMoAK
Anti-human CD138	MI15	ThermoFisher	Patients
Anti-human CD138	281-2	Biolegend	Patients
Anti-human CD4	SK3	Biolegend or Cytek	Patients
Anti-human CD25	BC96	Biolegend or BD Biosciences	Patients
Anti-human CD69	FN50	ThermoFisher, BD Biosciences or Biolegend	Patients, MMoAK, In vivo mouse model
Anti-human CD107a	H4A3	BD Biosciences or Biolegend	Patients
Anti-mouse CD45	30-F11	ThermoFisher	In vivo mouse model
Anti-human CD14	M5E2	BD Biosciences	In vivo mouse model
Anti-human CD19	HIB19	ThermoFisher	In vivo mouse model
Anti-human CD56	NCAM16.2	BD Biosciences	In vivo mouse model
Anti-human CD45	HI30	Biolegend	In vivo mouse model
Anti-human TCRab	IP26	Biolegend	In vivo mouse model
Anti-human CD4	OKT4	BD Biosciences	In vivo mouse model
Anti-human CD8	SK1	BD Biosciences, Biolegend or Cytek	RDL, MMoAK, Patients, in vivo mouse model
Anti-human CD25	M-A251	Biolegend, BD Biosciences	MMoAK, in vivo mouse model
Anti-cyno CD14	M5E2	BD Biosciences	In vivo cyno study
Anti-cyno CD4	M-T477	BD Biosciences	In vivo cyno study
Anti-cyno CD8	RPA-T8	BD Biosciences	In vivo cyno study
Anti-cyno CD20	2H7	BD Biosciences	In vivo cyno study
Anti-cyno CD69	L78	BD Biosciences	In vivo cyno study
Live/Dead fixable dyes	NA	ThermoFisher	RDL, patient
SYTOX dyes	NA	ThermoFisher	RDL, MMoAK
7-AAD	NA	ThermoFisher	RDL
FVS700	NA	BD Biosciences	In vivo mouse model
Anti-human CD10	HI10a	BD Biosciences	Patients
Anti-human CD117	A3C6E2	Biolegend	Patients
Anti-human CD19	HIB19	ThermoFisher	Patients
Anti-human CD3	SK7	Biolegend	Patients
Anti-human CD34	4H11	ThermoFisher	Patients
Anti-human CD38	polyclonal	Cytognos/MedTech	Patients
Anti-human CD45	2D1	Biolegend	Patients
Anti-human CD56	B159	BD Biosciences	Patients
Anti-human CD11b	M1/70	BD Biosciences	Patients
Anti-human CD11c	3.9	Biolegend	Patients
Anti-human PD-1	EH12.2H7	Biolegend	Patients
Anti-human TIM-3	7D3	BD Biosciences	Patients
Anti-human LAG-3	11C3C65	Biolegend	Patients
Anti-human CD4	OKT4	ThermoFisher	CD38 level on human immune cells
Anti-human CD8	SK1	Biolegend	CD38 level on human immune cells
Anti-human CD56	TULY56	ThermoFisher	CD38 level on human immune cells
Anti-human CD20	2H7	Biolegend	CD38 level on human immune cells
Anti-human CD14	MEM-15	ThermoFisher	CD38 level on human immune cells
Anti-human CD38	HIT2	ThermoFisher	CD38 level by flow-cytometry

205 **Supplementary Table 2: Culture media for *in vitro* and *ex vivo* assay**

Assay	Media composition
RDL, HD-PBMC	RPMI1640, 1% Penicillin/Streptomycin, 1%L-Glutamine, 10%heat inactivated fetal bovine serum (Hi FBS)
MMoAK	RPMI1640, 1% Penicillin/Streptomycin, 1% L-Glutamine, 50%Human Serum (HS) and 100U/ml hIL-2
MM Patient samples	RPMI1640, 1% Penicillin/Streptomycin, 1% L-Glutamine, 10%Human Serum (HS) and 3µg/ml hIL-6

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207 **Supplementary Table 3: Disease stage of patient samples included in the *ex vivo* study**

Patient samples Nb	Disease Stage	Treatment history
dara-naïve		
1	PCL	none
2	Smoldering MM	none
3	Smoldering MM	none
4	Newly diagnosed MM	none
5	Newly diagnosed MM	none
6	Newly diagnosed MM	none
7	Newly diagnosed MM	none
8	r/rMM- dara-naïve	(1) Carfilzomib, Cyclophosphamide, Dexamethasone, (2) Carfilzomib maintenance, (3) Melphalan-autograft
9	r/rMM- dara-naïve	(1) Velcade, Revlimid, Dexamethasone and Autograft
10	r/rMM- dara-naïve	(1) Velcade, Thalidomide, Dexamethasone, (2) Velcade, Endoxan, Dexamethasone, (3) Revlimid, Dexamethasone, Autograft
11	r/rMM- dara-naïve	(1) Velcade, Thalidomide, Dexamethasone, (2) Carfilzomib, Revlimid, Dexamethasone
dara-exposed <3 months		
12	PCL	(1) Thalidomide-Dexamethasone-Autograft, (2)Velcade-Thalidomide-Dexamethasone-Autograft, (3) daratumumab-Revlimid-Dexamethasone, (4)Velcade-Dexamethasone-daratumumab
13	r/rMM	(1) Velcade-Revlimid-Dexamethasone, (2) daratumumab-Ixazomib

dara-exposed >3 months		
14	r/rMM	(1) Dexamethasone-autograft, (2) Revlimid-Dexamethasone, (3) Velcade-Thalidomide-Dexamethasone, (4) Pomalidomide-Dexamethasone, (5) daratumumab, (6) Elranatanab
15	r/rMM	(1) Velcade-Revlimid-Dexamethasone, (2) daratumumab-KRD (Carfilzomib-Revlimid-Dexamethasone)

Nb: Number
PCL: Plasma Cell Leukemia
MGUS: Monoclonal gammopathy of undetermined significance
r/rMM: relapsed/refractory Multiple Myeloma
dara: daratumumab

Supplementary Table 4: Summary PK Parameters of ISB 1342 in Cynomolgus Monkeys: single dose study

Dose level (µg/kg)	C_0 (ng/mL)	C_{max} (ng/mL)	T_{max} (hr)	AUC_{0-t} (hr*ng/mL)	t_{last} (hr)	$AUC_{0-\infty}$ (hr*ng/mL)	$t_{1/2}$ (hr)	ADA detected
100	409	334	0.5	6852	252	8511[a]	52.6[a]	Day 15

[a] N=1

$AUC_{0-\infty}$ = area under the (serum) concentration time curve extrapolated out to infinity; AUC_{0-t} = area under the plasma concentration-time curve from time zero to time t; C_0 = initial concentration; NE: Not Estimable; $t_{½}$= terminal elimination half-life; t_{last} = time of the last measurable (positive) concentration; T_{max}= Time to reach maximum serum concentration following drug administration. ADA = Anti-Drug Antibodies.

Supplementary Material and Methods

Dara sharing statement

For original data, please contact Dr Mario Perro: mario.perro@ichnossciences.com

Affinity measurements by Surface Plasmon Resonance (SPR)

Binding of ISB 1342 to CD3εδ and CD38 was evaluated by SPR on a Biacore T200 instrument. For CD38 binding, biotinylated human CD38 AviTag-hisTag protein (Acrobiosystems) was immobilized on a Series S Biotin Capture sensor chip (CAP) according to the manufacturer's instructions and increasing concentrations of ISB 1342 were flushed onto the immobilized ligand. For CD3εδ binding, human CD3E&CD3D-his protein (Creative Biomart) was immobilized on a Series S CM5 sensor chip according to the manufacturer's instructions and increasing concentrations of ISB 1342 were flushed onto the immobilized ligand. Data were analysed in the Biacore evaluation software and fit to a 1:1 binding model to infer dissociation constants (K_D).

Flow cytometry analysis

Cytoflex or Cytoflex S (Beckman Coulter), iQue (Sartorius) or Aurora (Cytek) for FACS analysis and analysed using either FlowJo (Becton Dickinson), CytExpert (Beckman Coulter),

Spectroflo (Cytek), or Forecyt (Sartorius) software. Antibodies and live/dead dyes are listed in **Supplementary Table 1** for each assay.

Flow cytometry Cell-based Affinity assay (CBA)

Cells were incubated with increasing doses of ISB 1342, daratumumab (Darzalex®, Janssen Biotech Inc.) or control molecules in a 96-well plate at 4°C in the dark. A labelled anti-human Fc monoclonal antibody was then added to the mixture, for T-cells an anti-human CD38 (HIT2 or multi-epitope), was also added and incubation was performed at 4°C and in the dark. Cells were finally resuspended in FACS buffer containing viability dye (SYTOX) and acquisition was performed on a flow cytometer (Cytoflex or Cytoflex S). The Geometric Mean of Fluorescence Intensities (MFI) of the viable single cells (CD38- for T-cells) was extracted using FlowJo. The values of MFI from the control antibodies (ISB 1342_DUaDUa for ISB 1342, and ABC1 for daratumumab) were subtracted to the MFI values of ISB 1342 or daratumumab to generate the relative fluorescence intensity (RFI). The K_D was calculated with the RFI using a non-linear one site binding (hyperbola) regression.

Epitope mapping of h9G7

To map the epitope of h9G7, the extracellular domain of CD38 was divided in 19 13-amino acid long peptides and a final, 11-amino acid long peptide that were fused onto an IgG1 Fc via a linker. The binding of each peptide to h9G7 was evaluated by SPR on a Biacore T200 instrument.

Competition of ISB 1342 with daratumumab by BioLayer Interferometry (BLI)

Competition of ISB 1342 with daratumumab was assessed using BLI. Measurements were done on an OctetRED96e instrument (Sartorius) and analyzed using the Data Analysis version 11.1 software (Octet, Sartorius). Biotinylated recombinant human CD38-AviTag-HisTag (Acrobiosystems) was loaded onto Streptavidin (SA) Biosensor (Sartorius). The sensor was then dipped into a saturating solution of daratumumab or ISB 1342, followed by a mixture of both. An increase in signal upon dipping in the mixture was indicative of a lack of competition between ISB 1342 and daratumumab.

Affinity measurements to Fc receptors by Surface Plasmon Resonance

Binding of ISB 1342 and its Fc competent counterpart to the Fcγ Receptors was evaluated by SPR on a Biacore 8K instrument. For CD64 binding, biotinylated CD64 was immobilized onto a CAP chip according to the manufacturer's instructions and increasing concentrations of ISB 1342 were flushed onto the immobilized ligand in single-cycle kinetics mode. Data were analysed in the Biacore evaluation software and fit to a 1:1 binding model to infer K_D For CD16a, CD32a and CD32b binding, the antibody constructs were immobilized on a Protein G Series S CM5 chip prepared according to the manufacturer's instructions and increasing concentrations of the receptors were flushed onto the immobilized antibody constructs. The binding K_D to CD16a, CD32a and CD32b were measured using a steady state affinity model.

Soluble factors measurements

The quantification of cytokines in the supernatant of RDL after 48 hours of treatment was assessed by ELISA (BD OptEIA™, BD Biosciences) following manufacturer's instructions. Briefly, plates were coated with capture antibodies (anti-TNFα, anti-IFNγ anti-IL-6 or anti-IL-2) overnight at 4°C. Plates were then sequentially blocked, incubated with supernatant or standard and finally with detection antibody associated with horseradish peroxidase (HRP) enzyme. Addition of TMB substrate allowed release of a colorimetric product which was quantified using absorbance plate reader (Biotek Synergy).

Confocal live imaging

KMS-12-BM cells were stained with of CellTracker™ Blue Dye (10μM) and co-plated with T-cells from healthy donor labelled with CellTracker™ Orange Dye (2μM) at an E:T of 5:1. ISB 1342 (10nM) was labeled using Fab anti-Fc-AlexaFluor647 (Zenon™, Thermofisher) and added to the cells on ibiTreat pre-coated slides (Ibidi) for 5 hours at 37°C. Live microscopy was carried out using a Zeiss LSM800 inverted confocal microscope incubation system (Carl Zeiss). Images

were processed with Imaris software (Oxford Instruments) and composite is shown (blue for KMS-12-BM, green for T cells and white for ISB 1342).

Specific Antibody Bound per Cell (sABC)by flow cytometry

Expression levels of CD38 on KMS-12-BM, NCI-H929, RPMI 8226 and MOLP-8 cell lines were determined by flow-cytometry using QIFIKIT® reagents (Dako) according to manufacturer's instructions. An anti-human CD38 (HIT2; Thermofisher) was used as primary antibody at 10µg/ml and CD38 sABC (specific Antibody Binding Capacity) value was calculated using the QIFIKIT calibration standards and formulation provided by the kit.

CD38 expression level on human primary immune cells

Expression level of CD38 on primary immune cells were determined by flow-cytometry. Human PBMC from healthy donors were stained with labelled antibodies targetting CD56, CD20, CD14, CD4, CD8 and CD38. The geomean of fluorescence intensity of CD38 was exported for each subpopulation of immune cells.

Binding to human red blood cells

Human red blood cells (RBC) were stained with ISB 1342, daratumumab or their isotype controls at 80nM and detected with a labelled anti-human Fc secondary antibody. The geomean of fluorescence intensity reflecting the antibody binding was measured by flow-cytometry.

Antibody-Dependant Cellular Phagocytosis assay (ADCP)

Monocytes were isolated from healthy PBMC by immunomagnetic negative selection (Stemcell) following manufacturer's instructions. Isolated monocytes were culture for 7-10 days with RPMI1640 10%HiFBS, 1% Penicillin/Streptomycin (P/S), 1% L-Glutamine (Gln) supplemented with 50ng/ml rhM-CSF (Peprotech) to generate monocyte-derived-macrophages (MDM) used as effector cells. MDM were harvested using cell dissociation buffer (Thermofisher), stained with 2.5 µM CellTrace™ violet (Thermofisher) and resuspended in RPMI1640 10%HiFBS, 1% P/S, 1% Gln medium. Similarly, target tumor cell lines were labeled with 500ng/ml of pHrhodo red™ (Thermofisher). Cell lines were then co-cultured with MDM into a 96 well plate at an E:T of 1:3 in presence of increasing concentrations of daratumumab – starting at 80nM - for 2 hours and half at 37°C + 5% CO2 in a humidified atmosphere. Plates were acquired using CX5 high content image system (Thermofisher) and analyzed using HCS studio. Phagocytosis index was calculated as average number of phagocytosed cells for 100 macrophages.

Antibody-Dependant Cellular Cytotoxicity assay (ADCC)

NK cells were isolated from healthy PBMC by immunomagnetic negative selection (Stemcell) following manufacturer's instructions and rested overnight in RPMI1640 10%HiFBS, 1% P/S, 1% Gln. NK effector cells were then co-cultured into a 96-well plate with target tumor cell lines previously labelled with calcein AM 5 µM (Sigma). Cells were co-cultured at an E:T of 5:1 with increasing concentrations of daratumumab – starting at 80nM - for 4 hours and half at 37°C with 5% CO_2 in a humidified atmosphere. Calcein release was assessed in the supernatant using fluorescence plate reader (Biotek Synergy). Triton-lysed tumor cells was used as positive control for maximum release and tumor cells only as spontaneous release; percentage of cytotoxicity was calculated relative to maximum and spontaneous release.

Complement-Dependent Cytotoxicity assay (CDC)

Target tumor cell lines were labelled with calcein AM 5 µM (Sigma), resuspended in RPMI1640 1% P/S, 1% Gln and mixed with increasing concentrations of daratumumab – starting at 160nM - in a 96-well plate in presence of 50% Human Serum (HS; Sigma) for 4 hours and half at 37°C with 5% CO_2 in a humidified atmosphere. Calcein release was assessed in the supernatant using fluorescence plate reader (Biotek Synergy). Triton-lysed tumor cells was used as positive control for maximum release and tumor cells only as spontaneous release; percentage of cytotoxicity was calculated relative to maximum and spontaneous release.

T-cell fratricide in Re-directed Lysis (RDL) *in vitro* assay

A549 cell line expressing both CD38 and EGFR was co-cultured for 48 hours with hPBMCs at an effector to target ratio (E:T) of 10:1 with ISB 1342 or a CD3xEGFR BEAT® control molecule including the same CD3 binder than ISB 1342 and a high affinity for EGFR that are not expressed on hT-cells in contrast to CD38. The A549 cell killing was measured using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega) and normalized with the untreated and non-effector cells conditions. The proportion of T-cell expressing CD38 was measured by flow-cytometry.

***in vitro* HD-PBMC assay**

Human PBMC from healthy donors were cultured for 48 hours at $10*10^6$ cells/ml and then for an additional 48 hours at $1*10^6$ cells/ml in presence of increasing doses of ISB 1342 at 37°C with 5% CO_2 in a humidified atmosphere. The count of live immune cells per well, defined as viability dye negative and CD56+ for NK cells, CD20+ for B cells, CD14+ for myeloid cells and CD4+orCD8+ for T-cells, was measured by flow-cytometry and compared to the untreated condition.

***ex vivo* assay on T-ALL patient samples**

0.1 $x10^6$ BMMC or PBMC were incubated with ISB 1342 500nM or daratumumab 80nM for 18-24 hours at 37°C in IMDM, 1% P/S, 1% Glutamine, 10%FBS,10%Human Serum (HS) SCF (50 ng/ml, Bio-Techne), Flt3L (20 ng/ml, Peprotech), IL-7 (10 ng/ml, Bio-Techne), insulin (116 ng/ml, Sigma) and IL-3 (20ng/ml, Peprotech). The T-ALL blast killing was measured by flow cytometry as the decrease of the remaining live blasts count (based on viability dye staining) after treatment normalized with the untreated condition. The gating strategy to define tumor cells was adjusted to each sample, from parent gate *live* $CD11b^-CD11c^-NOT(CD3^{high}CD45^{high})$:

In-house patient sample ID	Source	Blasts gating strategy
B-T-ALL-13	BMMC	$CD10^+$ $CD38^{+/-}$
B-T-ALL-7	BMMC	$CD34^+$ $CD117^+$
B-T-ALL-1	BMMC	$CD45^{low}$ of $CD19^-$ $CD56^-$
P-T-ALL-1	PBMC	$CD45^{low}$ of $CD19^-$ $CD56^-$

***In vivo* efficacy mouse model: Daudi tumors**

The *in vivo* studies were performed with 6/7-week-old immunodeficient female NOD.CB17/AlhnRj-Prkdcscid/Rj (NOD/SCID) mice from Janvier labs and conducted according to the Swiss Animal Protection Law with authorization from the cantonal and federal veterinary authorities. For the Daudi study, 5.10^6 Daudi cells and 5.10^6 hPBMC were injected subcutaneously (s.c.). Treatments were injected intravenously (i.v.) 7 days after, when tumors reached an average volume of $120mm^3$ and then twice/week, for 3 weeks.

Indirect Coombs hemagglutination assay

Whole blood from healthy human donors were collected in EDTA tubes and centrifuged to remove plasma. Red blood cells (RBC) were resuspended in DG Gel Sol (Grifols) and treated with ISB 1342, daratumumab or control molecules for 30 minutes at 37°C, and then centrifuged in the DG Gel Coombs cards (Grifols). Any agglutinated RBCs are captured at the top of or along the gel column, and non-agglutinated RBCs descend to the bottom of the microtube forming a pellet. Extent of agglutination was scored from 0 (no agglutination) to 4 (complete agglutination).

Statistical analysis

Statistical analysis and graphs were generated using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). A non-linear one site binding (hyperbola)

regression was applied to calculate K_D in CBA assays. The percentage of killing and T-cell response were fitted with 4PL non-linear regression with variable slope and the maximum fitted response and the EC_{50} calculated when possible. EC50 values were excluded when the R^2 of the non-linear regression fitting curve was below 0.7, when the difference between the maximum and the minimum response was below 10% or when the calculated EC_{50} values were out of the range of the antibody concentration range tested. For the MMOAK experiments, EC50 and maximum killing values were not included when spontaneous killing (killing in absence of treatments compared to target only) was above 50%.

Normality of data was checked using the Kolmogorov–Smirnov test and non-parametric tests were used when the data did not follow normal distribution. Differences between individual treatments or groups were analysed by paired or unpaired t-test as appropriate. One or two-way analysis of variance (ANOVA) was used for multiple group comparison followed by a post hoc analysis where appropriate, using Tukey's test for comparisons between all groups when data followed normal distribution or a Dunnett's test for comparisons to a control group when data followed normal distribution or Kruskall-Wallis test when comparing to a control group and data did not follow normal distribution. The post test was selected to show relevant comparison between data groups that allow to answer specific scientific questions. P values ≤0.05 were considered significant.