

Ibrutinib enhances the bias of T-cell responses towards staphylococcal superantigens sustaining inflammation in chronic lymphocytic leukaemia

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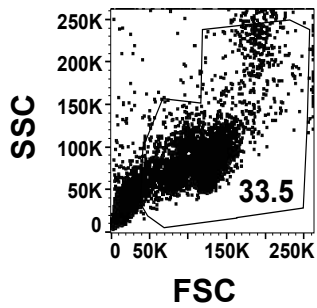
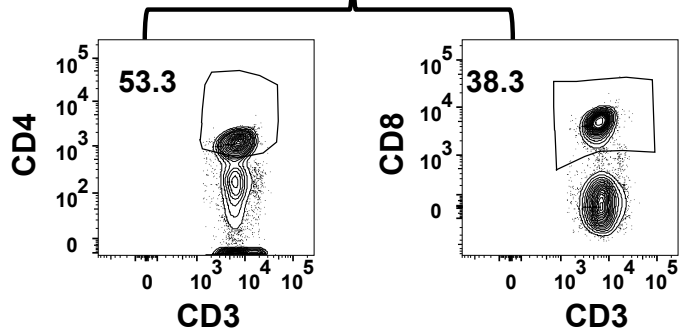
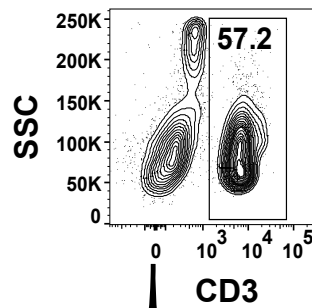
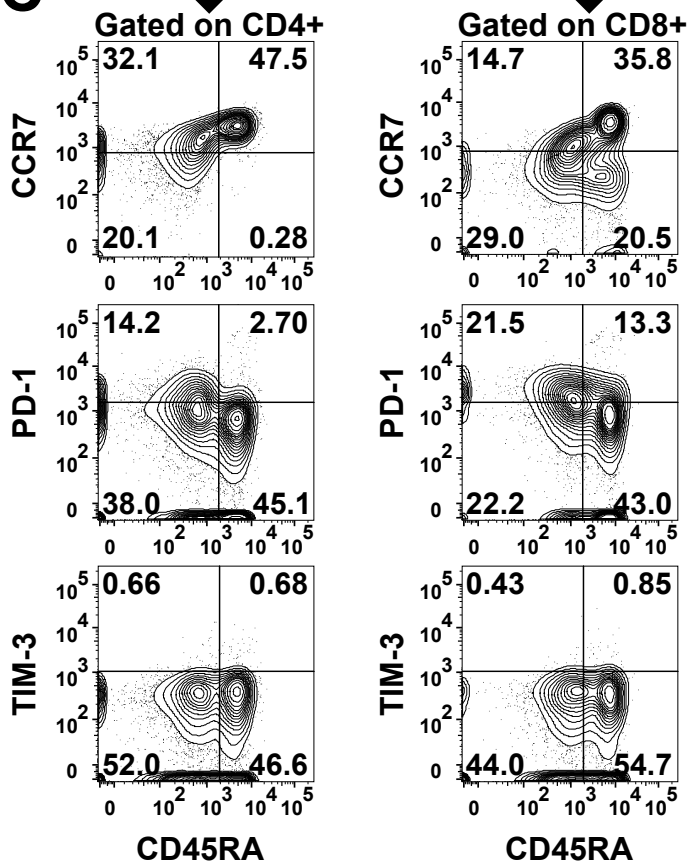
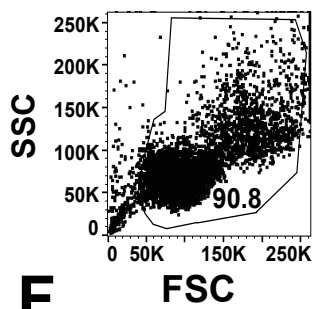
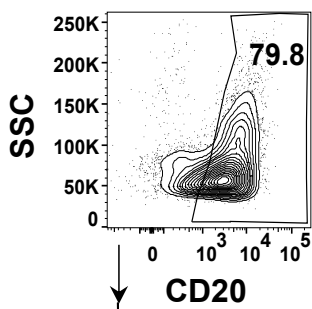
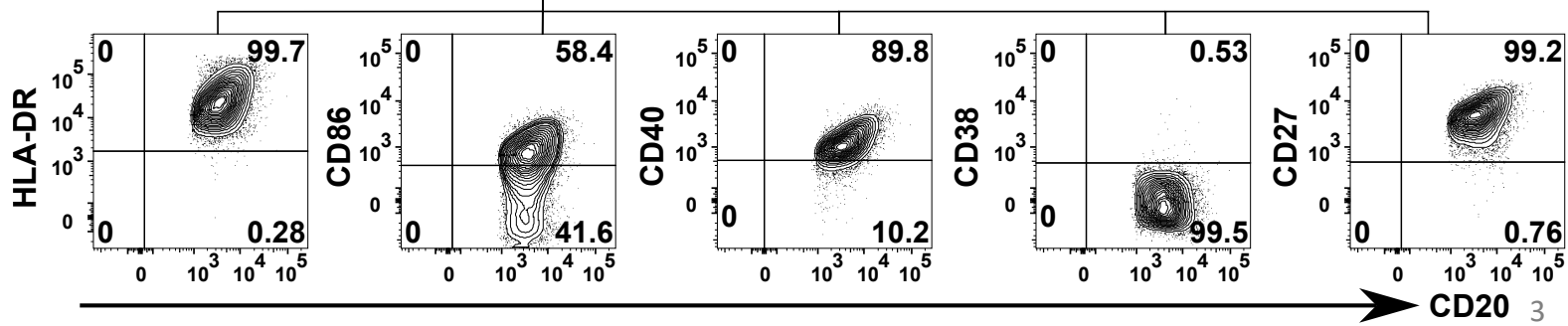
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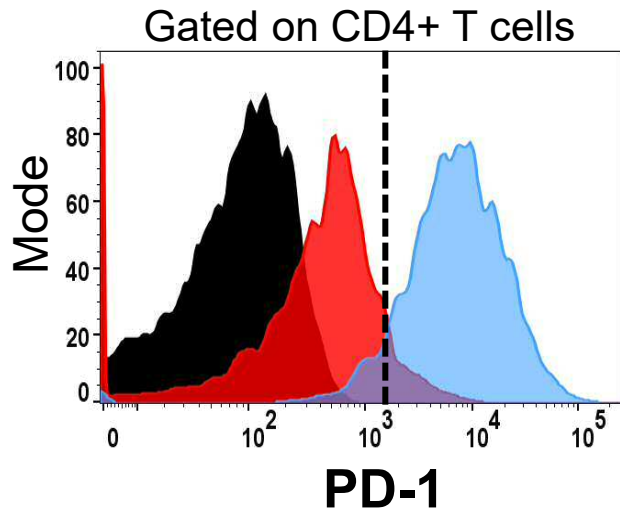
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A**B****C****D****E****F**

Supplementary Figure 1. Gating strategy used in the phenotypic analyses of T

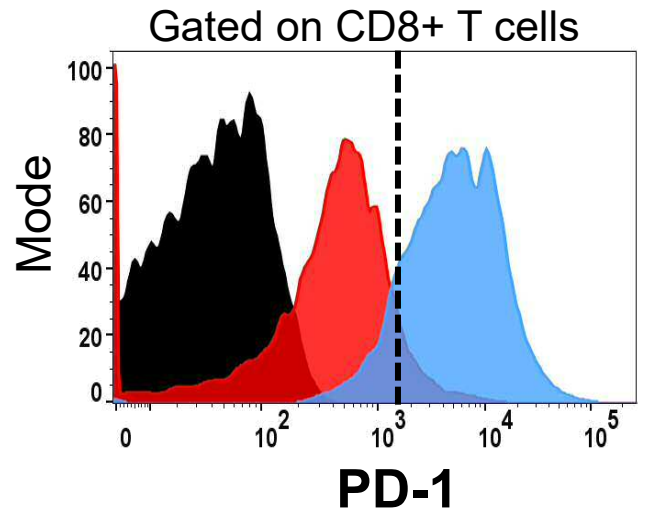
and B/CLL tumour cells. Freshly isolated, viable PBMCs (as by Trypan Blue exclusion assay; 2×10^6) derived from HDs or CLL patients were incubated with anti-FcR mAb and surface-stained with mAbs directed to the indicated surface markers in T (A-C) and B (D-F) cell flow-cytometry panels, as detailed in the Materials and Methods. Samples were then fixed and acquired on a BD LSR II Fortessa, setting up strictly defined rainbow profiles specific for each panel (required to remove day-to-day variability across the patient/donor recruitment period), and finally analysed using FlowJo™ software. **A)** The dot plot shows the viable lymphocyte gate by forward and side scatter (FSC vs SSC; *i.e.* size vs granularity) in HD, HDY-27M-013 sample (T cell panel), as example. **B)** The top contour plot shows how CD3⁺ T cells were identified in HDY-27M-013, by showing CD3 expression against SSC within gated viable lymphocytes. Thereafter, CD4⁺ CD3⁺ (left contour plot) and CD8⁺ CD3⁺ (right contour plot) T cells were identified in gated CD3⁺ viable T cells. **C)** Left and right contour plots respectively show the analysis of CD4⁺ and CD8⁺ T cells for the identification of: (i) naïve and memory T cell subsets using well known markers, CCR7 and CD45RA (top row), defining: naïve-like (Tn) CCR7⁺CD45RA⁺; central memory (Cm) CCR7⁺CD45RA⁻; effectors/effector memory (Eff/Em) CCR7⁻CD45RA⁻, and TEMRA (Temra) CCR7⁻CD45RA⁺ gates and (ii) the expression of checkpoint receptors: PD-1 (middle row) and TIM-3 (bottom row), gated against the memory marker CD45RA. **D)** Dot plot shows the viable lymphocyte gate by FSC vs SSC in an example CLL patient (P387, untreated CLL) for the B cell panel. **E)** Contour plot illustrates gated CLL tumour cells by showing CD20 expression against SSC within gated viable lymphocytes. **F)** Contour plots respectively show the expression of B cell activation/antigen-presentation marker, HLA-DR; co-stimulation markers, CD86 and

CD40; memory marker, CD27, and CLL negative prognosis marker, CD38, in gated viable CD20⁺ CLL tumour cells. The gates shown were used consistently across all samples analysed in the study.

A

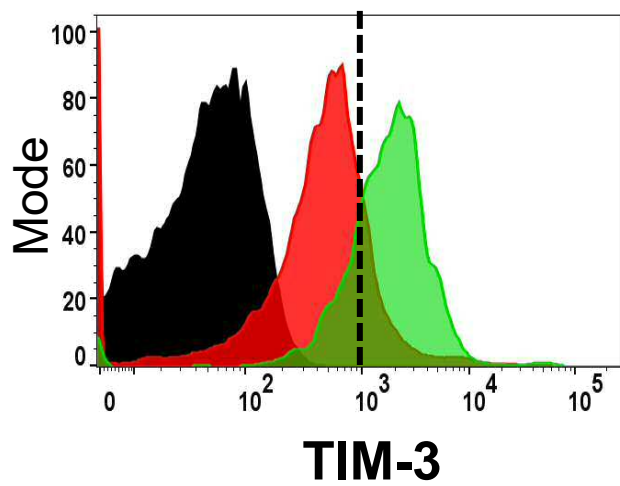
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■ Ex-vivo-HD

■ α CD3/28-HD**B**

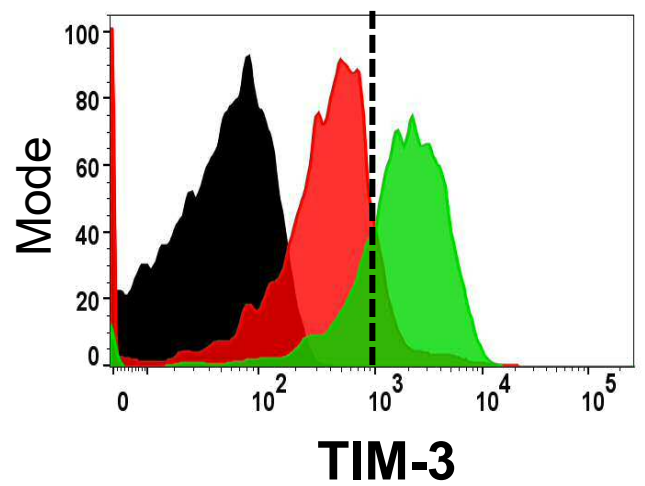
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■ Ex-vivo-HD

■ α CD3/28-HD**C**

■ CD4 or CD8-Single

■ Ex-vivo-HD

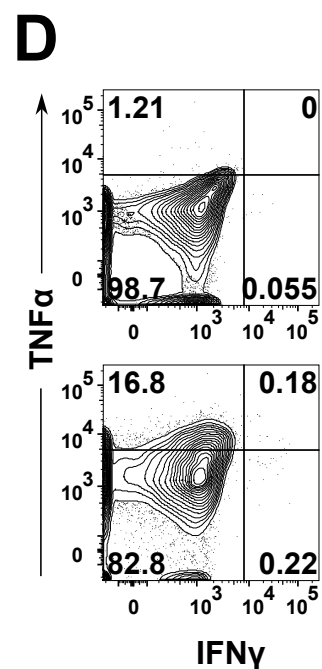
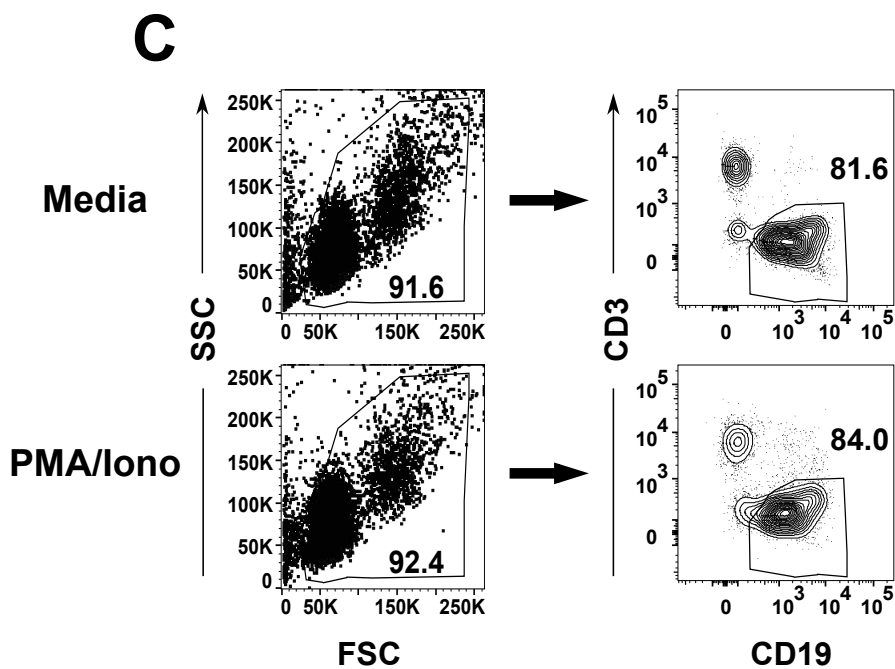
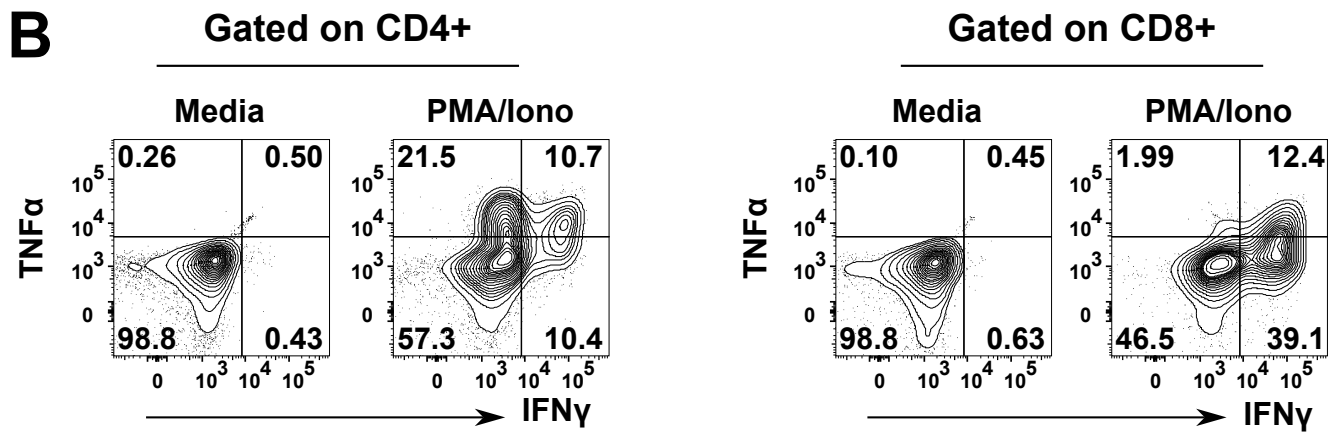
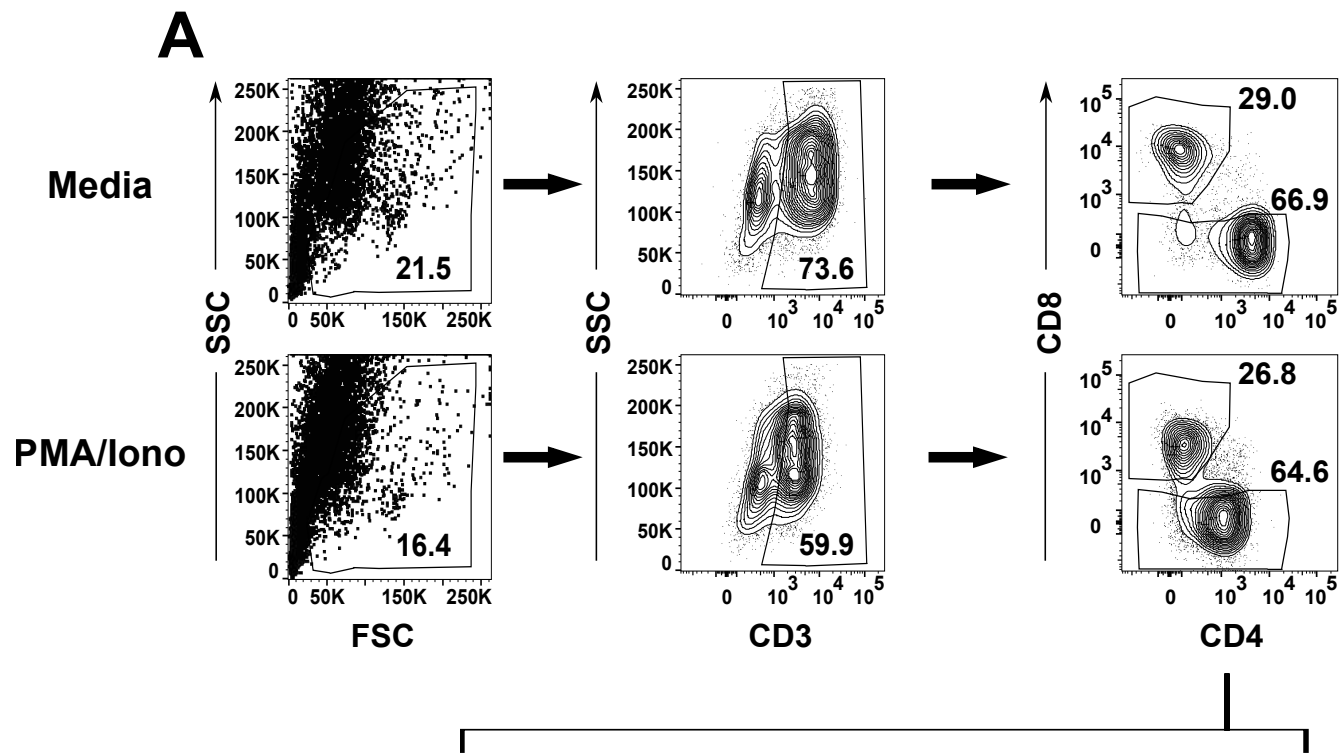
■ α CD3/28-HD**D**

■ CD4 or CD8-Single

■ Ex-vivo-HD

■ α CD3/28-HD

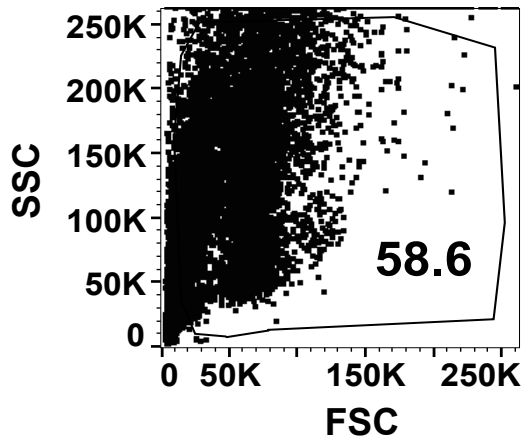
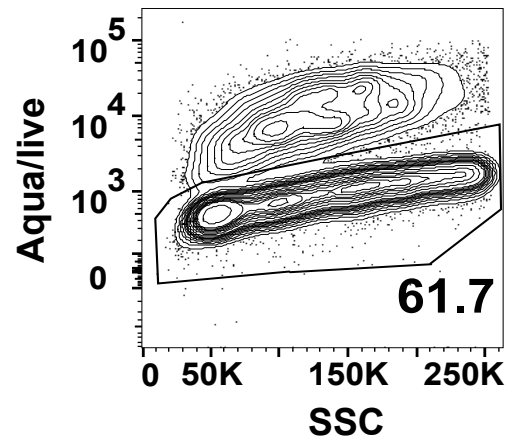
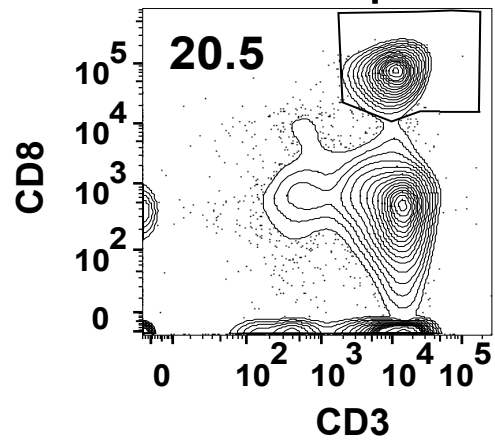
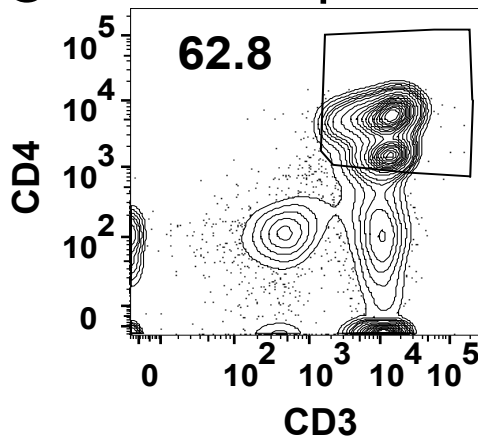
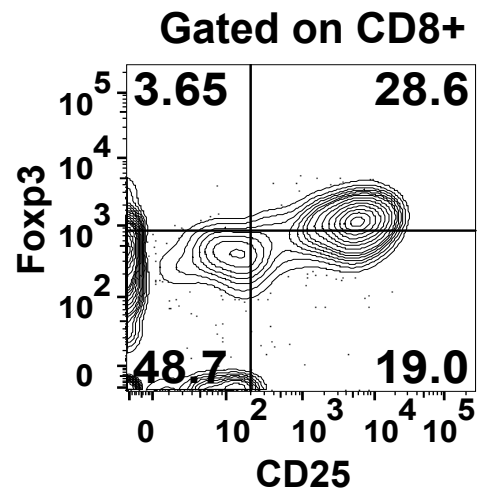
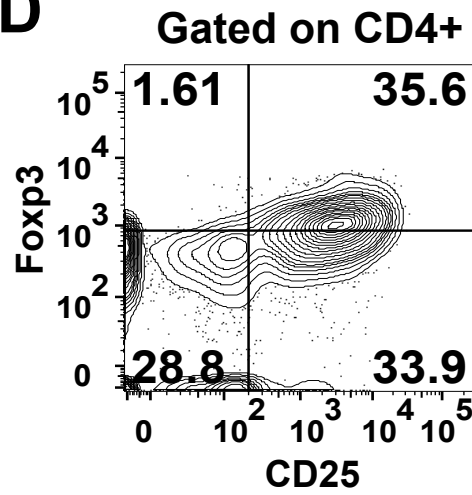
Supplementary Figure 2. Validation of PD-1+ and TIM-3+ gating strategy for CD4+ and CD8+ T cells. PBMCs from an example HD (HDY-33F-015) were immunophenotyped *ex vivo* (red shaded histograms) or after stimulation with anti-CD3 and anti-CD28 crosslinking monoclonal antibodies (α CD3/28 mAbs; light blue and green shaded histograms), as described in the Materials and Methods for five days. Then, cells were surface-stained with anti-CD3, anti-CD4, anti-PD-1, anti-TIM-3, anti-CD45RA, and anti-CCR7 mAbs prior to acquisition. Viable T cells from *ex-vivo* and day 5 acquisitions were further gated on CD3+ and CD4+ events (A, C) or CD3+ and CD8+ events (B, D), following the gating shown in Supplementary Figure 1A-B. **A-B)** Flow cytometric histogram overlays show PD-1 gating threshold (dashed line) in CD4+ (A) and CD8+ (B) T cells comparing expression *ex vivo* (red shaded histograms), after α CD3/28 stimulation (light blue shaded histograms) relative to CD4 (A) and CD8 (B) single staining controls (black histograms). **C-D)** Flow cytometric histogram overlays show TIM-3 gating threshold (dashed line) in CD4+ (C) and CD8+ (D) T cells comparing expression *ex vivo* (red shaded histograms), after α CD3/28 stimulation (green shaded histograms) relative to CD4 (C) and CD8 (D) single controls (black histograms). The gates shown were used consistently across all samples analysed in the study.



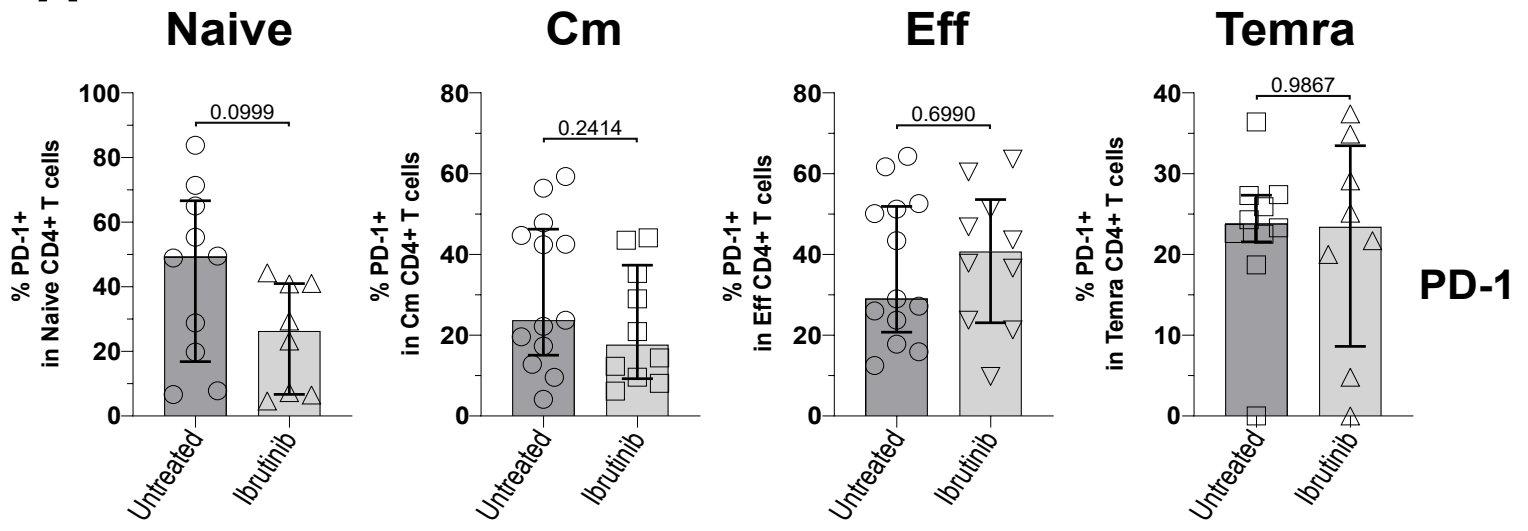
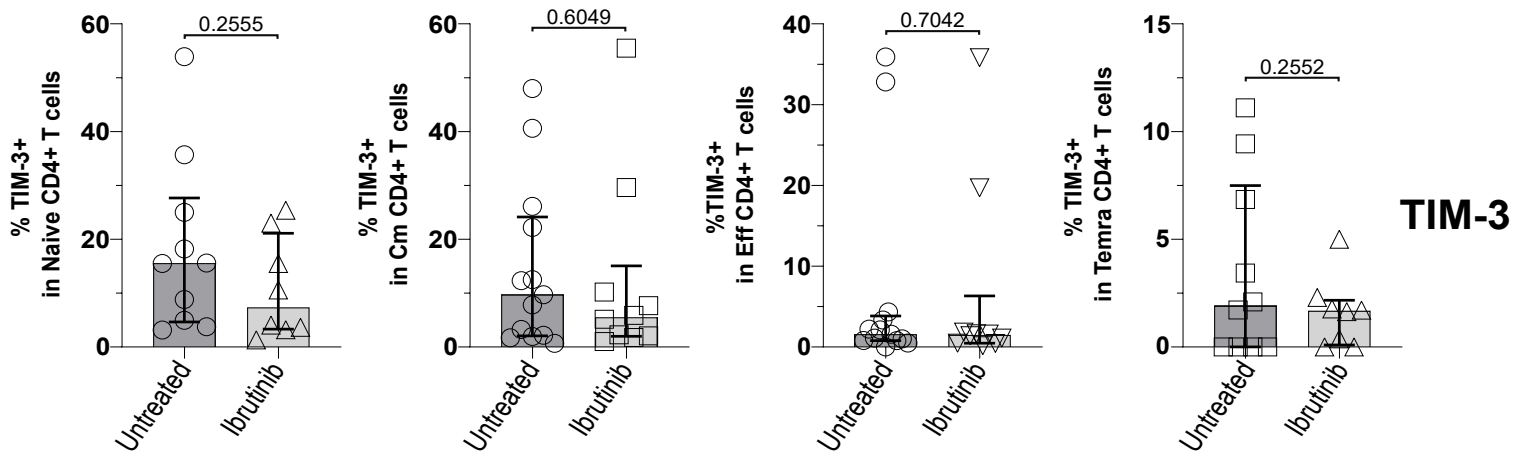
Supplementary Figure 3. Gating strategy for the intracellular cytokine staining

(ICS) analysis. PBMCs derived from CLL patients and HDs were stimulated *in vitro*, with α CD3 and α CD28 mAbs or bacterial superantigens, SEB and TSST-1 (refer to Materials and Methods for details), compared to control unstimulated cultures (Nil). After 5 days, cultured cells were briefly re-stimulated with PMA/Ionomycin (PMA/Iono) compared to control media (Media) in the presence of Brefeldin A (4 hrs). **A-B)** Cells were surface-stained with fluorescently-labelled anti-CD3, anti-CD4 and anti-CD8 mAbs; fixed; permeabilised; and intracellularly stained with IFN γ and TNF α mAbs in ICS. As done before in Reus et al. 2021, left dot plots in A show the identification of viable lymphocytes (by FSC vs SSC). Thereafter, CD3⁺ T cells were identified within gated viable lymphocytes (CD3 vs SSC; middle panels) and finally CD4⁺ and CD8⁺ T cells were identified within gated CD3⁺ T cells (right plots), accounting for the known downregulation of CD4 expression after PMA/Iono exposure. Contour plots in B depict the downstream gating analysis of IFN γ and TNF α secretion in gated CD4⁺ CD3⁺ (left panels) and CD8⁺ CD3⁺ (right panels) T cells upon stimulation with PMA/Iono, compared to the respective media negative controls (Media). **C-D)** Cells were surface stained with anti-CD19 and anti-CD3 mAbs, fixed and permeabilized, before ICS with anti-IFN γ and anti-TNF α mAbs in ICS. Left dot plots in C show the identification of viable lymphocytes (by FSC vs SSC), while right contour plots show the gating of CD19⁺ CD3⁻ B, or CLL tumour, cells within viable gated lymphocytes for the PMA/Iono and the respective control media conditions. **D)** Contour plots depict the downstream gating analysis of IFN γ and TNF α production within gated CD19⁺ CD3⁻ viable B (or CLL tumour) cells in the PMA/Iono and the respective control media conditions. For both T and B cell analyses, net cytokine secreted amounts were calculated by

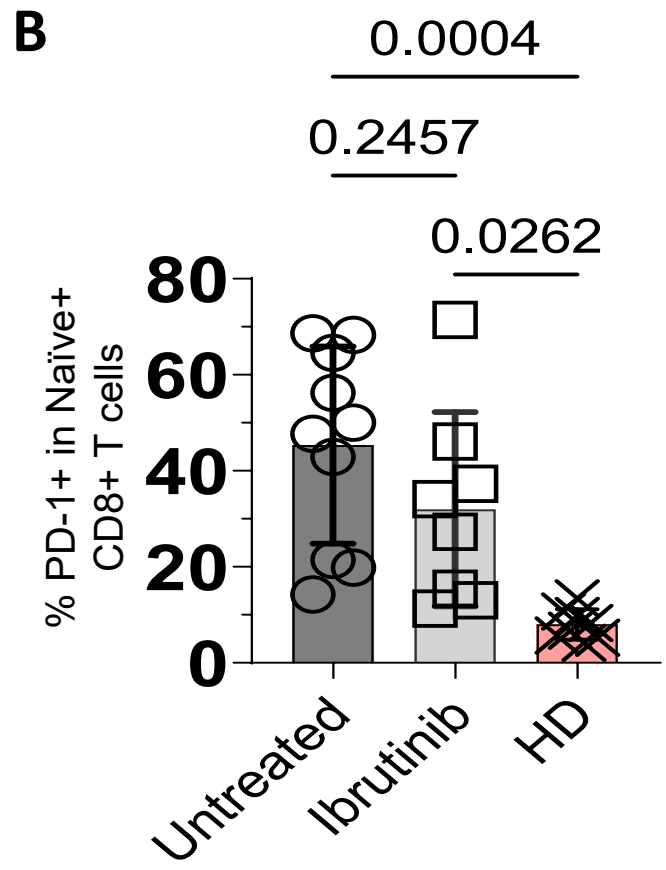
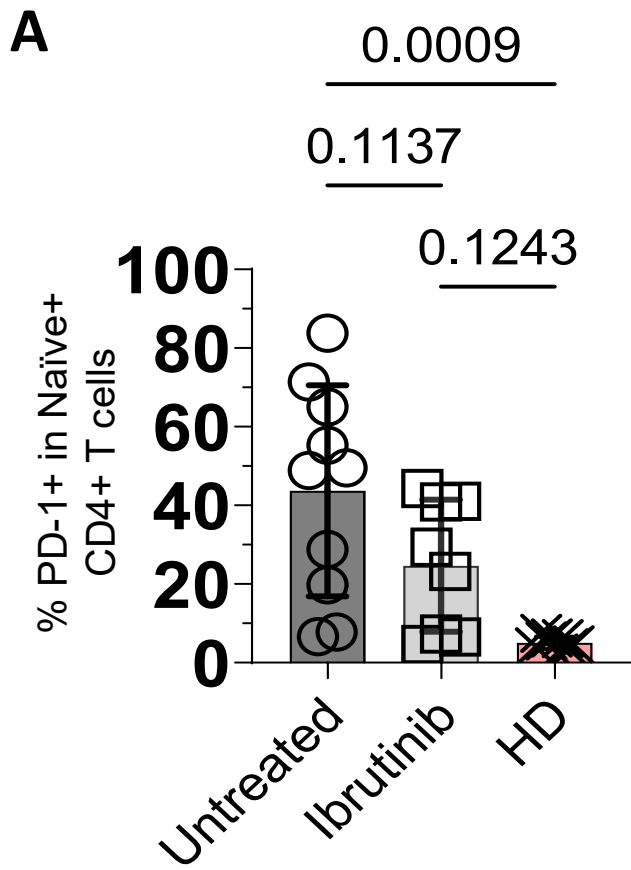
subtracting percentages detected in the control Media condition to the respective percentages in the PMA/Iono condition.

A**B****C****D**

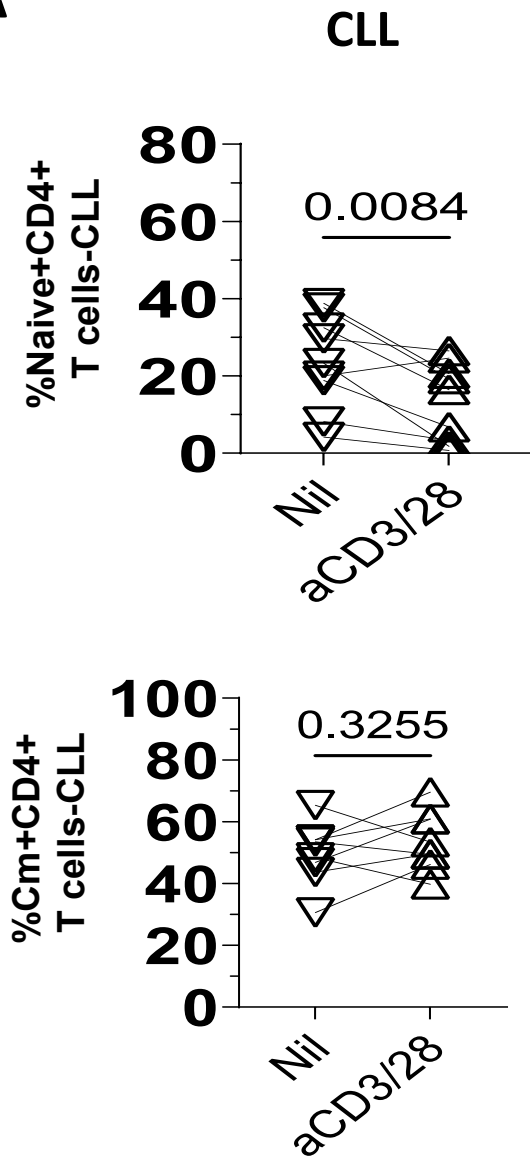
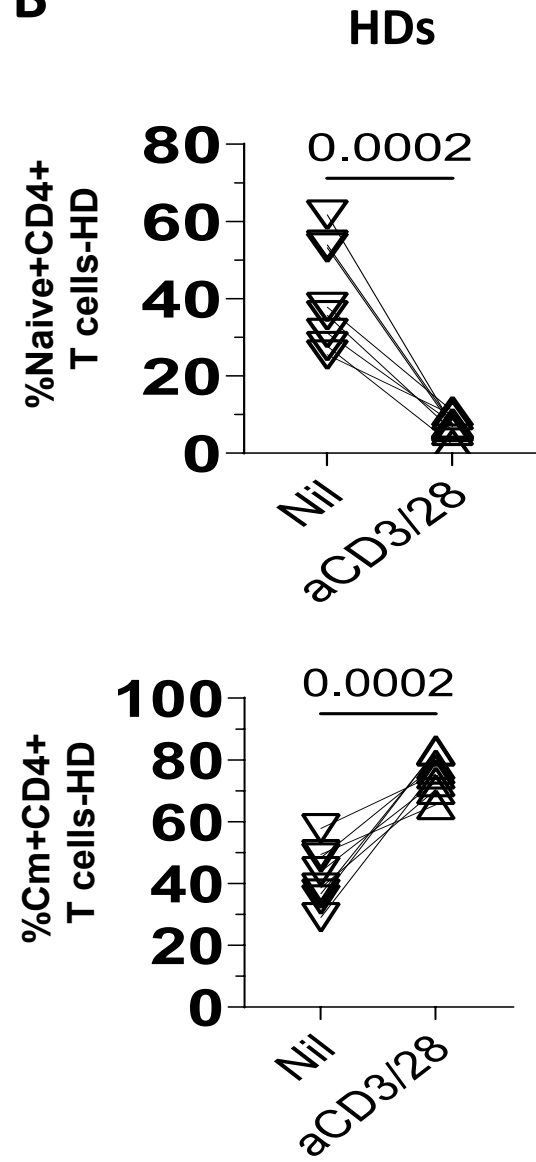
Supplementary Figure 4. Gating strategy for the detection of Foxp3⁺ CD25⁺ regulatory T (Treg) cells. PBMCs from HD, HDY-42F-014 are shown as an example. Cells were stimulated as described in Supplementary Figure 3 and the Materials and Methods for five days. Thereafter, cells were surface-stained with anti-CD4; anti-CD8; anti-CD25 and anti-CD3 mAbs and the Live/DeadTM Fixable Aqua fluorescent stain (thereafter referred to as Aqua/live stain), required in this protocol to identify viable cells given the known loss of FSC after permeabilization, and as done before (Taylor and Llewelyn, 2010). Cells were then fixed, permeabilized, and intracellularly stained with Foxp3 mAb. Samples were acquired on a BD LSR II Fortessa, using settings strictly defined and reproduced under a panel-specific rainbow profile. Data was analysed using FlowJoTM software. **A)** Dot plot illustrates the debris exclusion by defining a non-debris larger polygon gate in the FSC vs SSC plot. **B)** Viable lymphocytes within the non-debris gate are then identified by gating on cells that are negative for the Aqua/live stain vs SSC. **C)** CD4⁺ CD3⁺ (left contour plot) and CD8⁺ CD3⁺ (right contour plot) T cells are then identified within viable lymphocytes. **D)** Contour plots show the downstream gating analysis of Foxp3 against CD25 in gated CD4⁺ CD3⁺ (left) and CD8⁺ CD3⁺ (right) viable (Aqua/live⁻) T cells.

A**B**

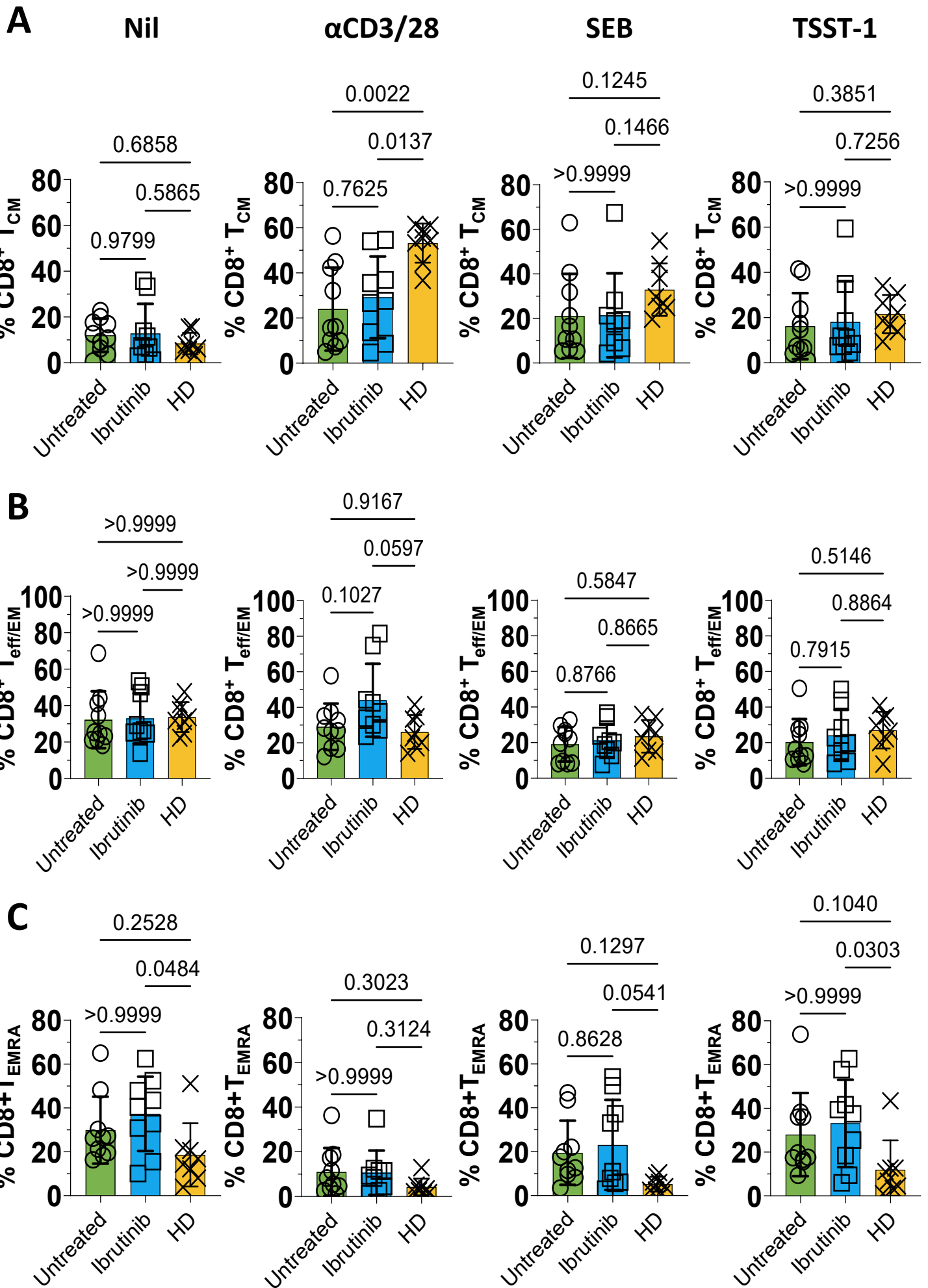
Supplementary Figure 5. CLL patients show similar levels of exhaustion-phenotype of CD4⁺ T cells, ex vivo, irrespective of ibrutinib treatment. Expression of the exhaustion markers/checkpoint receptors (PD-1 and TIM-3) was investigated within Tn (CCR7⁺CD45RA⁺) and memory subsets identified by using CCR7 and CD45RA (Cm; CCR7⁺CD45RA⁻, Eff; CCR7⁻CD45RA⁻, and Temra; CCR7⁻CD45RA⁺), after gating on viable CD3⁺ CD4⁺ T cells, as shown in Supplementary Figure 1. **A-B)** Scatter plots with medians (bars) ± IQR show percentages of PD1⁺ (A) and TIM-3⁺ (B) in gated on Naïve, Cm, Eff and TEMRA CD4⁺ T cell subsets (in order, from left to right), comparing levels of expression seen in ibrutinib-treated (n=8; light grey) and untreated (n=10; dark grey) CLL patients. Statistical significance was evaluated after assessing data normality (Shapiro-Wilk test), by using unpaired t or the Mann-Whitney U tests, respectively for parametric or non-parametric data.



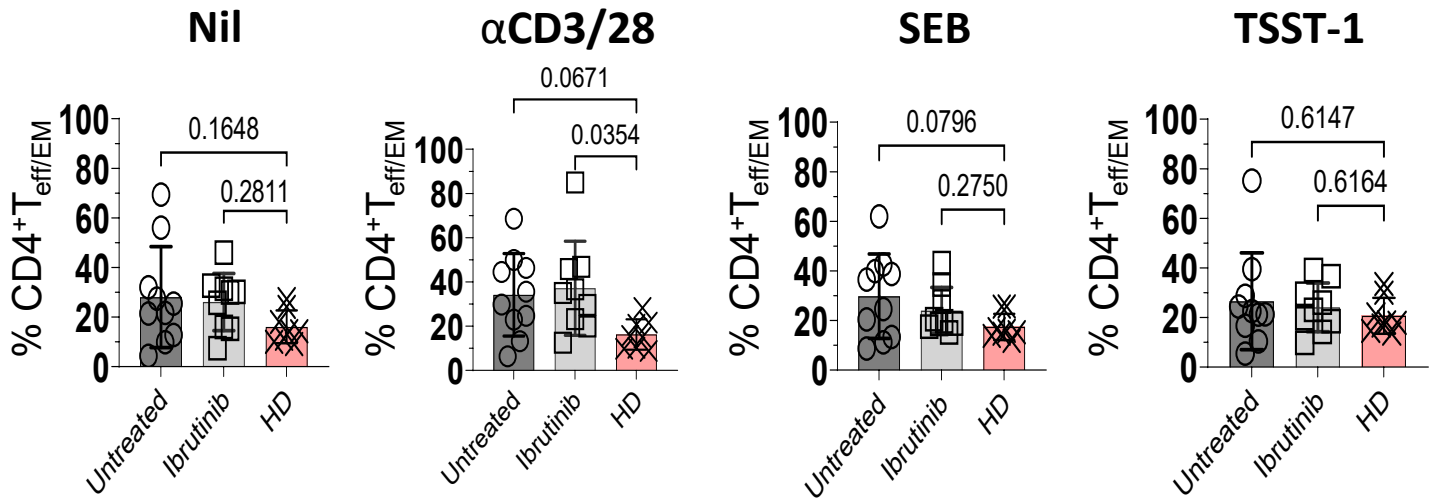
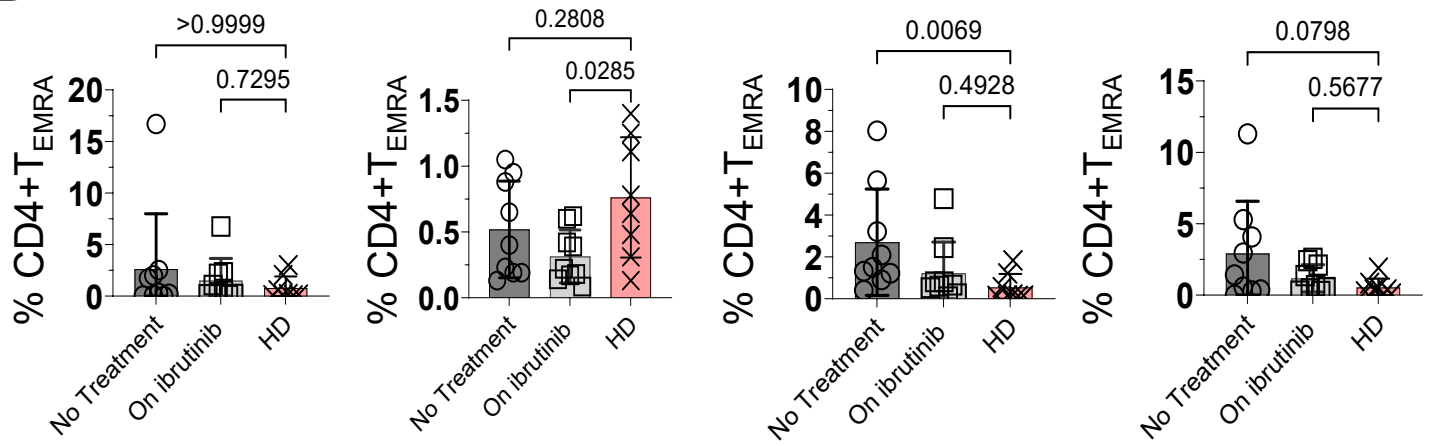
Supplementary Figure 6. Naïve-like T cells from CLL patients express significantly higher PD-1 levels than HD-derived counterparts. Fresh PBMCs were immunophenotyped *ex vivo*, comparing ibrutinib-treated (n=8; light grey) and untreated (n=10; dark grey) CLL patients to HDs (n=8; light red). Data normality was assessed with the Shapiro-Wilk test before evaluating significance with one-way ANOVA or the Kruskal-Wallis test with Tukey or Dunn's multiple comparison correction tests for parametric and nonparametric data respectively. **A-B)** Scatter plots with bars show percentages of PD-1⁺ within the naïve-like (CCR7⁺CD45RA⁺) subsets, after gating on viable CD3⁺ CD4⁺ (A) and CD3⁺ CD8⁺ (B) T cells. *P ≤ .05 and ***P ≤ .0005 were considered significant.

A**B**

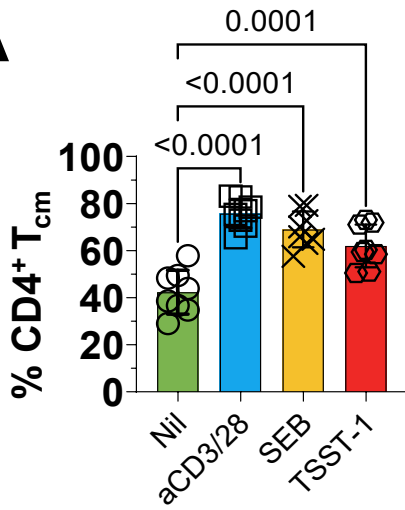
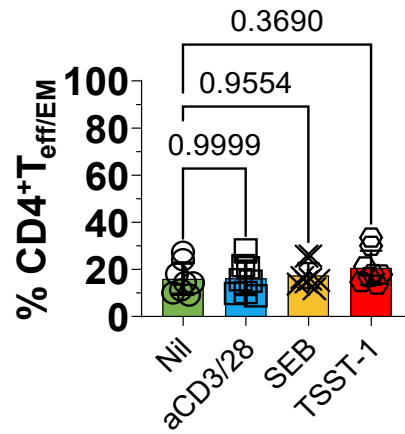
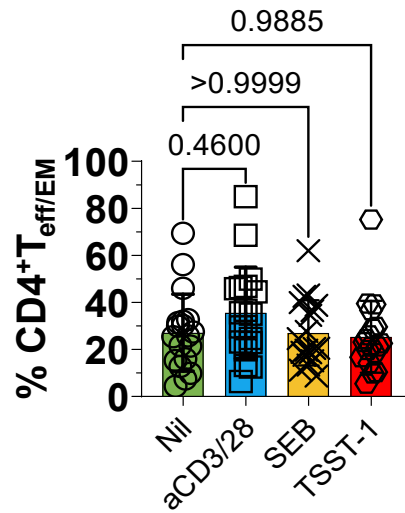
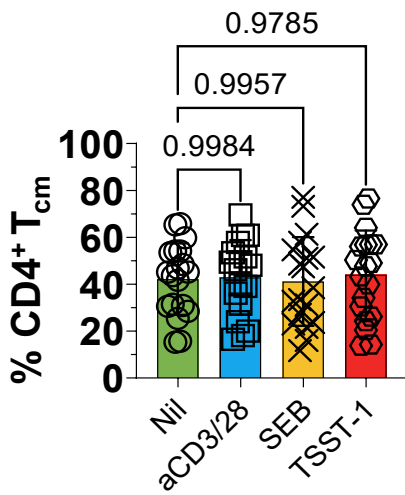
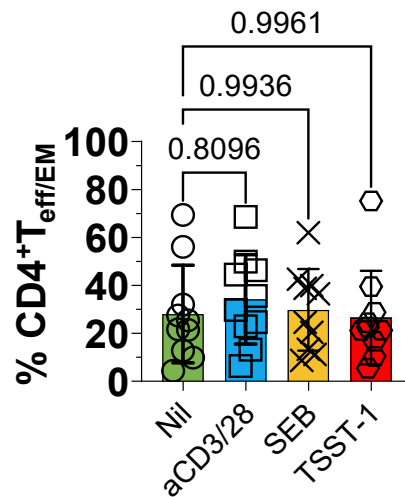
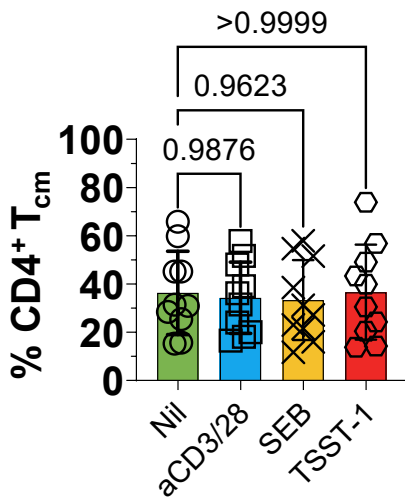
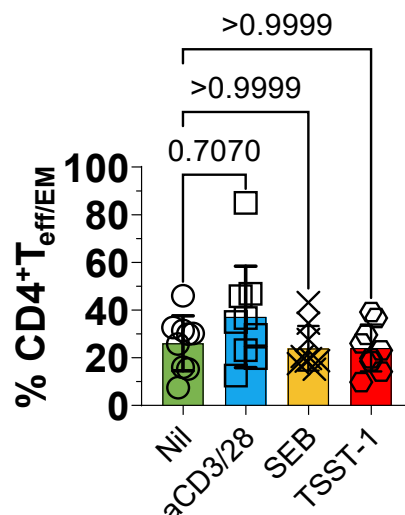
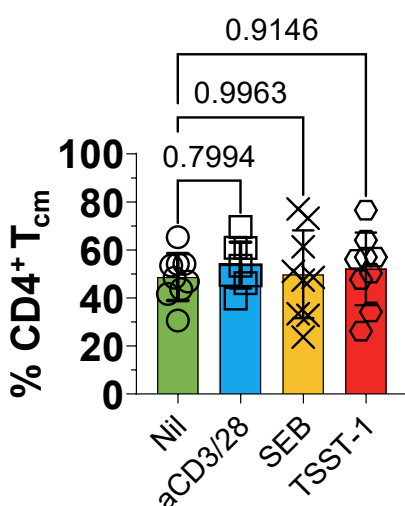
Supplementary Figure 7. The archetypal TCR-stimulation, α CD3/28 triggers stronger activation of Cm responses in cultures derived from HDs more than CLL patients, while reducing naive-like precursors. A-B) Plots show the percentage of naïve-like (top) and Cm (bottom) CD4⁺ T cells in cultures derived from CLL patients (A) and HDs (B) after α CD3/28 stimulation (upward triangles) compared to unstimulated controls (Nil, downward triangles). Statistical significance was determined using paired t tests, after assessing the data normality with Shapiro-Wilk tests. **, $P \leq .005$ and ***, $P \leq .0005$ were considered statistically significant.



Supplementary Figure 8. CD8⁺ T cell memory subset profiles in untreated and ibrutinib-treated CLL patients compared to HDs, following stimulations with α CD3/28 and SA SAgS. Fresh PBMCs from ibrutinib-treated (n=9, blue bars) and untreated (n=10, green bars) CLL patients or HDs (n=8, yellow bars) were stimulated *in vitro* for 5 days with α CD3/28 mAbs or staphylococcal superantigens, SEB and TSST-1 (refer to Materials and Methods for details), compared to unstimulated control cultures (Nil). Thereafter, cells were harvested, and surface stained, with anti-CD3, anti-CD8, anti-CD45RA, and anti-CCR7 mAbs. **A-C)** Scatter plots with bars show the percentages of Cm (A; CCR7⁺CD45RA⁻), Eff/Em (B; CCR7⁻CD45RA⁻) and Temra (C; CCR7⁻CD45RA⁺) in gated CD8⁺ T cells in the three experimental groups described above, for each of the culture conditions specified at the top. After assessment of data normality with Shapiro-Wilk tests, statistical significance was determined using the one-way ANOVA or the Kruskal-Wallis tests with Tukey or Dunn's multiple comparison correction tests for parametric and nonparametric data respectively. *P \leq .05 and **p \leq 0.005 were considered significant. As for CD4⁺ T cells (see Figure 2B), significantly higher accumulation of Cm CD8⁺ T cells was detected in α CD3/28 cultures derived from HDs rather than CLL patients. In contrast, overall, TEff/Em and Temra CD8⁺ T cell responses were not significantly impacted in the same cultures.

A**Gated on CD4⁺ T cells****B**

Supplementary Figure 9. CD4⁺ TEff/Em and Temra subset profiles in untreated and ibrutinib-treated CLL patients compared to HDs, following stimulations with α CD3/28 and SA SAg. Fresh PBMCs from ibrutinib-treated (n=9, light grey) and untreated (n=10, dark grey) CLL patients or HDs (n=8, light red) were stimulated *in vitro* for 5 days with α CD3/28 stimulatory mAbs or staphylococcal superantigens, SEB and TSST-1 (refer to Materials and Methods for details), compared to unstimulated control cultures (Nil). Thereafter, cells were harvested, and surface stained, with anti-CD3, anti-CD4, anti-CD45RA, and anti-CCR7 mAbs. **A-B)** Scatter plots with bars show the percentages of Eff/Em (A; CCR7⁻CD45RA⁻) and Temra (B; CCR7⁻CD45RA⁺) in gated CD4⁺ T cells in the three experimental groups described above, for each of the culture conditions specified at the top. After assessment of data normality with Shapiro-Wilk tests, statistical significance was determined by using the one-way ANOVA or the Kruskal-Wallis tests with Tukey or Dunn's multiple comparison correction tests for parametric and nonparametric data respectively. *P \leq .05 and **p \leq 0.005 were considered significant. Unlike the case of Cm CD4⁺ T cells (see Figure 2B), TEff/Em and Terma weakly changed in the same cultures representing relatively minor fractions by day 5.

A**B****Healthy Donors (HDs)****CLL patients (pooled)****Untreated CLL patients****Ibrutinib-treated CLL patients**

Supplementary Figure 10. Unlike CLL counterparts, HD-derived cultures show significant accumulation of Cm, but not Eff/Em CD4⁺ T cells upon α CD3/28, SEB and TSST-1 stimulations. Fresh PBMCs were cultured for 5 days and then immunophenotyped as described in Figure 2 and Materials and Methods. **A-B)** Scatter plots with bars show the percentages of Cm (A) and Eff/Em (B) in gated CD4⁺ T cells from HDs (n=8, top row), pooled CLL groups (n=19, second row), untreated (n=10, third row), and ibrutinib-treated (n=9, bottom row) CLL patients after α CD3/28 (blue bars), SEB (yellow bars), TSST-1 (red bars), compared to unstimulated controls (Nil) cultures (green bars). After evaluating data normality with Shapiro-Wilk tests, statistical significance was determined by using the one-way ANOVA or the Kruskal-Wallis tests with Tukey or Dunn's multiple comparison correction tests for parametric and nonparametric data respectively. ***p \leq 0.0005 was considered significant.

Total CD4+ T cells

Controls

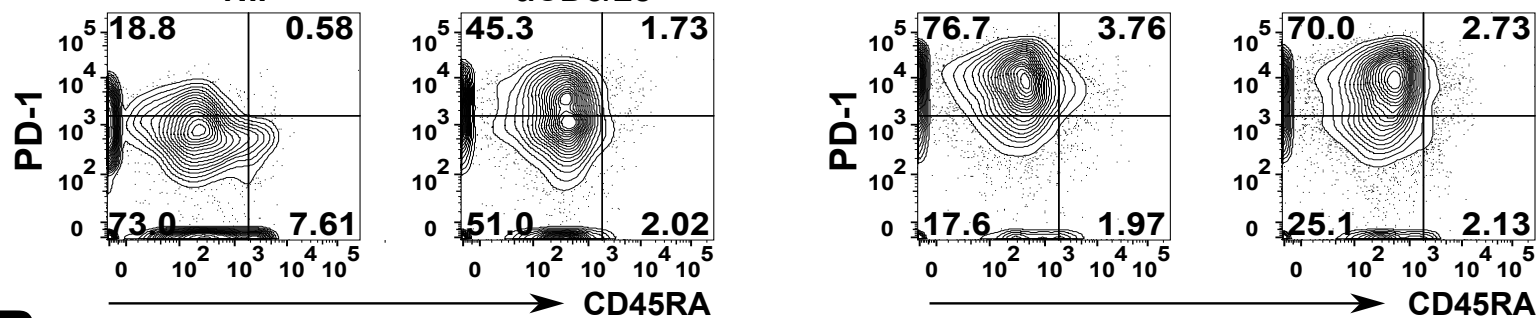
Superantigens

Nil

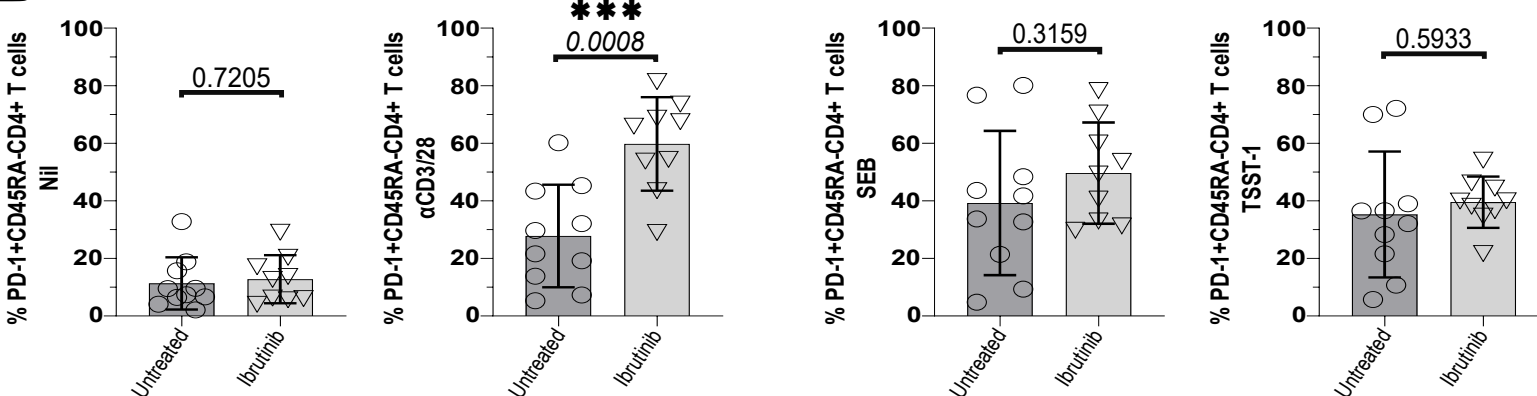
α CD3/28

SEB

TSST-1



B



C

CD4+ T cell memory subsets

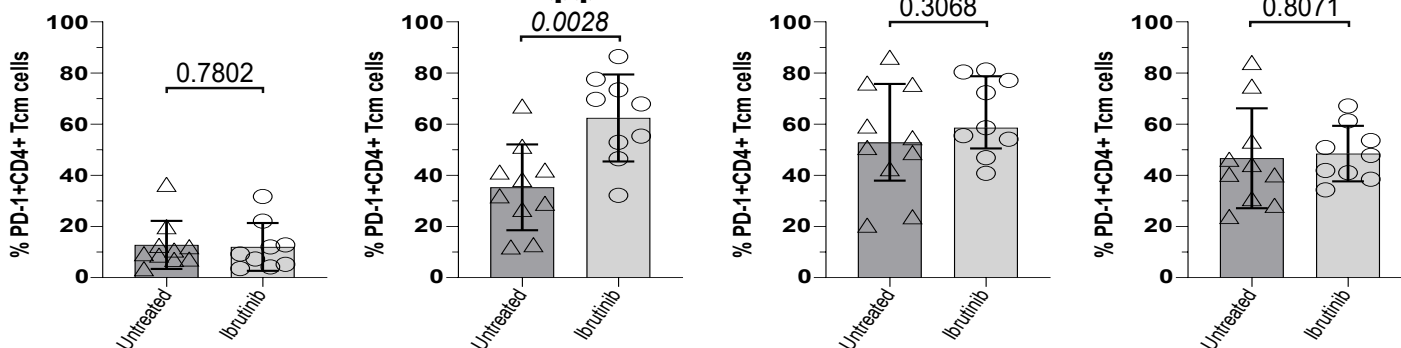
Nil

α CD3/28

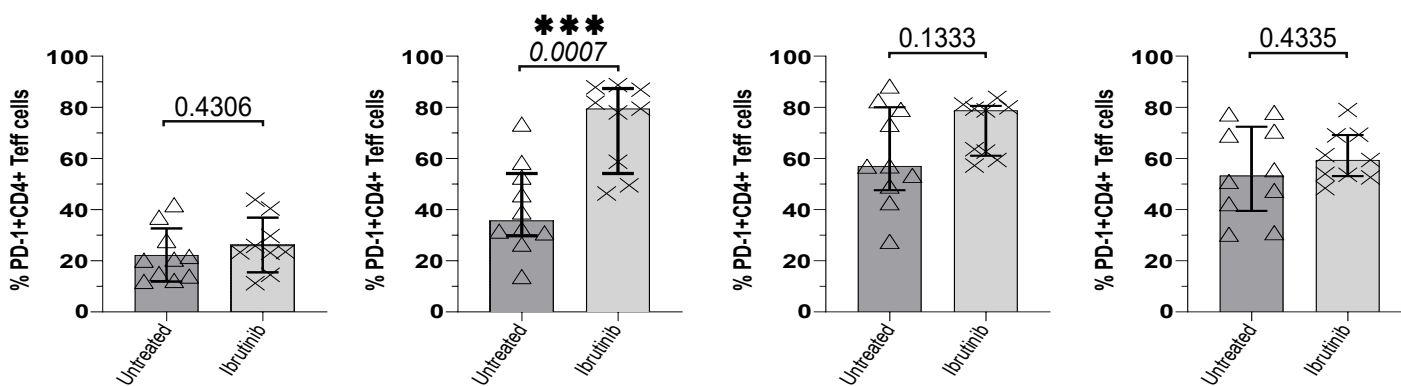
SEB

TSST-1

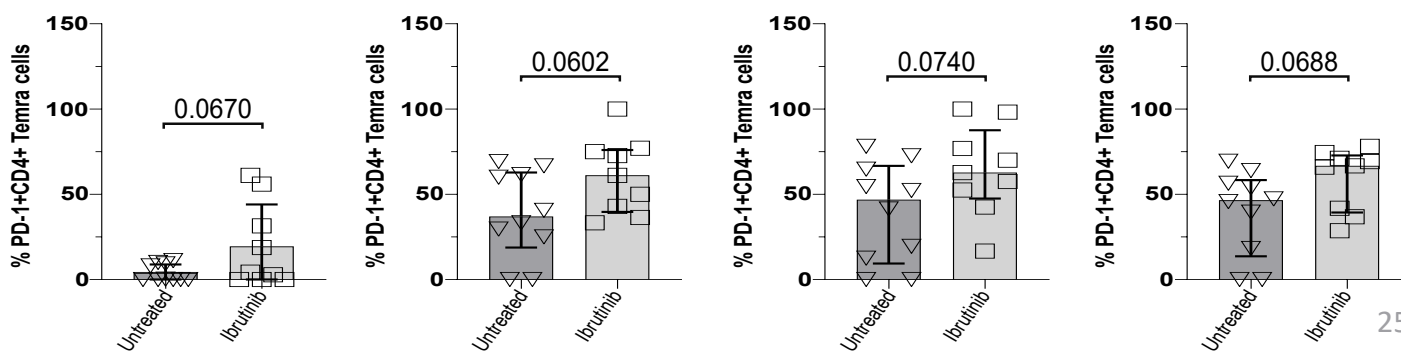
Cm



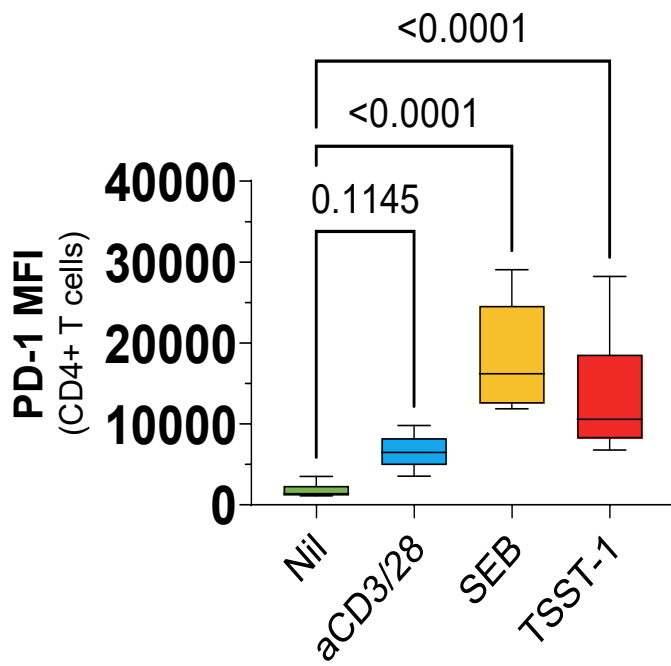
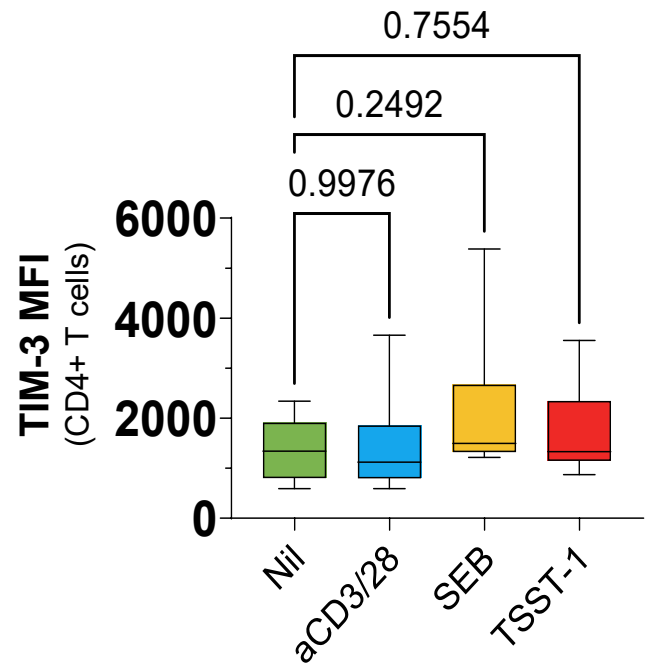
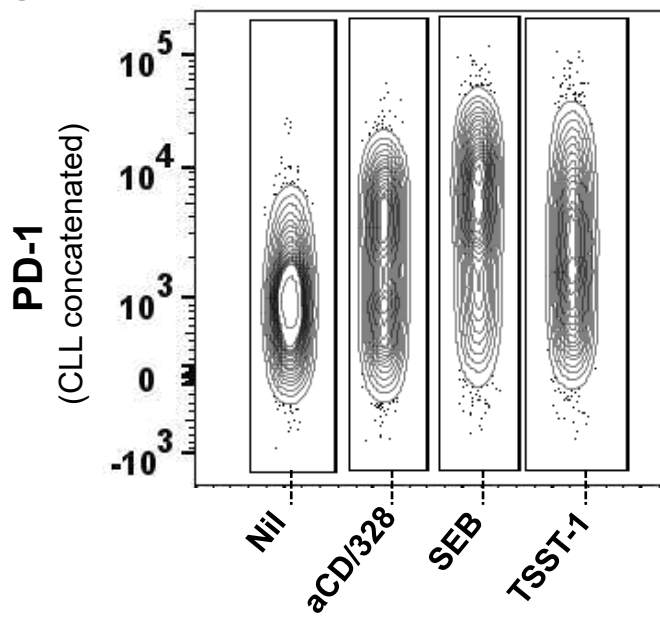
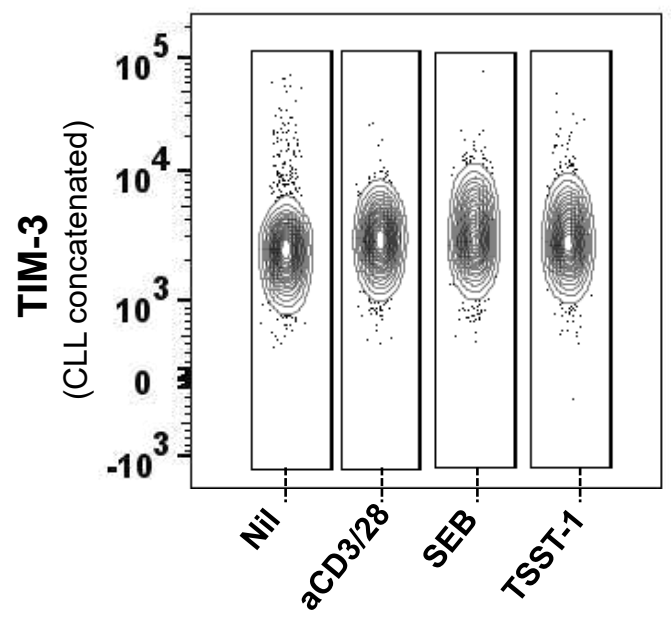
Eff



Temra



Supplementary Figure 11. Central and effector memory CD4⁺ T cells show elevated PD-1 expression upon α CD3/28 stimulation, preferentially in cultures derived from ibrutinib-treated CLL patients. PBMCs of ibrutinib-treated and untreated CLL patients were stimulated *in vitro*, as described in Supplementary Figure 3 and the Materials and Methods for five days. Thereafter, cells were harvested, and surface-stained with anti-CD3, anti-CD4, anti-CD45RA, anti-CCR7 and anti-PD-1 mAbs. Viable CD4⁺ T cells were identified as shown in Supplementary Figure 1. **A)** Example contour plots from an untreated CLL patient (P635) show surface expression of CD45RA (x-axis) and PD-1 (y-axis) within viable CD4⁺ T cells after 5d-stimulation with superantigens, SEB, and TSST-1 (left) compared to control conditions (right panels): α CD3/28 and unstimulated (Nil) cultures. **B)** Scatter plots with bars show the median percentages \pm IQR of PD-1⁺ cells within total memory CD45RA⁻ CD4⁺ T cells detected in the above cultures derived from either ibrutinib-treated (n=9) or untreated (n=10) CLL patients. **C)** Scatter plots with bars depict median percentages \pm IQR of PD-1⁺ cells within Cm (top row), Eff (middle row) and Temra (bottom row) CD4⁺ T cell subsets in the same cultures comparing ibrutinib-treated (n=9) and untreated (n=10) CLL patients. Statistical significance was determined after evaluating data normality with Shapiro-Wilk tests, by using the unpaired t or the Mann-Whitney U tests, respectively for parametric or non-parametric data. **P \leq 0.005 and ***P \leq 0.0005.

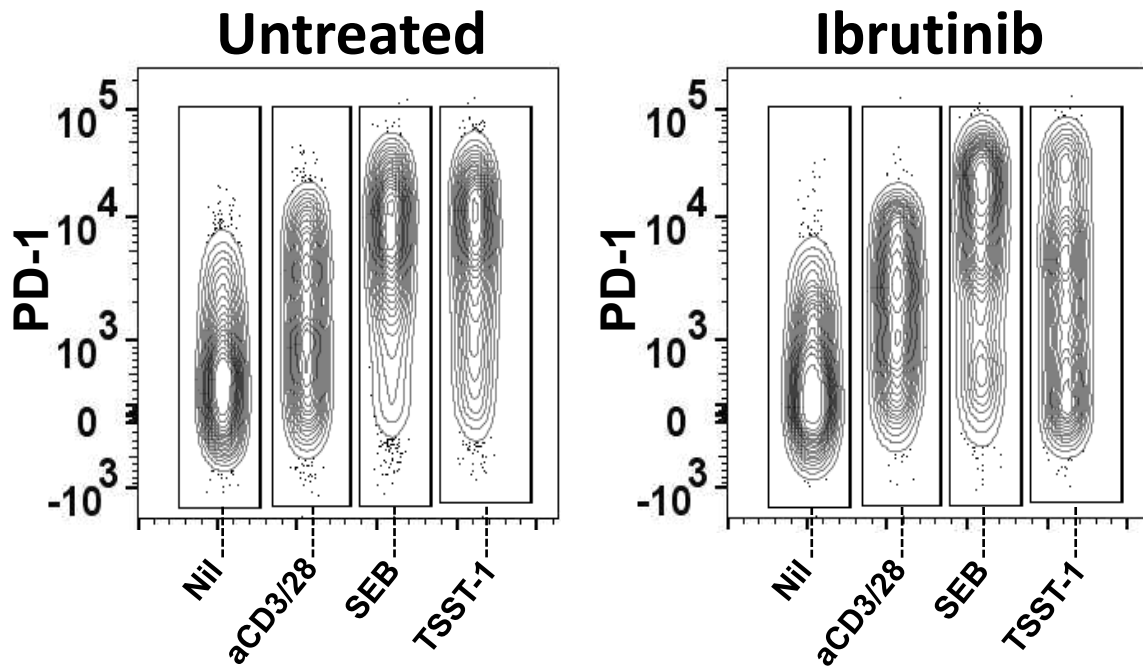
A**B****C****D**

Sample	Mean	SD
Nil	1365	138
αCD3/28	3480	130
SEB	7824	136
TSST-1	5502	192

Sample	Mean	SD
Nil	3145	147
αCD3/28	3136	50.6
SEB	3726	69.3
TSST-1	3317	72

Supplementary Figure 12. SEB and TSST-1 more than α CD3/28 stimulation promote higher levels of PD-1 and TIM-3 expression in CD4⁺ T cells when compared to unstimulated controls. PBMCs of CLL patients were stimulated *in vitro*, as described in Supplementary Figure 3 and the Materials and Methods for five days. Thereafter, cells were harvested, and surface-stained with anti-CD3, anti-CD4, anti-CD45RA, anti-CCR7, anti-PD-1 and anti-TIM-3 mAbs. Viable CD4⁺ T cells were identified as shown in Supplementary Figure 1. After acquisition, for each experimental culture condition, 7200 events from individual CLL patient samples (n=17) were concatenated using the FlowJO™ version 10. Thereafter, a concatenated file of the four concatenated culture conditions was created and further analysed in comparison to statistically pooled data. **A-B)** Box plots show the MFI of PD-1 (A) and TIM-3 (B) expression in total CD4⁺ CD3⁺ T cells in statistically pooled analyses (median \pm min & max range) of cultures with α CD3/28 (blue bars), SEB (yellow bars) and TSST-1 (red bars) relative to Nil cultures (green bars) derived from CLL patients. Statistical significance was determined after evaluating the data normality with Shapiro-Wilk test using the one-way ANOVA test or the Kruskal-Wallis test with Tukey or Dunn's multiple comparison correction tests for parametric and nonparametric data respectively. ***, P \leq .0005 was considered statistically significant. **C-D)** Concatenation flow cytometry plots show the concatenated MFIs of PD-1 (C) and TIM-3 (D) in total CD4⁺ CD3⁺ T cells, for each culture condition tested: α CD3/28, SEB and TSST-1 stimulation, compared to unstimulated control cultures (Nil). The colour-coded tables show the mean and SD of each concatenated parameter for the stimulations mentioned above. Violet shades indicate low to high levels of expression, respectively from light to dark tones. Overall, concatenated analyses agreed with statistically pooled data analyses.

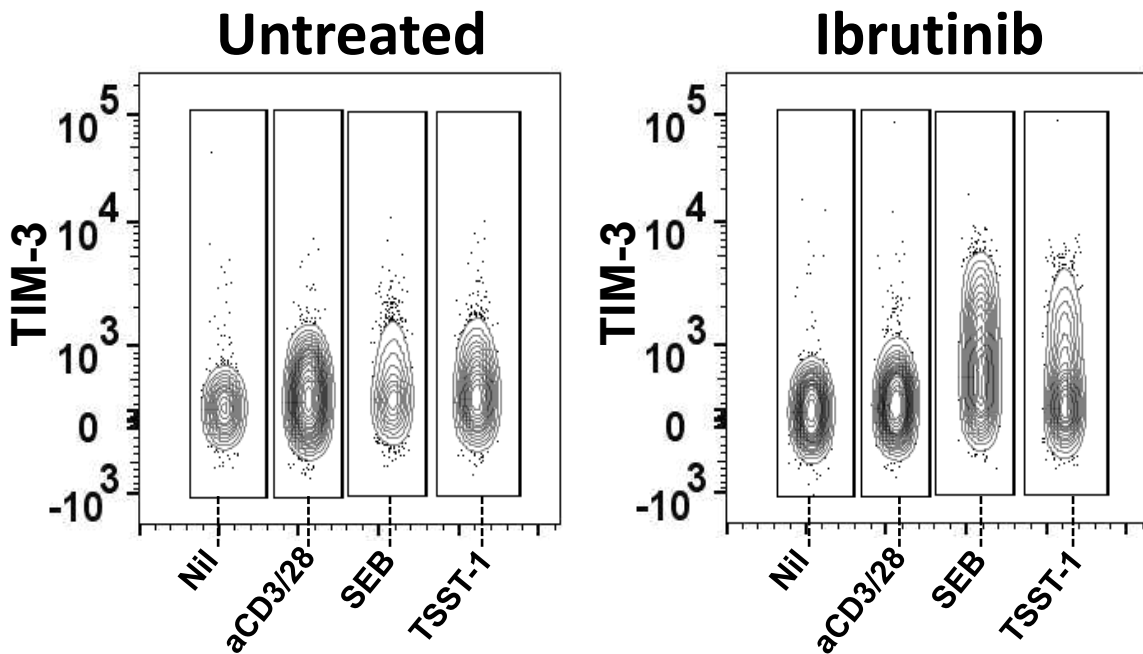
A



Sample	Mean	SD
Nil	614	189
α CD3/28	2361	170
SEB	8409	183
TSST-1	4833	161

Sample	Mean	SD
Nil	345	239
α CD3/28	3541	116
SEB	5858	124
TSST-1	2859	184

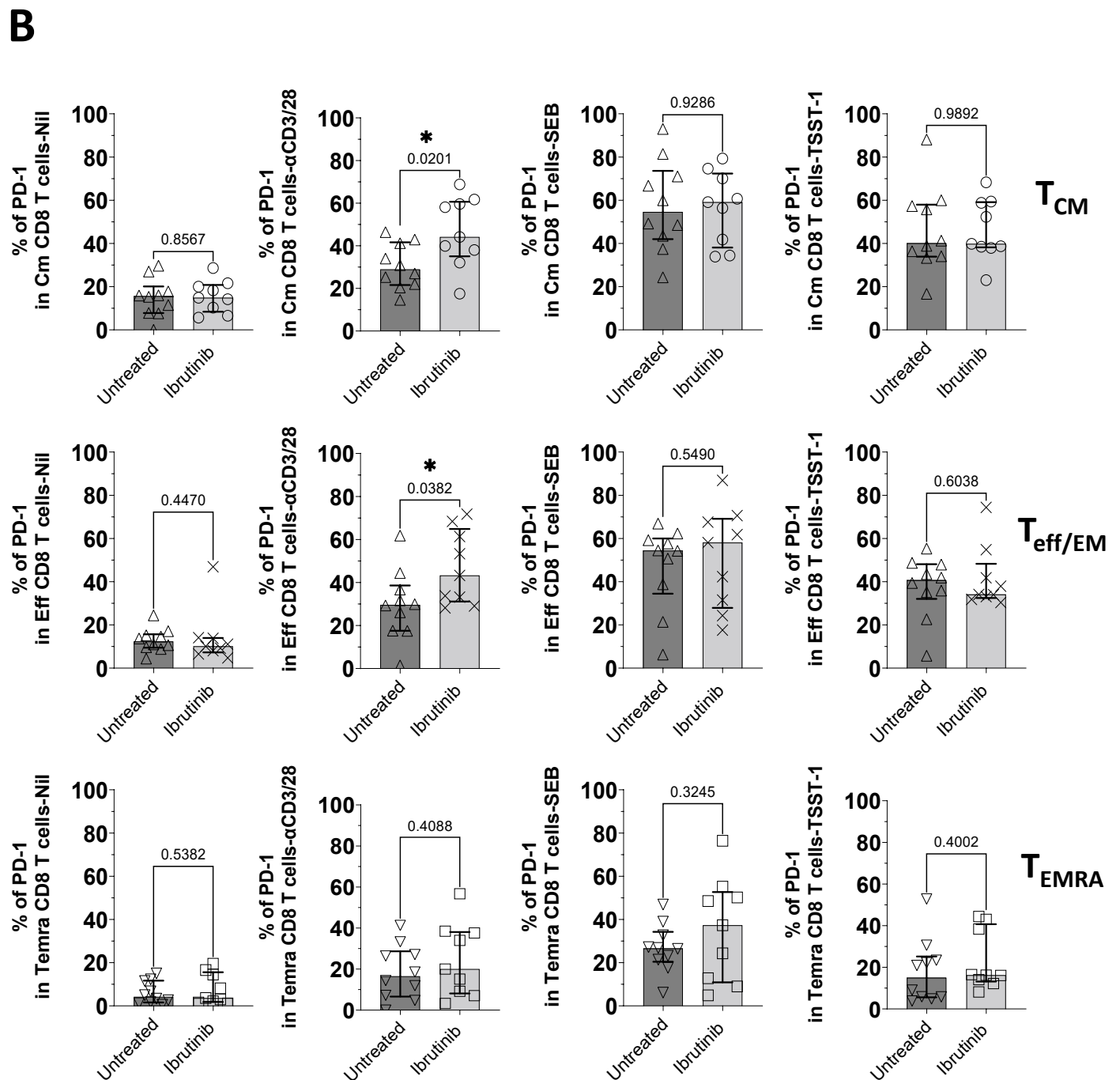
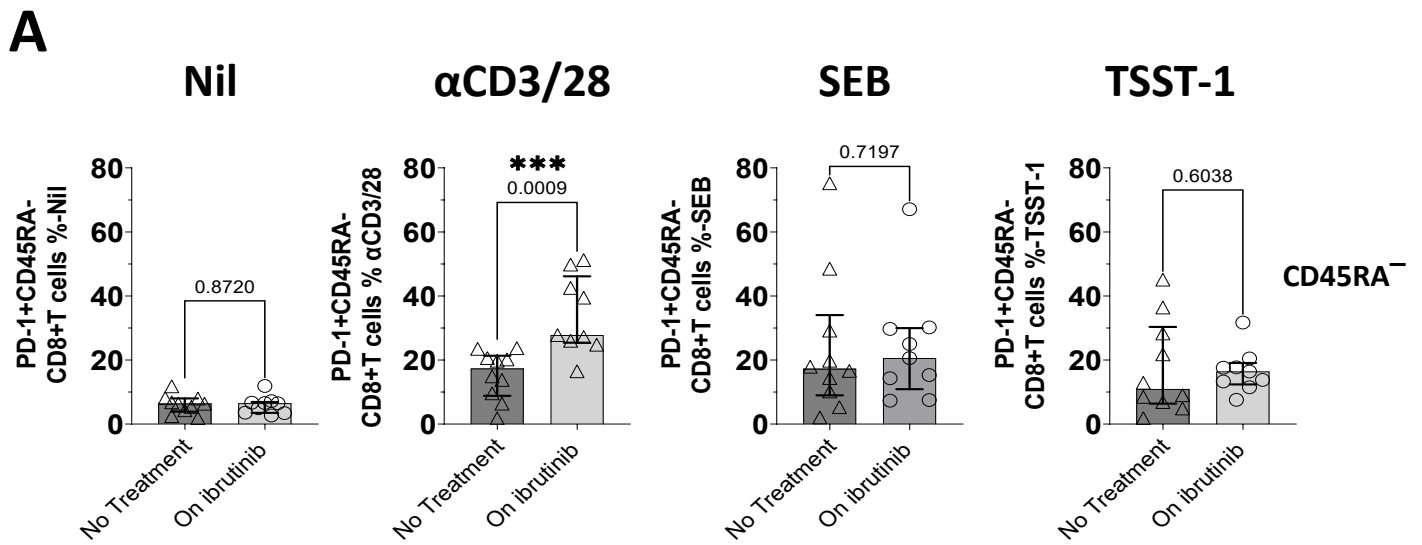
B



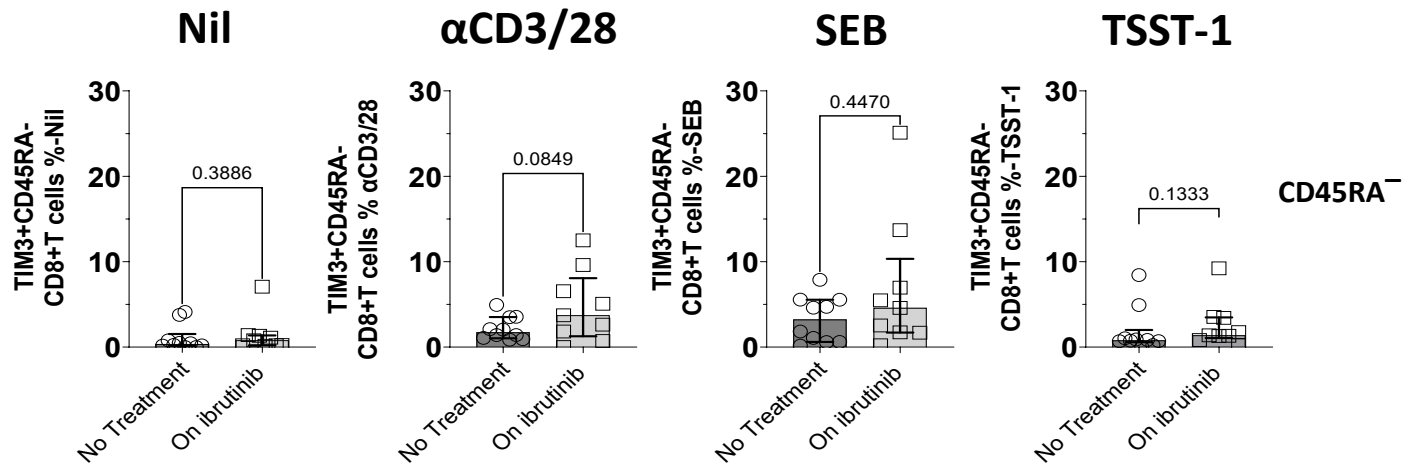
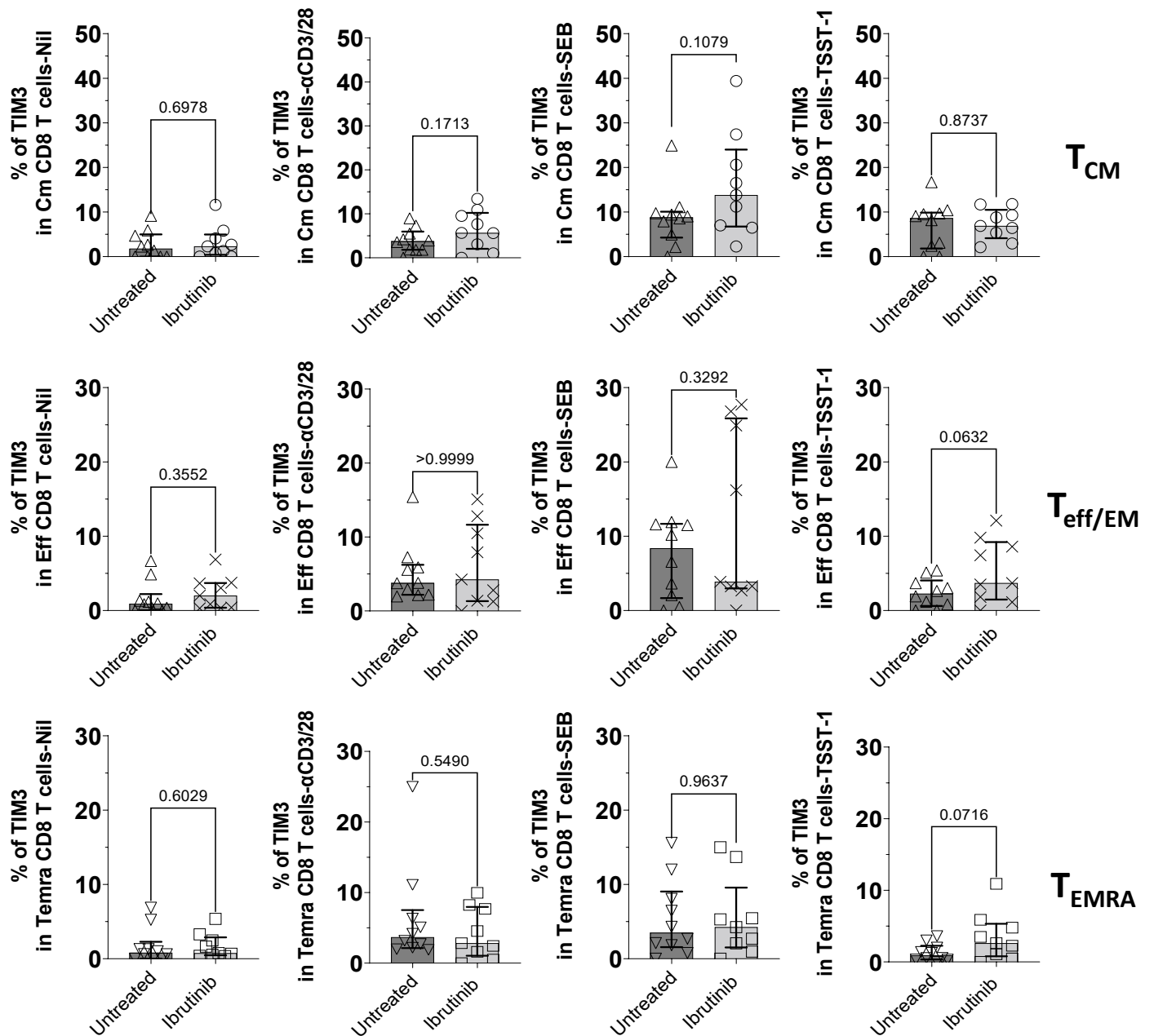
Sample	Mean	SD
Nil	245	707
α CD3/28	340	156
SEB	504	166
TSST-1	423	127

Sample	Mean	SD
Nil	162	574
α CD3/28	278	411
SEB	1105	116
TSST-1	738	228

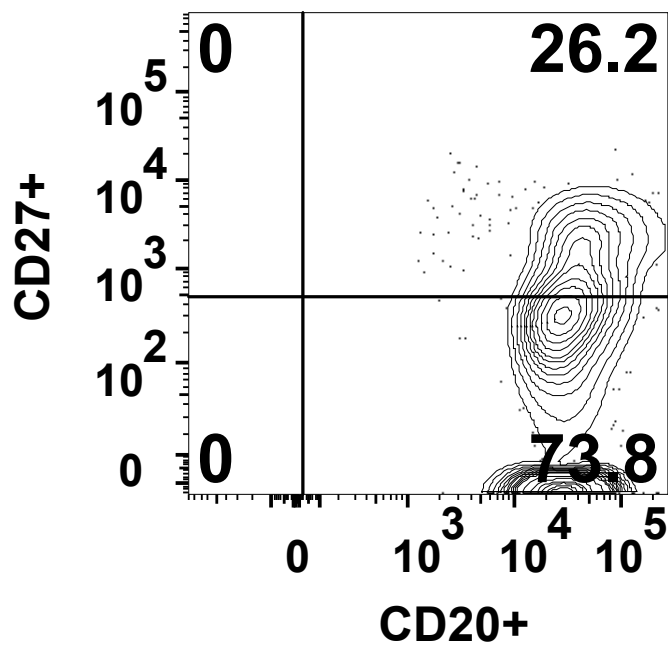
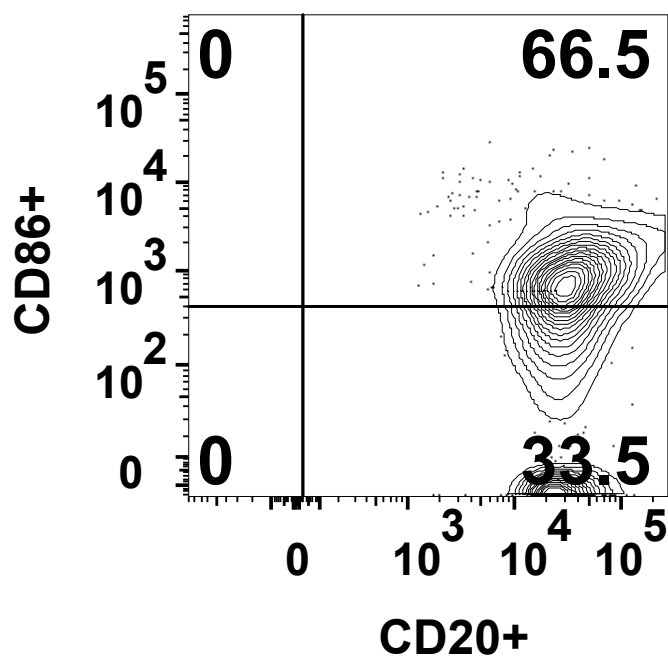
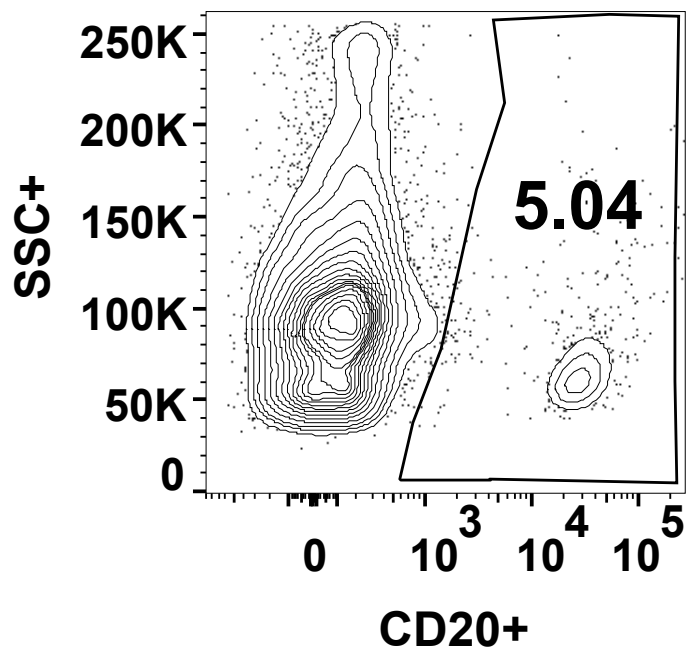
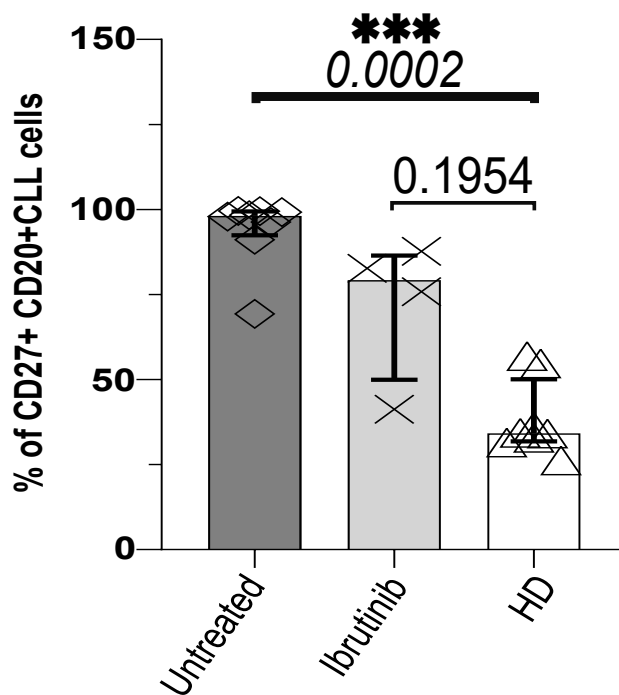
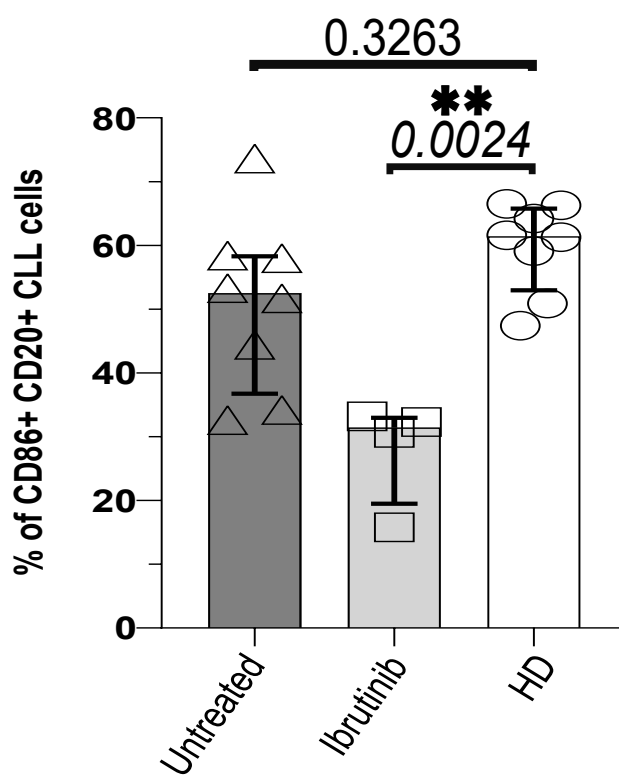
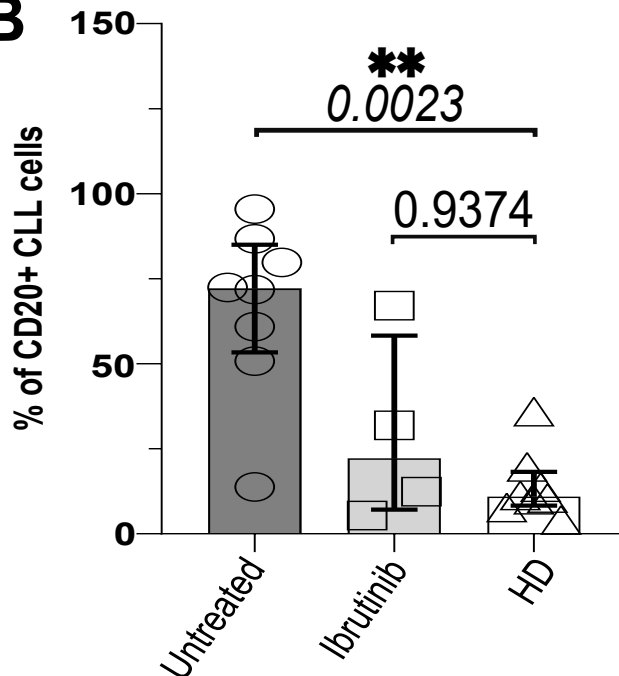
Supplementary Figure 13. Concatenated MFI analyses reveal higher TIM-3 expression on CD4⁺ T cells from ibrutinib-treated rather than untreated CLL patients, upon SEB and TSST-1 stimulations. PBMCs of CLL patients were stimulated and then immunophenotyped as described in Supplementary Figure 12. After acquisition, for each experimental culture condition, 8000 events from individual untreated (n=8) and ibrutinib-treated (n=9) CLL patient samples were concatenated using the FlowJO™ version 10. Thereafter, for each patient sub-cohort, concatenated files of the four concatenated culture conditions were created and further analysed. **A-B)** Concatenation flow cytometry plots of untreated (left panels) and ibrutinib-treated (right panels) CLL patients show the concatenated MFIs of PD-1 (A) and TIM-3 (B) in total CD4⁺ CD3⁺ T cells, for each culture condition tested: αCD3/28 (blue), SEB (yellow) and TSST-1 (red) stimulation, compared to unstimulated control cultures (Nil; green). The colour-coded tables show the mean and SD of each concatenated parameter for the stimulations mentioned above. Violet shades indicate low to high levels of expression, respectively from light to dark tones.



Supplementary Figure 14. Central and effector memory CD8⁺ T cells show increased PD-1 expression upon α CD3/28 stimulation, preferentially in cultures derived from ibrutinib-treated CLL patients. PBMCs of ibrutinib-treated and untreated CLL patients were stimulated *in vitro*, as described in Supplementary Figure 3 and the Materials and Methods for five days. Thereafter, cells were harvested, and surface stained, with anti-CD3, anti-CD8, anti-CD45RA, anti-CCR7 and anti-PD-1 mAbs. Viable CD8⁺ T cells were identified as shown in Supplementary Figure 1. **A)** Scatter plots with bars show the median percentages \pm IQR of PD-1⁺ cells within total memory CD45RA⁻ CD8⁺ T cells detected in the above cultures derived from either ibrutinib-treated (n=9, light grey) or untreated (n=10, dark grey) CLL patients. **B)** Scatter plots with bars depict median percentages \pm IQR of PD-1⁺ cells within Cm (top row), Eff/Em (middle row) and Temra (bottom row) CD8⁺ T cell subsets in the same cultures comparing ibrutinib-treated (n=9, light grey) and untreated (n=10, dark grey) CLL patients. Statistical significance was determined after evaluating data normality with Shapiro-Wilk tests, by using the unpaired t or the Mann-Whitney U tests, respectively for parametric or non-parametric data. *P \leq 0.05 and ***P \leq 0.0005.

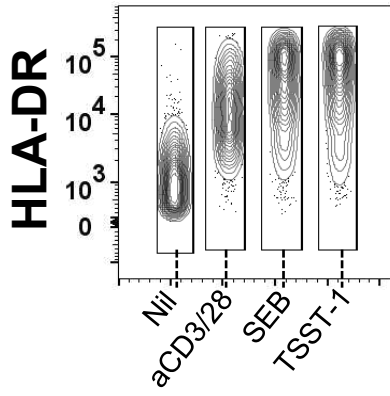
A**B**

Supplementary Figure 15. Ibrutinib-treated and untreated CLL patients show similar TIM-3 percentage expression in CD8⁺ T cell memory subsets after α CD3/28 and SA SAg stimulations. PBMCs of ibrutinib-treated and untreated CLL patients were stimulated *in vitro*, as described in Supplementary Figure 3. At day 5 of culture, cells were harvested and surface stained with anti-CD3, anti-CD8, anti-CD45RA, anti-CCR7 and anti-TIM-3, as indicated in the Materials and Methods section. **A)** Scatter plots with bars show the percentage of TIM-3⁺ within total memory CD45RA⁻ CD8⁺ T cells detected after 5d-stimulation with α CD3/28, SEB, and TSST-1 compared to unstimulated control cultures (Nil) from either ibrutinib-treated (n=9, light grey) or untreated (n=10, dark grey) CLL patients. **B)** Scatter plots with bars demonstrate the percentage of TIM-3⁺ cells within Cm (top row), Eff/Em (middle row) and Temra (bottom row) CD8⁺ T cell subsets in the same cultures comparing ibrutinib-treated (n=9, light grey) and untreated (n=10, dark grey) CLL patients. Statistical significance was determined after evaluating the data normality with Shapiro-Wilk test using the unpaired T test or the Mann-Whitney test for parametric or non-parametric data respectively.

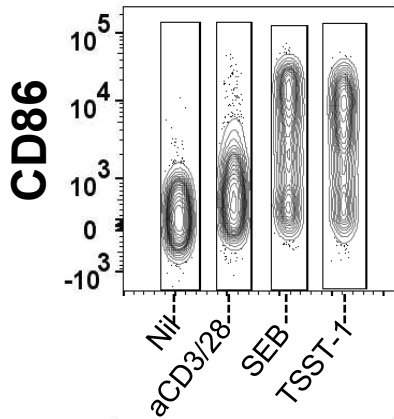
A**B**

Supplementary Figure 16. Expression of CD86 and CD27 in CD20⁺ B cells comparing CLL patient sub-cohorts to HDs. A) Example contour plots from HDY-74M-017 show: (i) gated CLL tumour cells, in the CD20⁺ vs SSC top contour plot); (ii) expression of B cell co-stimulation marker, CD86 on gated CD20⁺ B/CLL cells (middle contour plot); and (iii) expression of the memory marker, CD27 on gated CD20⁺ B/CLL cells (bottom contour plot). **B)** Scatter plots with bars show median percentages \pm IQR of CD20⁺ B (if from HDs) or CLL tumour cells (top panel), and median percentages \pm IQR of CD86⁺ (middle panel) and CD27⁺ (bottom panel) cells, within gated CD20⁺ B/CLL tumour cells, comparing levels across ibrutinib-treated (n=4) and untreated (n=8) CLL patients and HDs (n=8), *ex-vivo*. Statistical significance was determined after assessing data normality with the Shapiro-Wilk test, by using the one-way ANOVA test or the Kruskal-Wallis test with Dunnett or Dunn's multiple comparison correction tests for parametric or non-parametric data, respectively. *P \leq 0.05; **P \leq 0.005; ***P \leq 0.0005.

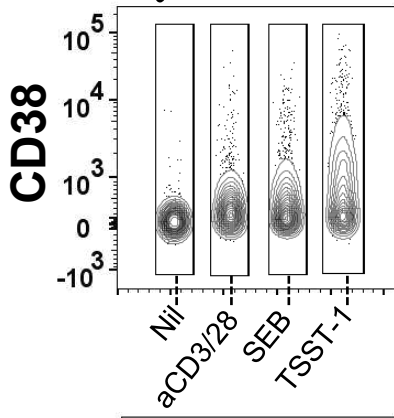
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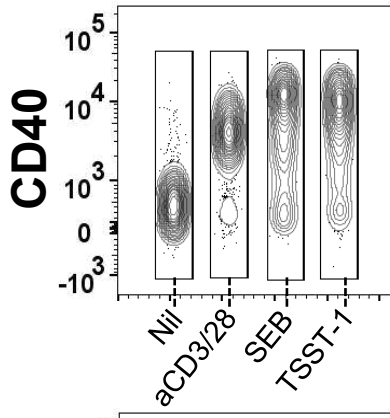
HLA-DR concatenated MFIs		
Sample	Mean	SD
Nil	1867	253
αCD3/28	24821	144
SEB	57983	100
TSST-1	65140	96.9



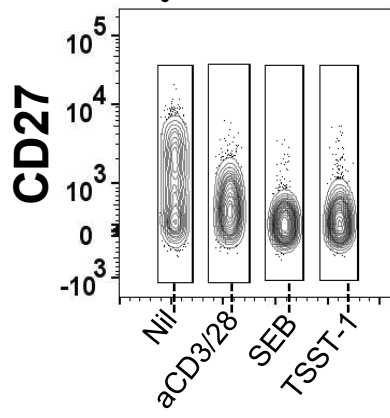
CD86 concatenated MFIs		
Sample	Mean	SD
Nil	243	230
αCD3/28	1250	252
SEB	7479	129
TSST-1	6371	122



CD38 concatenated MFIs		
Sample	Mean	SD
Nil	79.8	299
αCD3/28	465	386
SEB	632	399
TSST-1	1605	310

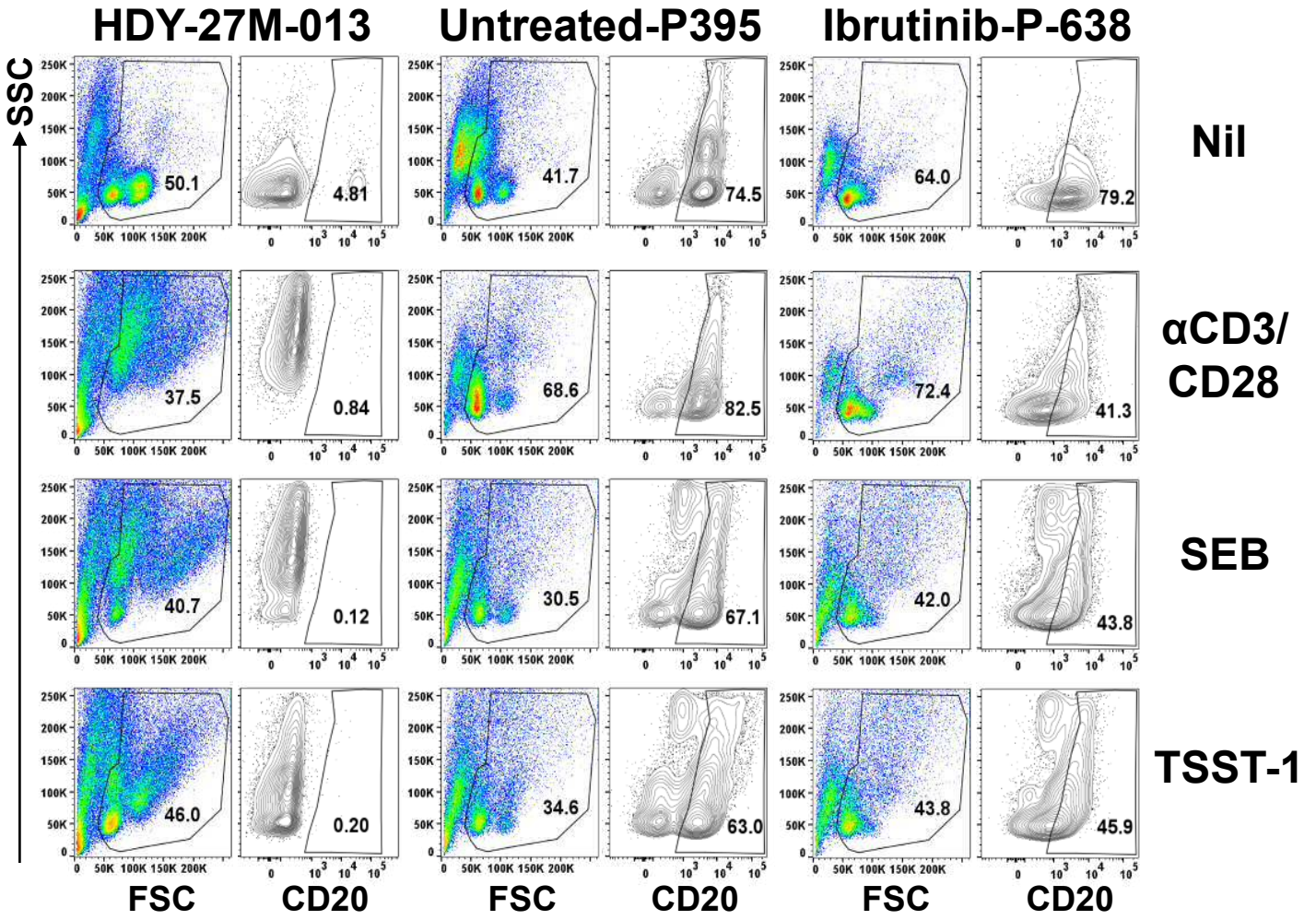


CD40 concatenated MFIs		
Sample	Mean	SD
Nil	566	141
αCD3/28	4463	68.8
SEB	8388	87.8
TSST-1	8199	82.8



CD27 concatenated MFIs		
Sample	Mean	SD
Nil	1508	114
αCD3/28	556	98.1
SEB	186	183
TSST-1	271	171

Supplementary Figure 17. Concatenated MFI analyses reveal that exposure to staphylococcal SAGs potentiates the pro-inflammatory phenotype of CLL tumour cells, *in vitro*. PBMCs of CLL patients were stimulated *in vitro*, as described in Supplementary Figure 3 and the Materials and Methods. At d5, cells were harvested, and surface-stained with anti-CD20, anti-HLA-DR, anti-CD86, anti-CD38, anti-CD40, and anti-CD27 mAbs. Viable CLL cells were identified as shown in Supplementary Figure 1D-F. After acquisition, for each experimental culture condition, 4450 events from individual CLL patient samples (n=10) were concatenated using the FlowJO™ version 10. Thereafter, a concatenated file of the four concatenated culture conditions was created and further analysed in comparison to statistically pooled data shown in Figure 7A. **A)** Concatenation flow cytometry plots of CLL patients show the concatenated MFIs of HLA-DR, CD86, CD38, CD40 and CD27 (in order, from top to bottom) in total CD20⁺ CLL cells, for each culture condition tested: αCD3/28 (blue; a T-cell activator, hence promoting activation of T-helper cells that support B cells), SEB (yellow) and TSST-1 (red) stimulation compared to unstimulated control cultures (Nil; green). The colour-coded tables show the mean and SD of each concatenated parameter for the stimulations mentioned above. Violet shades indicate low to high levels of expression, respectively from light to dark tones.

A

Supplementary Figure 18. CD20⁺ CLL-B cells of CLL patients resist more in cultures with T-cell stimulants than B cells from HDs. Freshly isolated PBMCs (2×10^6 per condition) from HDs and untreated and ibrutinib-treated CLL patients were stimulated in 24-well plates, using stimulatory antibodies: α CD3 (0.125 μ g/ml) and α CD28 (2 μ g/ml) or staphylococcal SAGs (SEB and TSST-1; at 2.5 μ g/ml) or left unstimulated (Nil) for five days. On day 5, cultured cells were harvested and surface stained with the monoclonal antibody anti-CD20. Viable B and CLL-B cells were identified using the gating strategy described in Supplementary Figure 1D-F. **A)** Flow cytometric analysis shows the lymphocyte/CLL cell gate (FSC vs. SSC) and the gate for the detection of CD20⁺ B/CLL cells (CD20⁺ vs. SSC) eventually surviving/activating in the cultures. Example plots from HDs (HDY-27M-013; left columns), untreated (P395; middle columns), and ibrutinib-treated (P638; right columns) CLL patients are provided. HD B cells do not benefit from the exposure to staphylococcal SAGs or α CD3/28 and do not survive in culture with these stimulants, significantly. In contrast, CLL cells (especially when derived from untreated patients) well survive in the cultures with staphylococcal SAGs/ α CD3/28 and go on to activate (as shown in Supplementary Figure 17 and Figure 7A-B). Statistical analysis of this dataset is provided in Figure 7C.