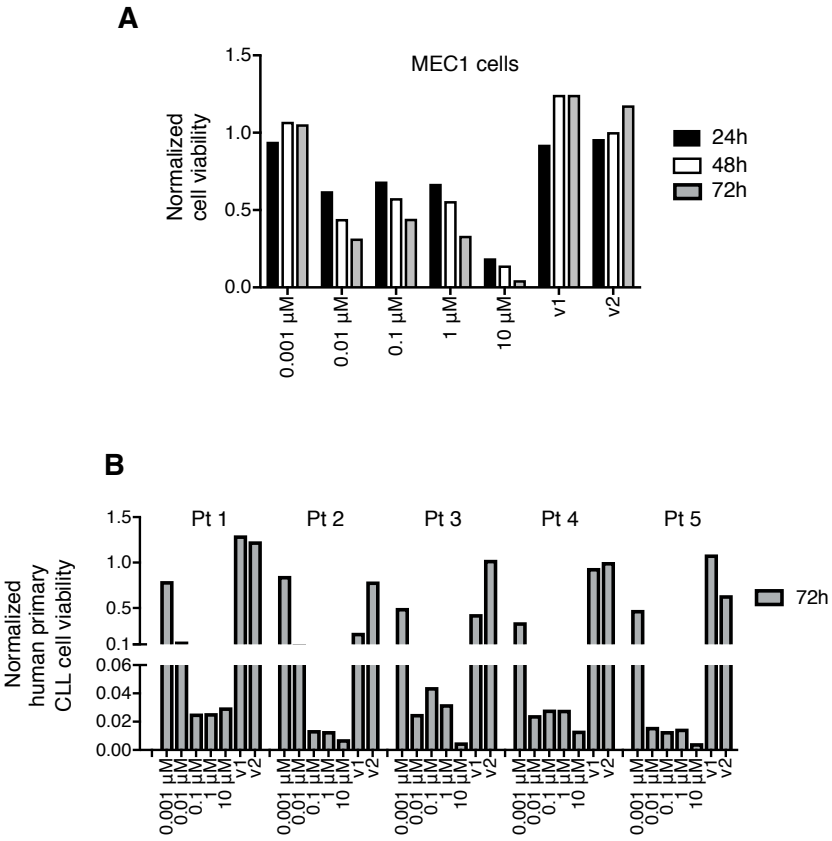
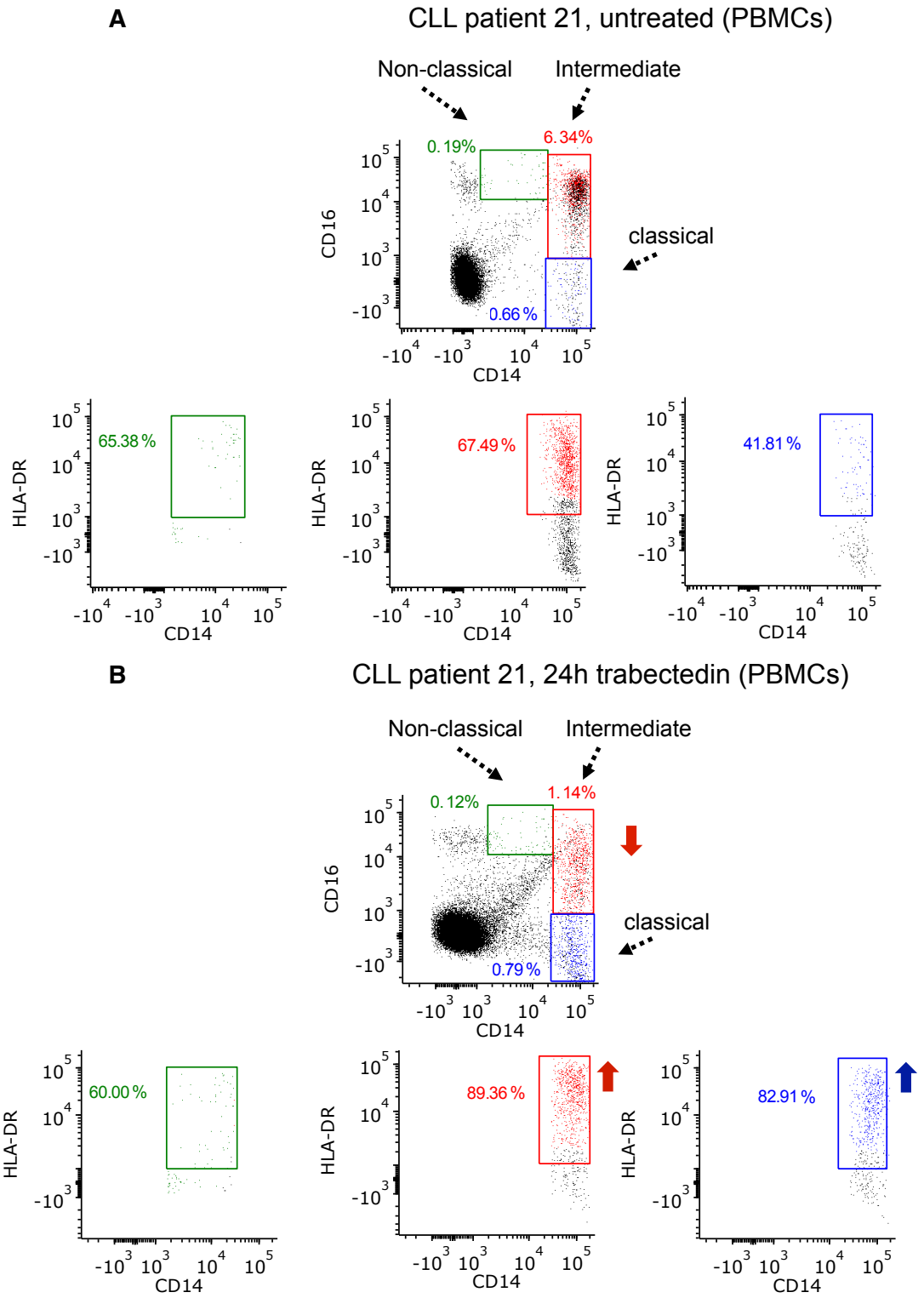


Supplementary Figure S1. Effect of trabectedin on CLL cells.

(A) MEC1 cells were plated in 96-well plates alone (control), with increasing concentrations of trabectedin (0.001 µM, 0.01 µM, 0.1 µM, 1 µM, 10 µM) or with Vehicle (v1, v2 DMSO) and a luminescent assay was performed 24, 48 and 72 hours later to evaluate MEC1 cells' sensitivity to the drug. Cell viability of each sample has been normalized to its control.

(B) Human primary CLL cells obtained from CLL patients (n=5, patients 1-5, Supplementary Table S1) were plated in 96-well plates alone (control), with increasing concentrations of trabectedin (0.001 µM, 0.01 µM, 0.1 µM, 1 µM or 10 µM) or with Vehicle (DMSO v1, v2) and subjected to a luminescent assay at 72 hours to assess the cells' sensitivity to the drug. Cell viability of each sample has been normalized to its control.

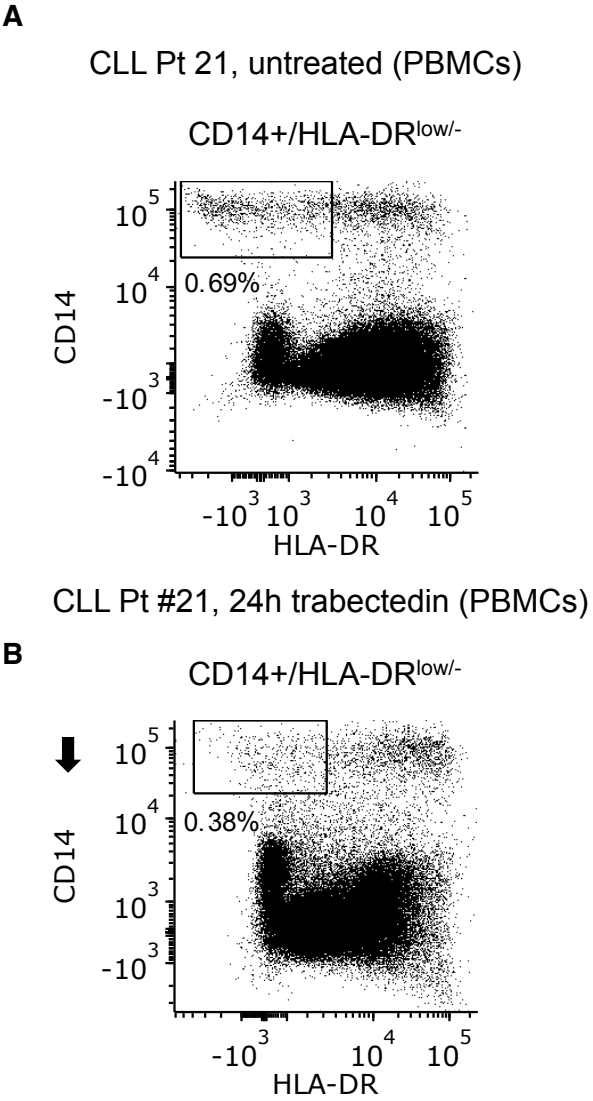
Supplementary Figure S2



Supplementary Figure S2. Human monocyte subsets upon trabectedin treatment

(A) Live PBMCs were gated through LIVE/DEAD Aqua Cell Stain (not shown). After the exclusion of neutrophils (through CD66b molecule, not shown) and then the exclusion of NK/NKT cells, T cells, and B cells through lineage cocktail including CD56, CD3, CD19, CD20 (not shown), monocytes were visualized as $CD14^{+}$ $CD16^{++}$ non-classical, $CD14^{++}$ $CD16^{+}$ intermediate and $CD14^{++}$ $CD16^{-}$ classical subsets in the absence or (B) presence of 0.01 μM of trabectedin (24h). Representative PBMCs from CLL patient #181 are described.

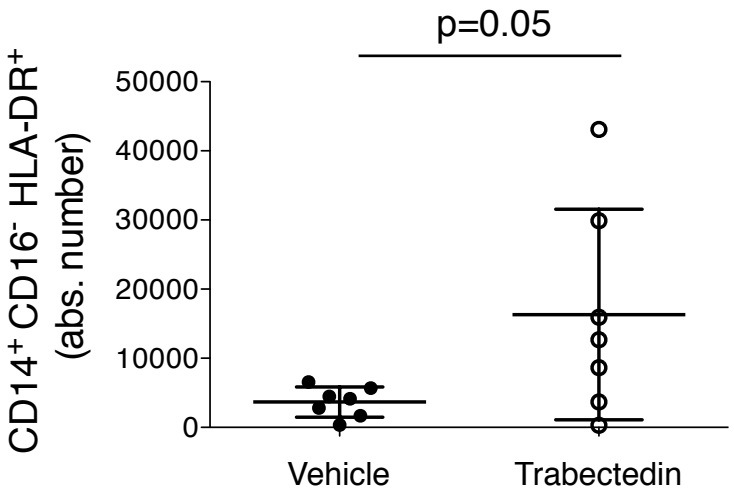
Supplementary Figure S3



Supplementary Figure S3. Human M-MDSCs upon trabectedin treatment

(A) Live PBMCs were gated through LIVE/DEAD Aqua Cell Stain (not shown). HLA-DR downregulation was defined for each sample by FMO control (not shown). $CD14^{+}$ $HLA\text{-}DR^{-/low}$ M-MDSCs were identified and are described in the plot in absence or (B) presence of 0.01 μM of trabectedin (24h). Representative PBMCs from CLL patient 181 are described.

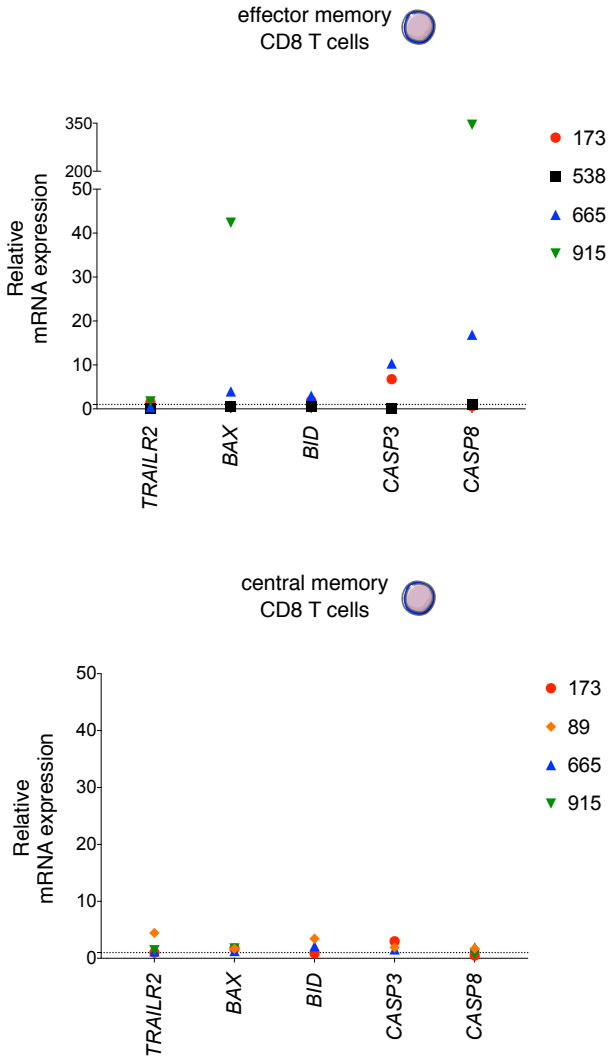
Supplementary Figure S4



Supplemental Figure S4. Classical HLADR$^+$ monocytes upon trabectedin treatment

Classical CD14^{++}CD16$^-$ HLADR$^+$ myeloid cell absolute number after 24h treatment (n=7) with 0.01μM of trabectedin was calculated in treated and untreated CLL patient samples.

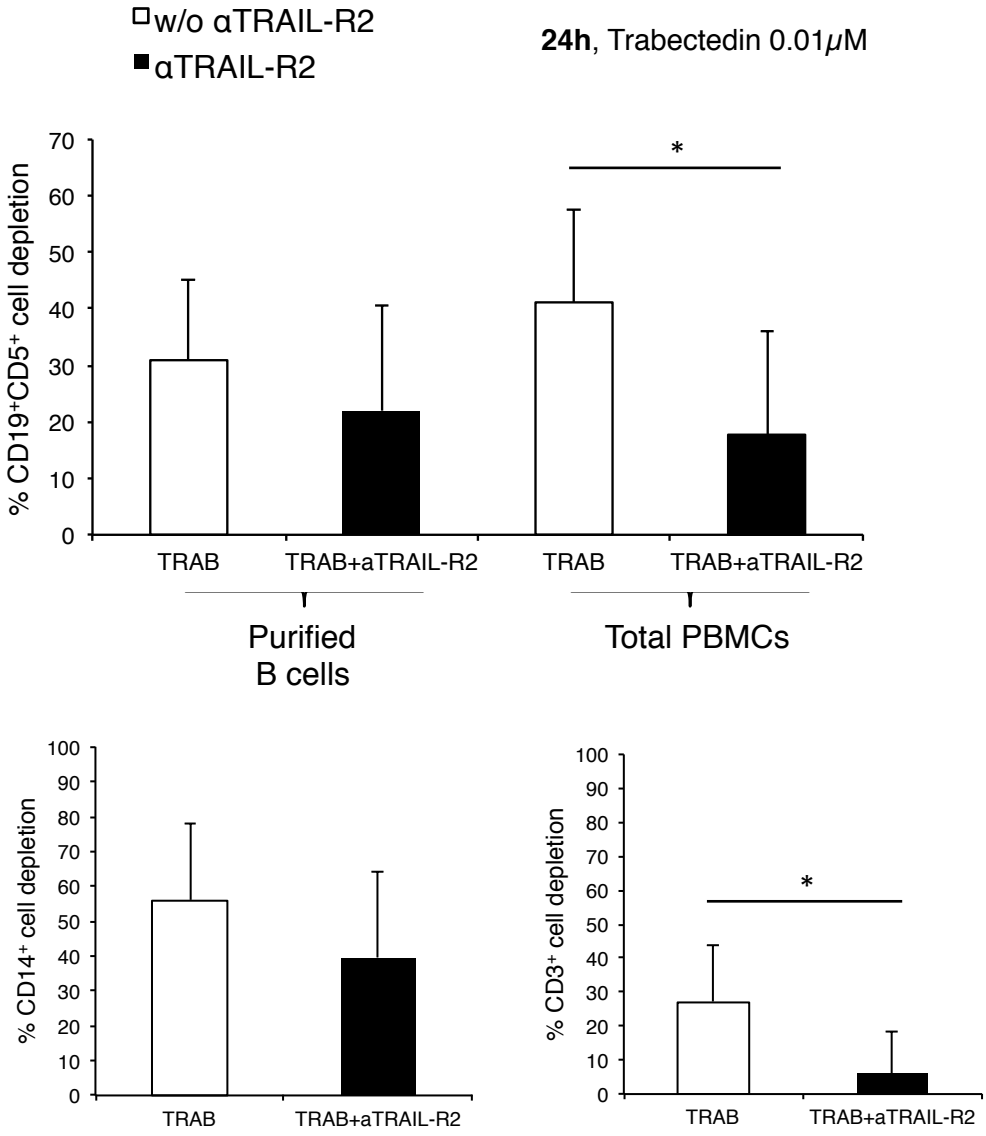
Supplementary Figure S5



Supplementary Figure S5. Trabectedin-induced TRAIL cell death pathway in CD8⁺ memory T cells from CLL patients.

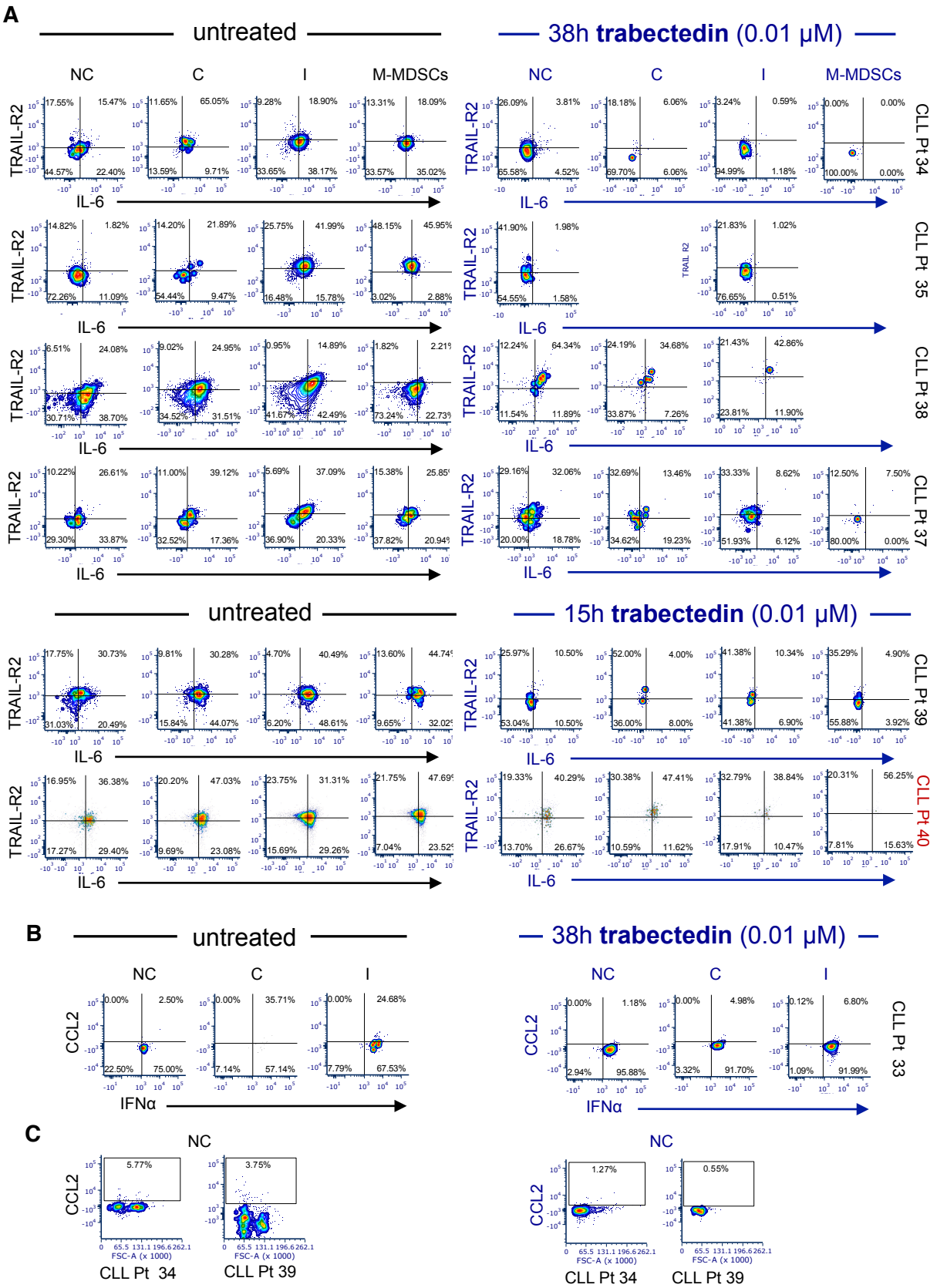
Relative mRNA expression of (**A**) *TRAILR2*, *BAX*, *BID*, *CASP 3*, *CASP 8* in in hCD8⁺ CD45RA⁻CD45RO⁺CD62L⁻ T_{EM} and hCD8⁺ CD45RA⁻CD45RO⁺CD62L⁺ T_{CM} separated by FACS from PBMCs (n=6, patients 173, 538, 665, 915, 089 (Supplementary Table S2) and plated in 6-well plates alone (black bars) or with 0.01 μM of trabectedin (white bars) for 15 h. Three technical replicates were analyzed for each sample. Data were normalized to β-actin expression. Gene expression was determined by calculating the difference (ΔCt) between the threshold cycle (Ct) of each gene and that of the reference gene and was expressed as the mean of 3 replicates ± SEM. Then the relative quantification values were calculated as the fold change expression of the gene of interest over its expression in the selected cell type reference sample, i.e., the untreated sample (considered as the calibrator sample), by the formula $2^{-\Delta\Delta Ct}$. Finally the treated sample relative mRNA expression was normalized to the vehicle. Samples with undetermined Ct values are not included.

Supplementary Figure S6



Supplementary Figure S6. Cell depletion upon TRAIL-R2 blockade
hCD19$^+$ CD5$^+$ cell (plated alone or with total PBMCs) depletion after 24h treatment (n=7) with 0.01μM of trabectedin, with (black bar) or without (white bar) blocking anti-TRAIL-R2 moAb (1μg/ml) was calculated by the following formula: 100 - % remaining cells, where % remaining cells = (Absolute number in treated samples/Absolute number in untreated samples) ×100. (*$p<0.05$), Student's t test. hCD14$^+$ and hCD3$^+$ cell depletion after 24h treatment (n=7) with 0.01μM of trabectedin, with (black bar) or without (white bar) blocking anti-TRAIL-R2 moAb (1μg/ml) was calculated as: 100 - % remaining cells, where % remaining cells = (Absolute number in treated samples/Absolute number in untreated samples) ×100. (*$p<0.05$), Student's t test.

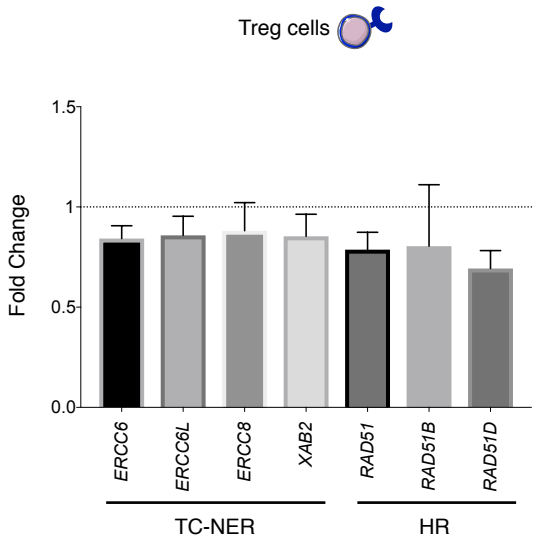
Supplementary Figure S7



Supplementary Figure S7. The immunomodulatory activity of trabectedin on human primary CLL cells at protein level

(A) Flow cytometry detection of TRAIL-R2 expression and intracellular staining flow cytometry analysis of IL-6 production by non classical hCD14^{+}CD16^{++}, classical hCD14^{++}CD16^{-}, intermediate hCD14^{++}CD16^{+} and M-MDSCs hCD14^{+}HLADR$^{low/-}$ myeloid cells from PBMCs of six CLL patients after 38h treatment with 0.01μM of trabectedin. Patient (Pt) 39 and 40 carrying 17p deletion were treated 15h with 0.01μM of trabectedin. The percentage of TRAIL-R2 expressing cells and IL-6 producing cells is indicated. (B) Intracellular staining flow cytometry analysis of CCL2 and IFNα production by non classical hCD14^{+}CD16^{++}, classical hCD14^{++}CD16^{-}, intermediate hCD14^{++}CD16^{+} monocytes from PBMCs of CLL Pt 33 after 38h treatment with 0.01μM of trabectedin. The percentage of CCL2 and IL-6 producing cells is indicated. (C) Intracellular staining flow cytometry analysis of CCL2 production by non classical hCD14^{+}CD16^{++} monocytes from PBMCs of CLL Pt 34 and 39 after 38h treatment with 0.01μM of trabectedin. The percentage of CCL2 producing cells is indicated.

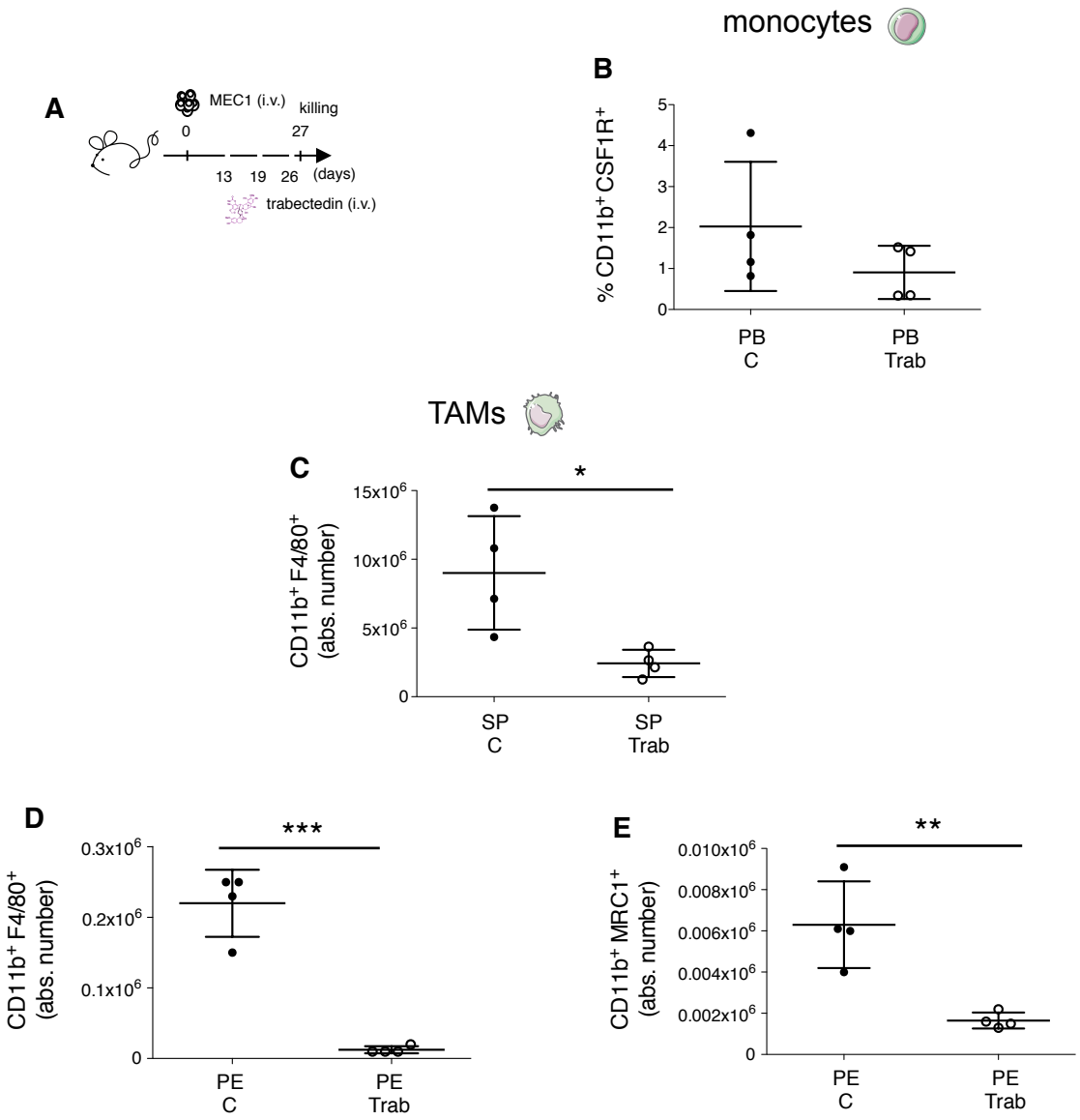
Supplementary Figure S8



Supplementary Figure S8. Trabectedin-induced transcription-coupled nucleotide excision (TC-NER) and homologous recombination (HR) repair pathways

Fold-change of TC-NER gene (*ERCC6, ERCC6L, ERCC8, XAB2)* and HR gene (*RAD51, RAD51B, RAD51D)* mRNA expression was measured via qRT-PCR in $CD4^{+}CD25^{+}CD127^{low/-}$ Tregs separated by FACS from PBMCs (n=5, patients 173, 538, 665, 915, 089, Supplementary Table S2) and plated in 6-well plates alone or with 0.01 μM of trabectedin for 15 h. Three technical replicates were analyzed for each sample. Data were normalized to β-actin expression. Gene expression was determined by calculating the difference (ΔCt) between the threshold cycle (Ct) of each gene and that of the reference gene and was expressed as the mean of 3 replicates ± SEM. Finally the treated sample relative mRNA expression was normalized to the vehicle. Fold change has been calculated as the mean ± SD of five CLL patients. Samples with undetermined Ct values are not included.

Supplementary Figure S9

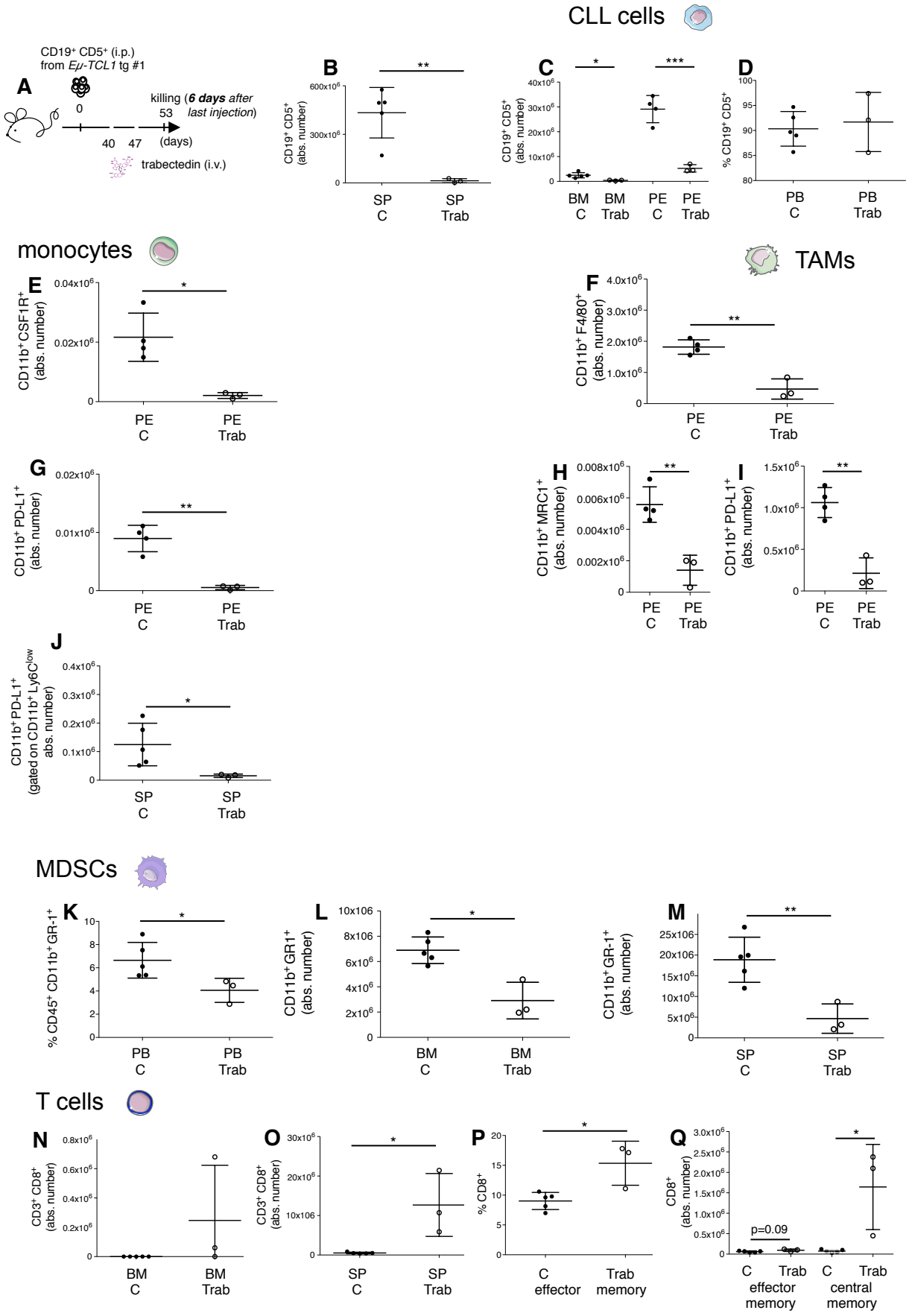


Supplementary Figure S9. Effect of trabectedin on myeloid cells in the xeno-transplantation system

(A) i.v. MEC1 transplanted (day 0) Rag2$^{-/-}$γc$^{-/-}$ mice were left uninjected (n=4, black circles) or treated i.v. (days +13, +19, +26) with 0.15mg/Kg of trabectedin (n=4, white circles) and killed at day 27

(B) The mean value of the relative contribution of CD11b^{+} CSF1R^{+} cells gated on CD45^{+} in PB is shown in graph. (C) The mean value of the absolute number of CD11b^{+} F4/80^{+} cells gated on CD45^{+} in SP and (D) PE is shown in graphs. (E) The mean value of the absolute number of CD11b^{+} MRC1^{+} cells to the whole macrophage pool (CD11b^{+} F4/80^{+}) gated on CD45^{+} in PE is shown in graph. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test.

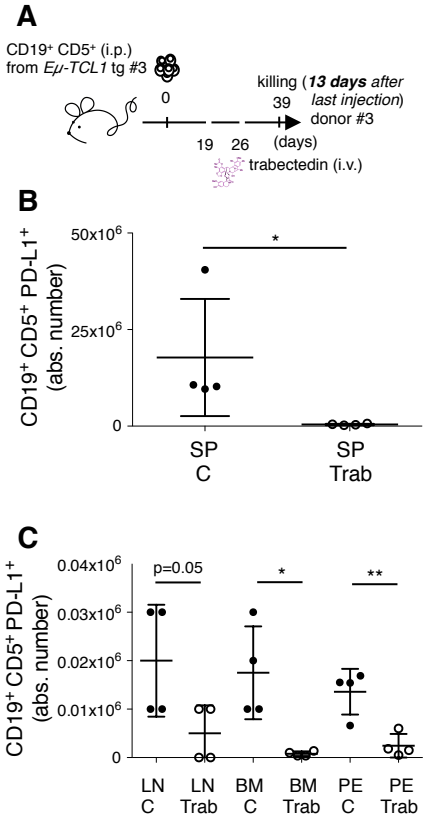
Supplementary Figure S10



Supplementary Figure S10. Effect of trabectedin on leukemic cells and on the microenvironment of the TCL1 tg transplantation system

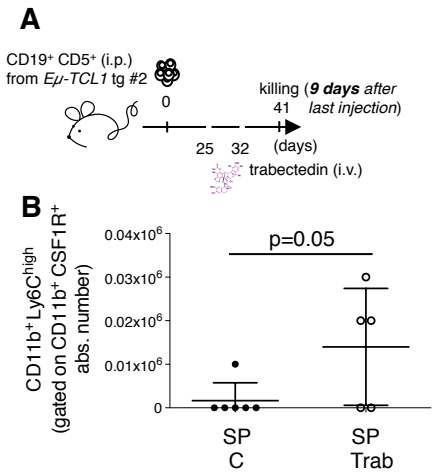
(A-Q) C57BL/6 mice transplanted i.p. with leukemic B cells from *Eμ-TCL1* transgenic mouse donor #1, left untreated (n=4, black circles) or treated (day +40, +47) with 0.15mg/Kg i.v. of trabectedin (n=3, white circles) were killed at day 53 (6 days after the last injection of trabectedin) and analyzed by flow cytometry. (B) The mean value of the absolute number of $CD19^+$ $CD5^+$ cells gated on $CD19^+$ in SP, (C) in BM and PE and (D) the relative contribution of $CD19^+$ $CD5^+$ cells to the whole B cell pool in PB are shown in the graphs. (E) The mean value of the absolute number of $CD11b^+$ $CSF1R^+$ cells gated on $CD45^+$ in PE is shown in graph. (F) The mean value of the absolute number of $CD11b^+$ $F4/80^+$ cells gated on $CD45^+$ in PE is shown in graph. (G) The mean value of the absolute number of $CD11b^+$ $PD\text{-}L1^+$ cells to the whole monocyte pool ($CD11b^+$ $CSF1R^+$) gated on $CD45^+$ in PE is shown in graph. (H) The mean value of the absolute number of $CD11b^+$ $MRC1^+$ cells and (I) of the absolute number of $CD11b^+$ $PD\text{-}L1^+$ cells to the whole macrophage pool ($CD11b^+$ $F4/80^+$) gated on $CD45^+$ in PE is shown in graphs. (J) The mean value of the absolute number of $CD11b^+$ $PD\text{-}L1^+$ cells to the $CD11b^+$ $Ly6C^{low}$ monocyte subset gated on $CD45^+$ in the SP is shown in graph. (K) The mean value of the relative contribution of $CD11b^+$ $Gr1^+$ cells gated on $CD45^+$ in PB is shown in graph. (L-M) The mean value of the absolute number of $CD11b^+$ $Gr1^+$ cells gated on $CD45^+$ in BM and SP is shown in graphs. (N) The mean value of the absolute number of $CD3^+$ $CD8^+$ T cells in the BM and (O) in the SP is shown in the graphs. (P) The mean value of the relative contribution of $CD44^+CD62L^{low/neg}$ effector memory $CD8^+$ T cells in PB is shown in graph. (Q) The mean value of the absolute number of $CD44^+$ $CD62L^+$ central and $CD44^+$ $CD62L^{low/neg}$ effector memory $CD8^+$ T cells in BM is shown in the graph. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's t test.

Supplementary Figure S11



Supplementary Figure S11. PD-L1 expression of leukemic cells in the TCL1 tg transplantation system

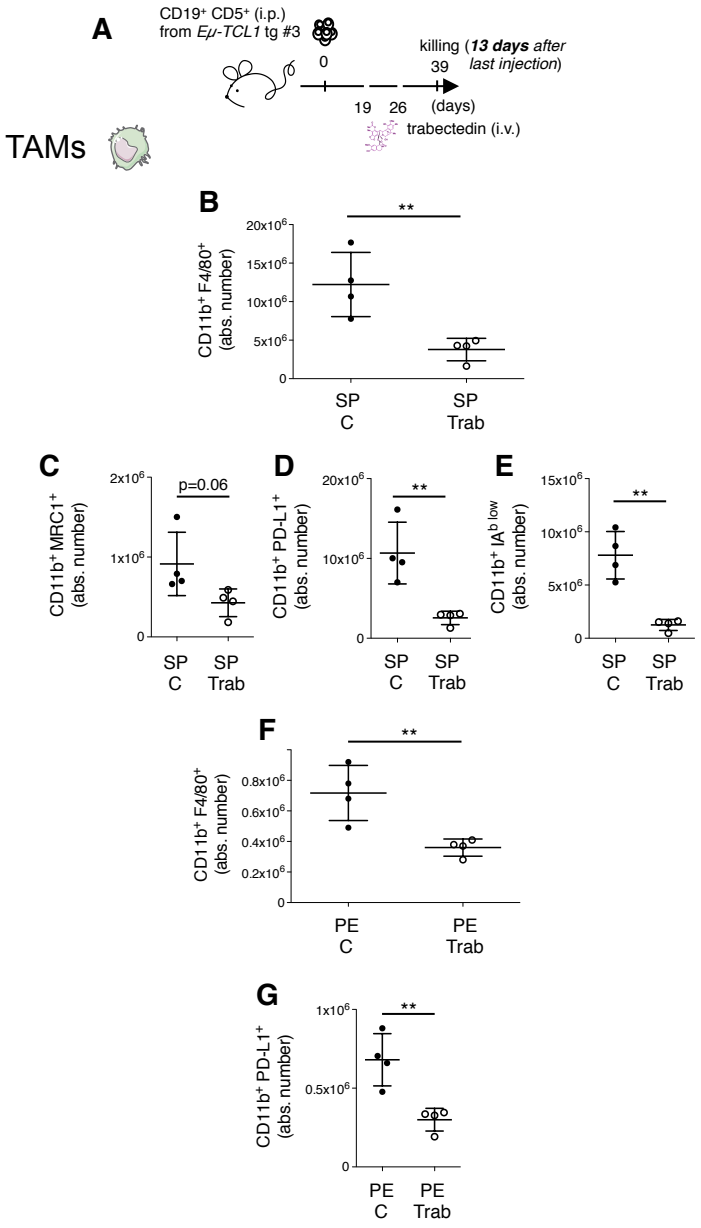
(A) C57BL/6 mice transplanted i.p. with leukemic B cells from *Eμ-TCL1* transgenic mouse donor #3, left untreated (n=4, black circles) or treated (day +19, +26) with 0.15mg/Kg i.v. of trabectedin (n=4, white circles) were killed at day 39 (13 days after the last injection of trabectedin) and analyzed by flow cytometry. (B) The mean value of the absolute number of PD-L1$^+$ cells to the whole CD19$^+$ CD5$^+$ B cell pool in SP, (C) LN, BM and PE is shown in the graphs. *p < 0.05, **p < 0.01, ***p < 0.001, Student's t test.



Supplementary Figure S12. Effect of trabectedin on the murine Ly6C^{high} myeloid subset

(A) C57BL/6 mice transplanted i.p. with leukemic B cells from *Eμ-TCL1* transgenic mouse donor #2, left untreated (n=6, black circles) or treated (day +25, +32) with 0.15mg/Kg i.v. of trabectedin (n=5, white circles) were killed at day 41 (9 days after the last injection of trabectedin) and analyzed by flow cytometry. (B) The mean value of the absolute number of CD11b$^+$ Ly6C^{high} cells to the whole monocyte subset (CD11b$^+$ CSF1R$^+$) gated on CD45$^+$ in the SP is shown in graph.

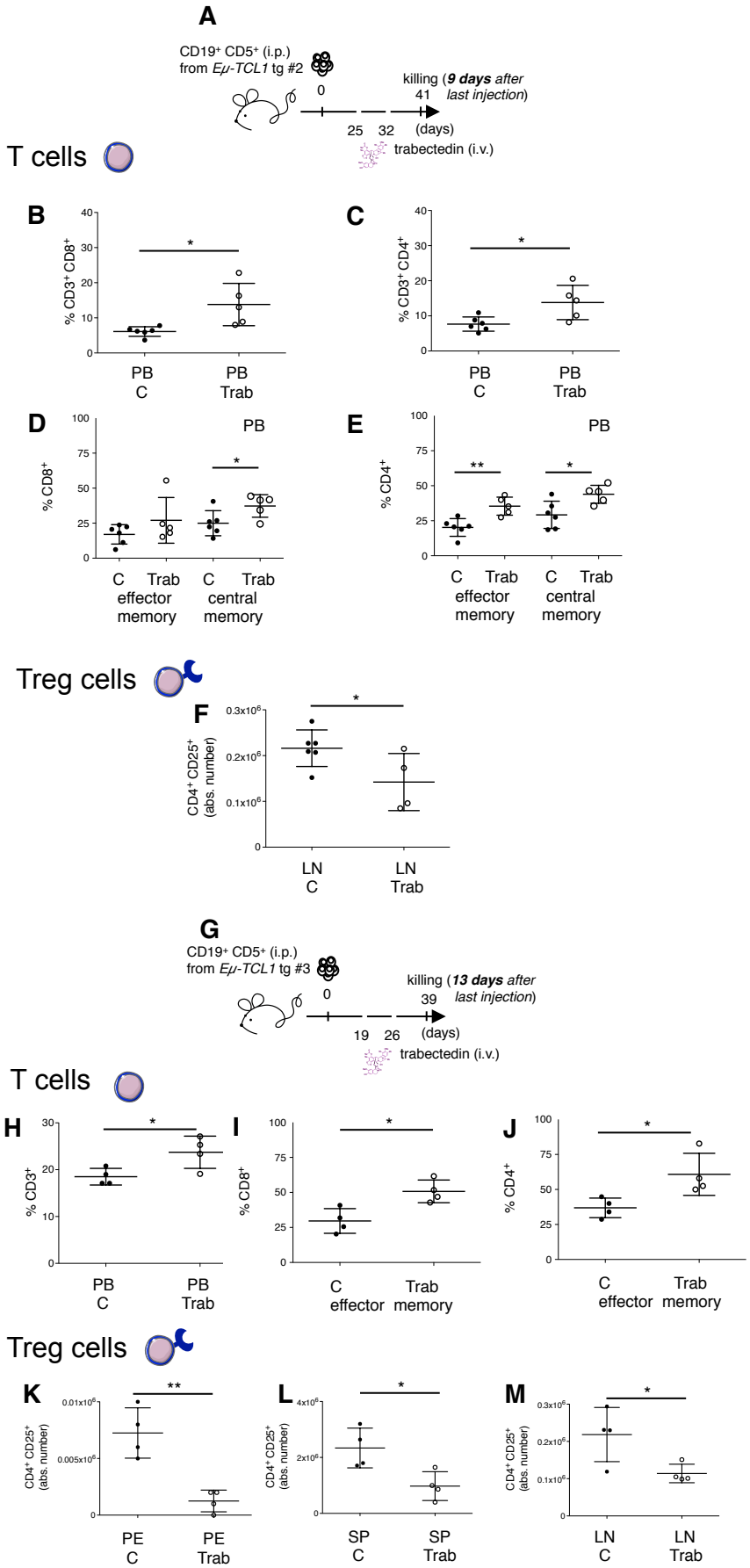
Supplementary Figure S13



Supplementary Figure S13. Effect of trabectedin on TAMs in the TCL1 tg transplantation system

(A) C57BL/6 mice transplanted i.p. with leukemic B cells from *Eμ-TCL1* transgenic mouse donor #3, left untreated (n=4, black circles) or treated (day +19, +26) with 0.15mg/Kg i.v. of trabectedin (n=4, white circles) were killed at day 39 (13 days after the last injection of trabectedin) and analyzed by flow cytometry. (B) The mean value of the absolute number of $CD11b^+$ $F4/80^+$ cells gated on $CD45^+$ in SP is shown in graph. (C) The mean value of the absolute number of $CD11b^+$ $MRC1^+$ cells and (D) of $CD11b^+$ $PD\text{-}L1^+$ cells and (E) of $CD11b^+$ $IA^{b\ low}$ cells to the whole macrophage pool ($CD11b^+$ $F4/80^+$) gated on $CD45^+$ in SP is shown in graphs. (F) The mean value of the absolute number of $CD11b^+$ $F4/80^+$ cells gated on $CD45^+$ in PE is shown in graph. (G) The mean value of the absolute number of $CD11b^+$ $PD\text{-}L1^+$ cells to the whole macrophage pool ($CD11b^+$ $F4/80^+$) gated on $CD45^+$ in PE is shown in graph. *$p < 0.05$, **$p < 0.01$, Student's t test.

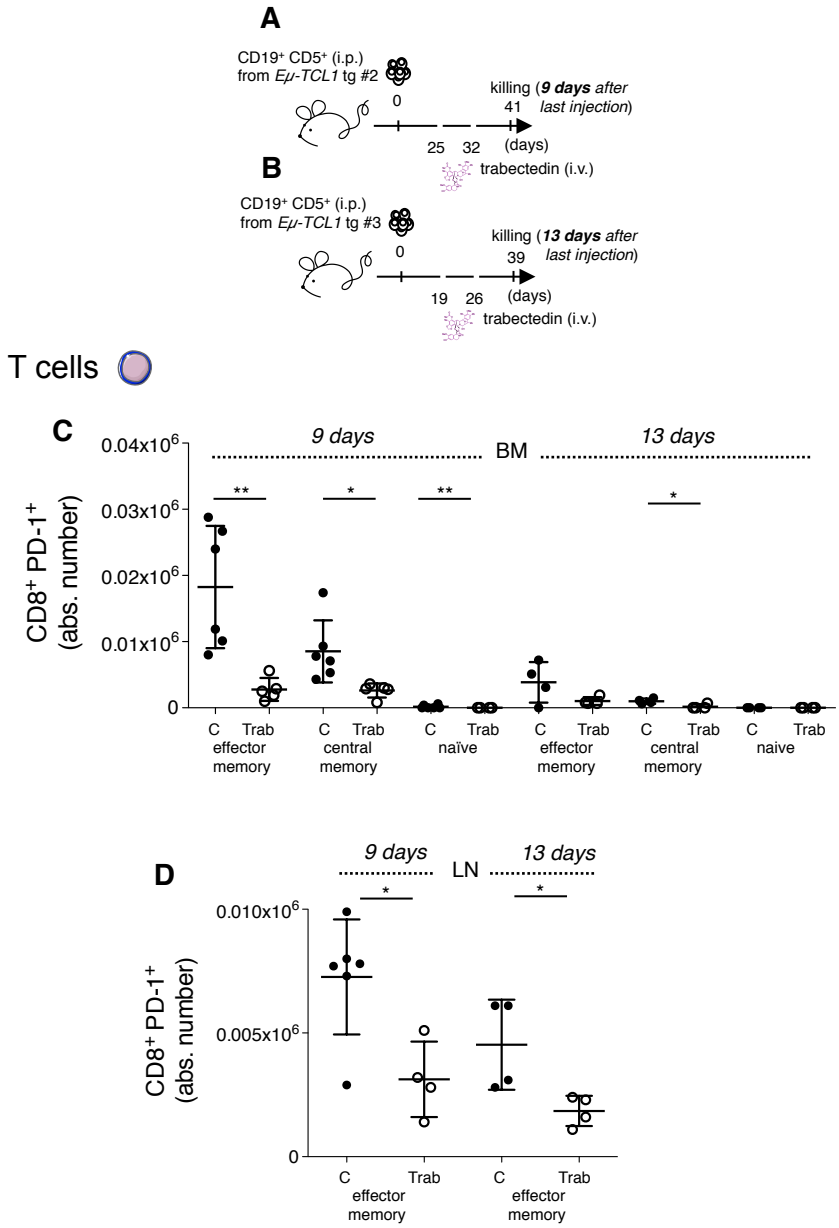
Supplementary Figure S14



Supplementary Figure S14. Impact of trabectedin on T cells in the TCL1 tg transplantation system

(A) C57BL/6 mice transplanted i.p. with leukemic B cells from *Eμ-TCL1* transgenic mouse donor #2, left untreated (n=6, black circles) or treated (day +25, +32) with 0.15mg/Kg i.v. of trabectedin (n=5, white circles) were killed at day 41 (9 days after the last injection of trabectedin) and analyzed by flow cytometry. (B) The mean value of the relative contribution of $CD3^+$ $CD8^+$ and (C) $CD3^+$ $CD4^+$ T cells in the PB of mice described in (A) is shown in the graphs. (D) The mean value of the relative contribution of $CD44^+CD62L^{low/neg}$ effector memory and $CD44^+$ $CD62L^+$ central memory $CD8^+$ T cells in PB of mice described in (A) is shown in graph. (E) The mean value of the relative contribution of $CD44^+CD62L^{low/neg}$ effector memory and $CD44^+$ $CD62L^+$ central memory $CD8^+$ T cells in PB of mice described in (A) is shown in graph. (F) The mean value of the absolute number of $CD4^+$ $CD25^+$ T cells in the LN of mice described in (A) is shown in the graph. (G) C57BL/6 mice transplanted i.p. with leukemic B cells from *Eμ-TCL1* transgenic mouse donor #3, left untreated (n=4, black circles) or treated (day +19, +26) with 0.15mg/Kg i.v. of trabectedin (n=4, white circles) were killed at day 39 (13 days after the last injection of trabectedin) and analyzed by flow cytometry. (H) The mean value of the relative contribution of $CD3^+$ in the PB of mice described in (G) is shown in the graph. (I) The mean value of the relative contribution of $CD44^+CD62L^{low/neg}$ effector memory $CD8^+$ T cells and of (J) $CD44^+CD62L^{low/neg}$ effector memory $CD4^+$ T cells in PB of mice described in (G) is shown in graphs. (K) The mean value of the absolute number of $CD4^+$ $CD25^+$ T cells in the PE, in the (L) SP and in the (M) LN of mice described in (G) is shown in the graphs. *$p < 0.05$, **$p < 0.01$, Student's t test.

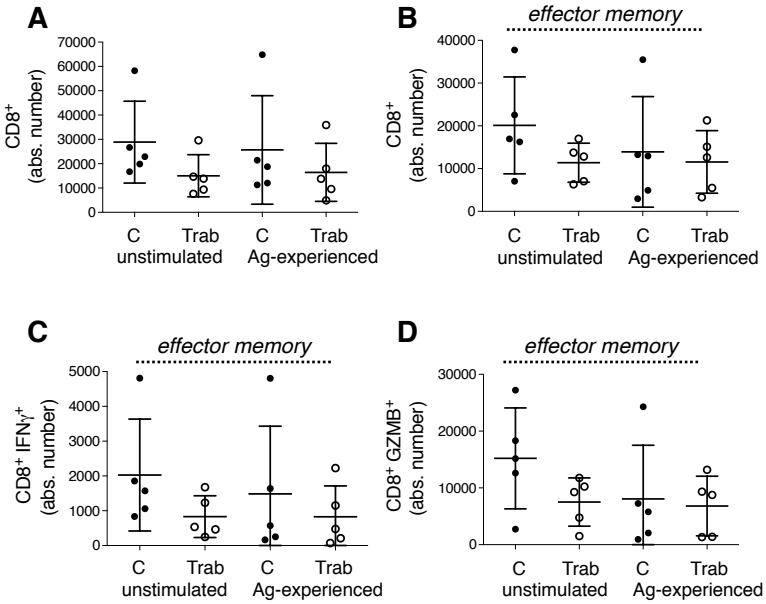
Supplementary Figure S15



Supplementary Figure S15. Impact of trabectedin on PD-1⁺ T cells in the TCL1 tg transplantation system

(A) C57BL/6 mice transplanted i.p. with leukemic B cells from *Eμ-TCL1* transgenic mouse donor #2, left untreated (n=6, black circles) or treated (day +25, +32) with 0.15mg/Kg i.v. of trabectedin (n=5, white circles) were killed at day 41 (9 days after the last injection of trabectedin) and analyzed by flow cytometry. (B) C57BL/6 mice transplanted i.p. with leukemic B cells from *Eμ-TCL1* transgenic mouse donor #3, left untreated (n=4, black circles) or treated (day +19, +26) with 0.15mg/Kg i.v. of trabectedin (n=4, white circles) were killed at day 39 (13 days after the last injection of trabectedin) and analyzed by flow cytometry. (C) The mean value of the absolute number of $CD8^+$ $PD\text{-}1^+$ cells gated on $CD44^+CD62L^{low/neg}$ effector memory, $CD44^+$ $CD62L^+$ central memory and $CD44^-$ $CD62L^+$ naïve $CD8^+$ T cells in the BM and (D) in the LN of mice described in (A) and (B) is shown in graphs. *p < 0.05, **p < 0.01, Student's t test.

Supplementary Figure S16



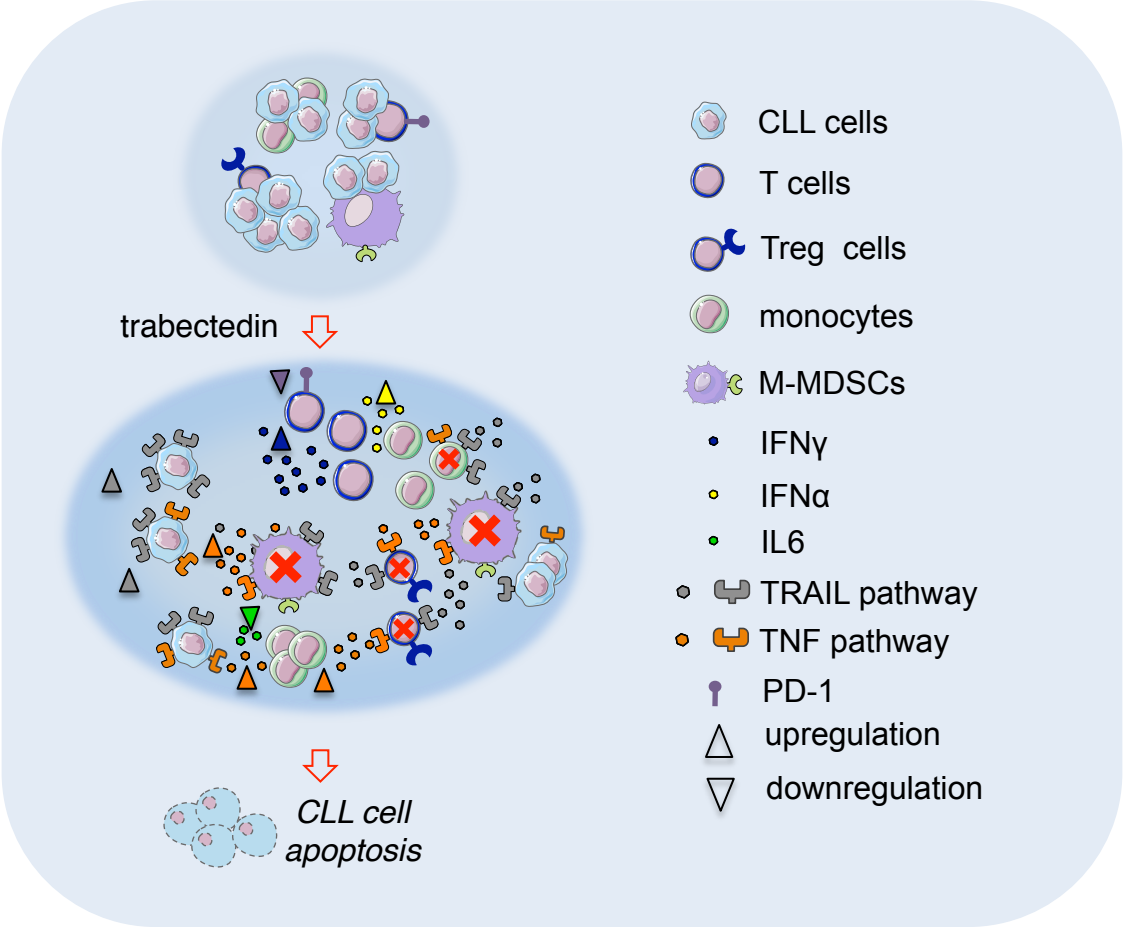
Supplementary Figure S16. The impact of trabectedin on CD8⁺ memory T cells from CLL patients

$CD8^+$ T lymphocytes (including T_{EM} and T_{CM}) from PBMC of patients 45-49 (n=5) were separated by fluorescence activated cell-sorting, co-incubated in vitro with heated-MEC1 cells (at effector target ratio 2:1) to induce tumor-antigen specific T cell response (Ag-experienced) in absence (C) or presence (Trab) of 0.01μM trabectedin for 15h.

(A-B) The mean value of the absolute number of $CD8^+$ T cells and T_{EM} cells in absence or presence of trabectedin is shown in graphs.

(C-D) The mean value of the absolute number of $CD8^+$ T_{EM} cells producing IFNy and GRANZYME B is shown in graphs.

Supplementary Figure S17



Supplementary Figure S17. Schematic model of CLL cell and microenvironment cell molecular interaction
CLL cells interact with cellular elements of the microenvironment including T cells, M-MDSCs, monocytes and macrophages. Trabectedin targets both CLL cells and immunosuppressive cells such as M-MDSCs and Treg cells mainly through the TRAIL and TNF apoptotic pathways. Trabectedin has also an immunomodulatory activity on myeloid and lymphoid cells and it modifies the RNA/protein expression levels of selected cytokines involved in the antitumor activation of macrophages, and B lymphocyte control of proliferation and T cell cytotoxic response. Trabectedin interferes with PD-1/PD-L1 immune checkpoint.