Targeting CD38 is lethal to Breg-like chronic lymphocytic leukemia cells and Tregs but restores CD8+ T-cell responses

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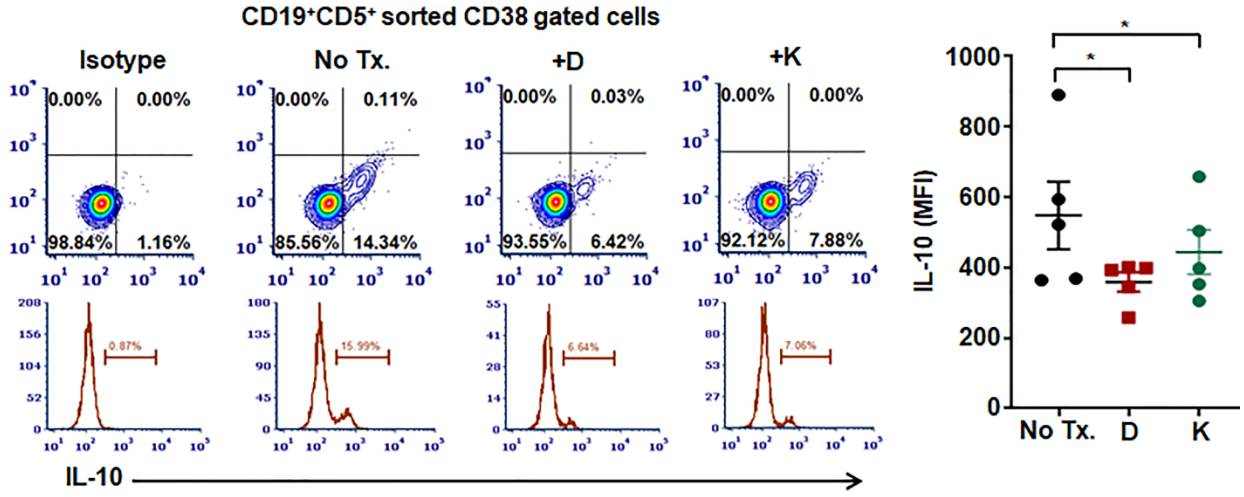
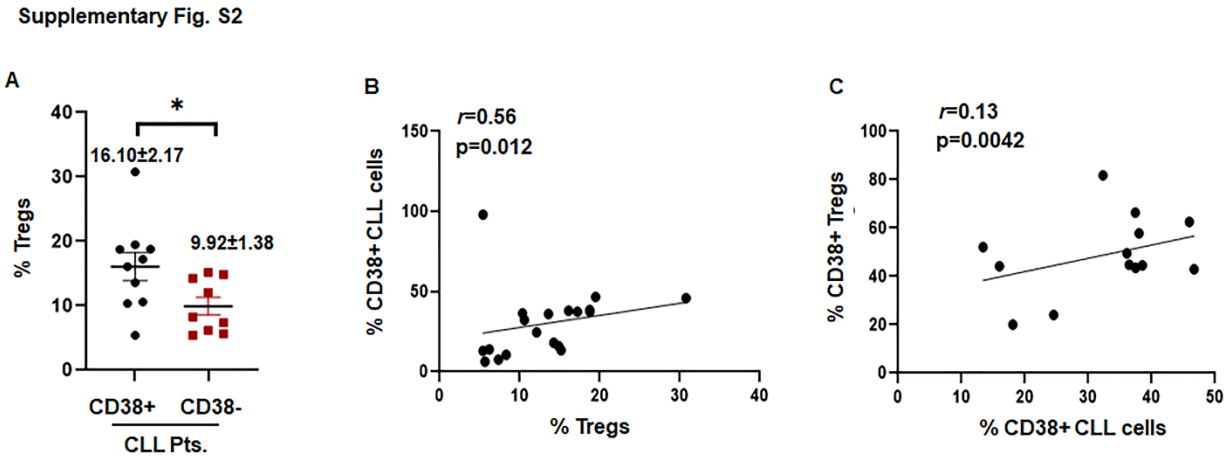
Supplementary Figure Legends and Supplementary Materials and Methods

Supplementary Fig. S1



Supplementary Fig. S1.

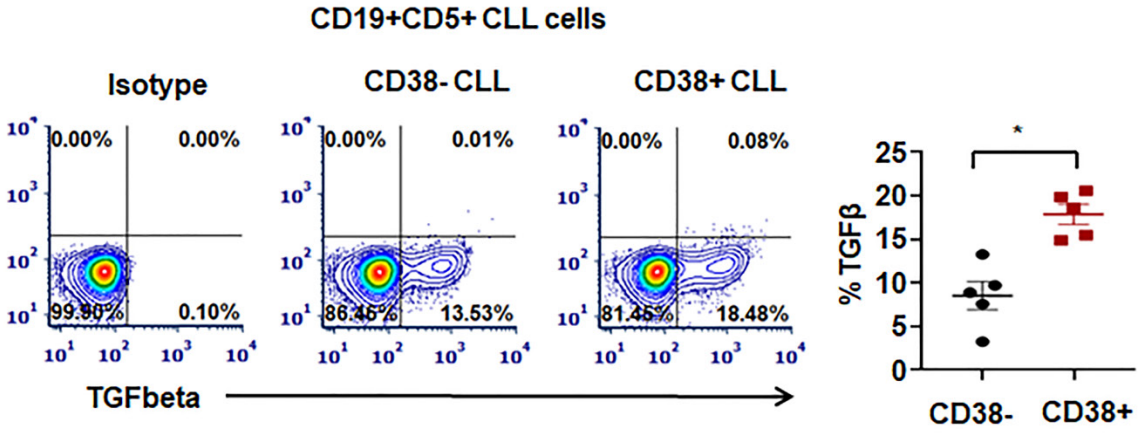
Intracellular IL-10 is decreased within CLL cells that are exposed to CD38-targeting agents : The effect of daratumumab (D, 1µg/mL, 72hr) or kuromanin (K, 30µM, 72hr) on intracellular IL-10 levels was examined in CD19+CD5+ CLL cells (n=5). Magnetic bead-isolated CD19+CD5+ cells, were treated with daratumumab or kuromanin. 4h before termination of experiments, brefeldin was used as a protein transport blocker. Cells were permeabilized and fixed with fix-perm solution and labelled with anti-IL10 antibody. The percentage of IL-10 positive cells and mean fluorescence intensity (MFI) were analyzed in an equal number of CD38 gated cells (10,000 cells/sample); with data represented as % IL-10 positive cells in scatter flow diagram, histogram depicting MFI of IL-10 and MFI of IL10 ± SEM in dot plot. Each experiment was performed at least thrice in duplicate. *$p<0.05$



Supplementary Fig. S2.

The proportion of Tregs and CD38+Tregs correlates with the % of CD38+ CLL cells: **(A).** A comparative analysis of Treg cell frequency in CD38+ and CD38- CLL patients (n=9/group) was performed and data was represented as % Treg cells ± SEM in dot plot, *p<0.05. **(B)** A Spearman correlation analysis was done between % CD38+CLL cells vs. % Treg cells and **(C).** % CD38+CLL cells vs. % CD38+Treg cells**.** Data were represented as dot plot and correlation as r value and p value was determined via two tailed ANOVA analysis.

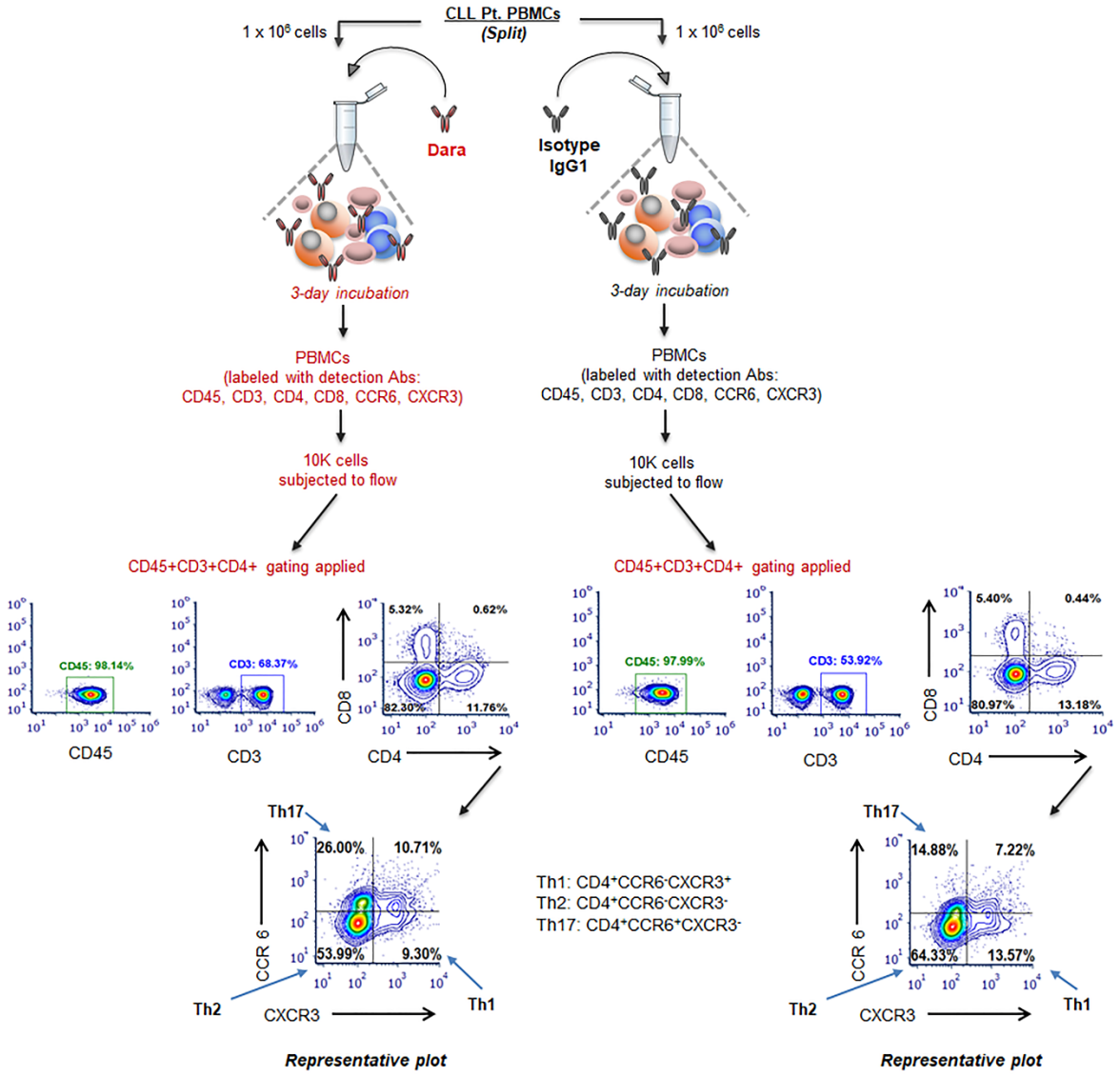
Supplementary Fig. S3



Supplementary Fig. S3.

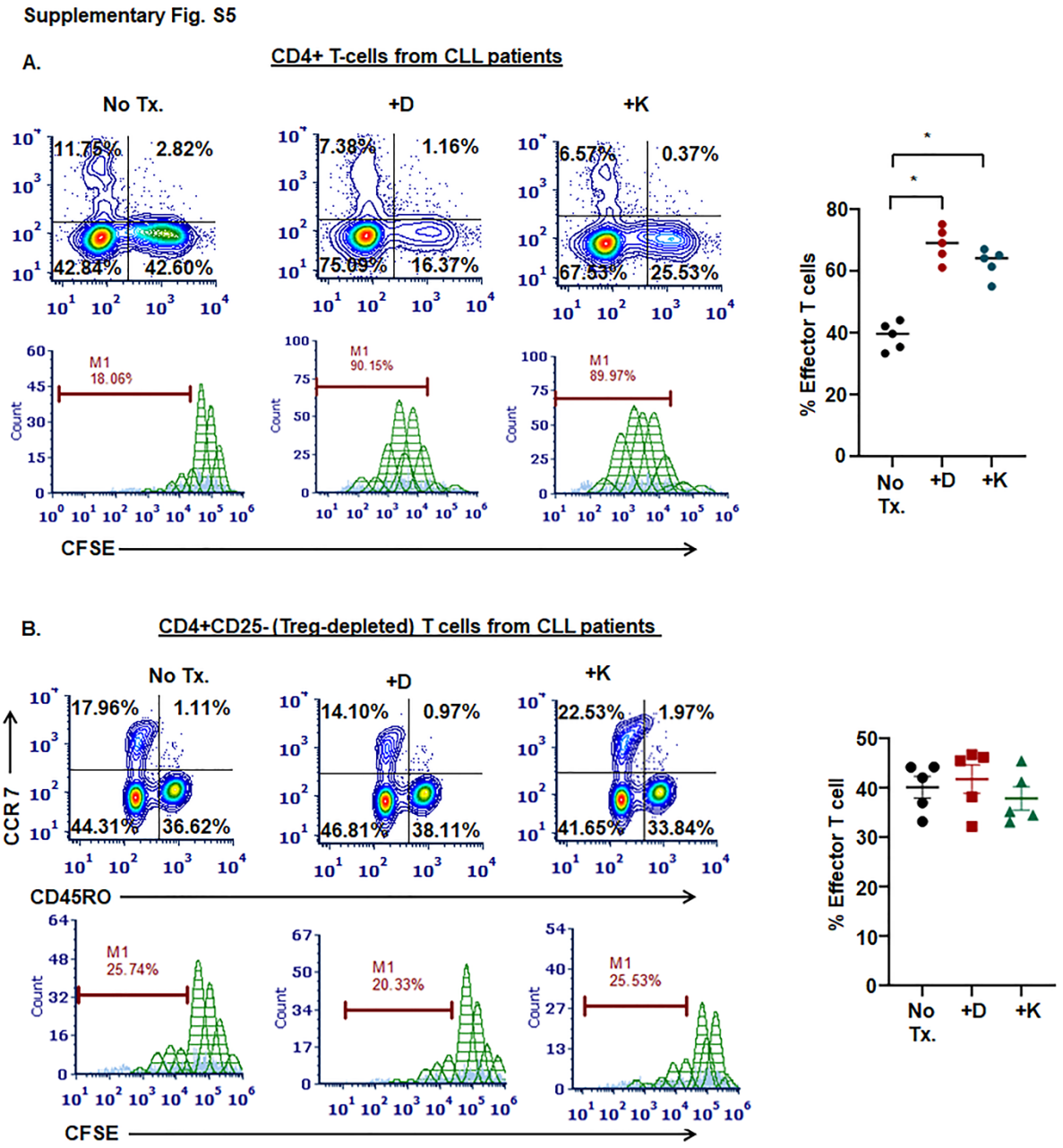
Presence of TGFβ+ CLL cells in patients: The baseline intracellular levels of TGFβ was measured in CLL cells from patients with CD38+ or CD38- disease (n=5/group). CD19+CD5+ CLL cells were blocked with brefeldin-A for 4 h and were permeabilized and fixed with fix-perm solution and labelled with anti-TGFβ antibody. The % TGFβ positive cells were analyzed and data were represented as % positive cell in scatter flow diagram, % positive TGFβ ± SEM in dot plot. Each experiment was performed at least thrice in duplicate. *$p<0.05$

Supplementary Fig. S4



Supplementary Fig. S4.

Workflow of experiment to determine proportion of Th1, Th2 and Th17 cells in PBMCs from CLL patients treated with or without daratumumab: PBMCs from CLL patients were obtained and split into 2 aliquots ($1x10^6$ cells, each aliquot). One aliquot was treated with Dara and the other with isotype IgG1 control Ab. After 72hr, PBMCs were labeled with detection antibodies (CD45, CD3, CD4, CD8, CCR6, CXCR3) and 10,000 cells were subjected to flow cytometry. Gating strategies were applied as shown and cells were then further gated on markers for Th1, Th2 and Th17 cells and 10,000 events analyzed to determine % of each cell type.



Supplementary Fig. S5.

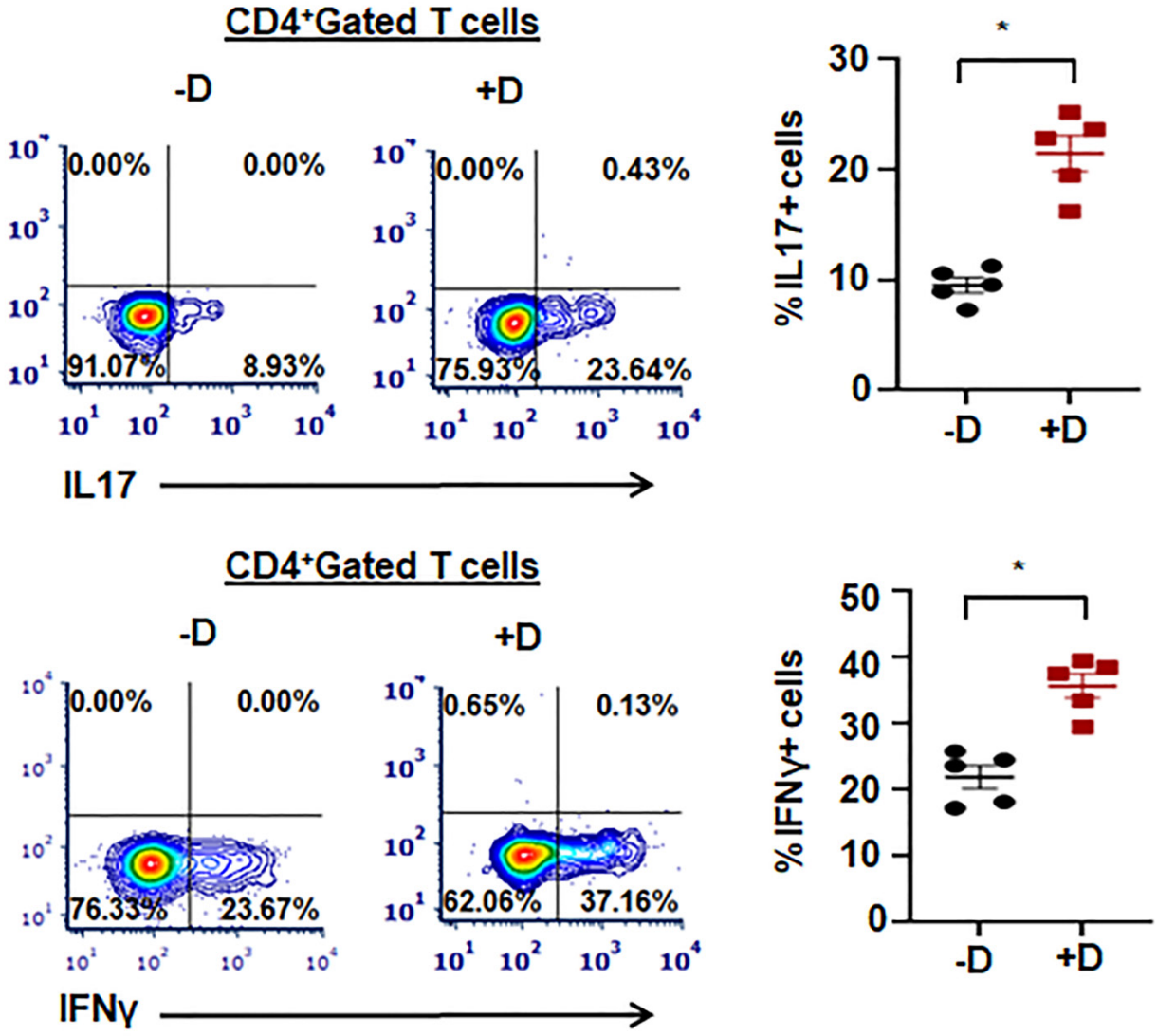
Effect of CD38-targeting agents on CD4+ effector T cell proliferation: A. The effect of daratumumab or kuromanin on CD4+T effector cell proliferation was measured. CD4+ cells were isolated from CLL patients (n=5) using magnetic bead-based cell separation, followed by labeling with celltrace CFSE. These cells were then treated with daratumumab (D, 1µg/mL, 72hr) or kuromanin (K, 30µM, 72hr). The cell proliferation was measured using a flow cytometer. The % CD4+CCR7-CD45RO- cells were analyzed

(Flow scatter plots are the representative) for proliferation and data were represented as % effector cells proliferated ± SEM in dot plot. Each experiment was performed at least thrice in duplicate. *p<0.05. B. (**B**) CD4+T effector cell proliferation was also measured in a CD4+CD25- T-cell mixture (n=5, same patients as in panel A) that was depleted of Tregs and then exposed to daratumumab (D, 1µg/mL, 72hr) or kuromanin (K, 30µM, 72hr). Methodology and analysis was identical to that in panel A. The % CD4+CCR7-CD45RO- cells were analyzed (Flow scatter plots are the representative) for proliferation and data were represented as % effector cells proliferated ± SEM in dot plot. Each experiment was performed at least thrice in duplicate.

Supplementary Fig. S6.

Increase in Th17 cell proportion is not due to a direct effect of daratumumab but indirectly related to the presence of CLL cells exposed to daratumumab in a PBMC mixture. The effect of daratumumab (D, 1µg/mL, 72hr) in modulating % of Th cell subsets was examined in the PBMCs as well as CD19-CD5- fraction of cells from patients with CLL (n=5). These cells were categorized as such based on the following immunophenotypic markers: $CD3^{+}CD8^{-}CD4^{+}CCR6^{-}CXCR3^{+}$ (Th1); $CD3+CD8-CD4^{+}CCR6^{-}CXCR3^{-}$ (Th2); $CD3+CD8-CD4^{+}CCR6^{+}CXCR3^{-}$(Th17). Daratumumab altered the % of Th17 cells from patients with CLL (n=5) in the presence of CD19+CD5+ CLL cells (upper panel) but not without CLL cells (lower panel) significantly, p=0.024. Contour plots are representative and compiled data are presented as mean ± SEM with individual data points overlaid. Each experiment was performed at least twice in duplicate. *p<0.05.

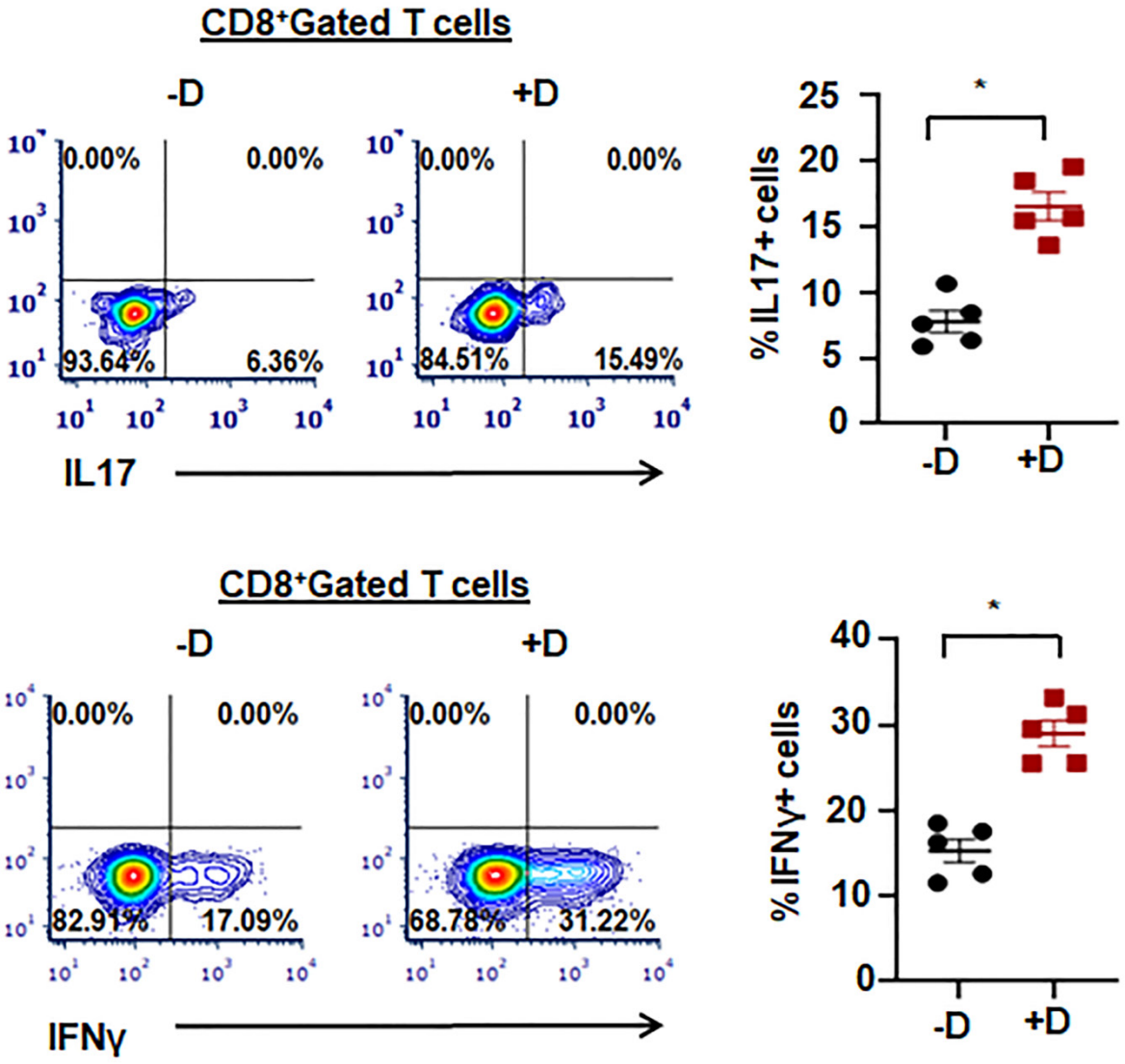
Supplementary Fig. S7



Supplementary Fig. S7.

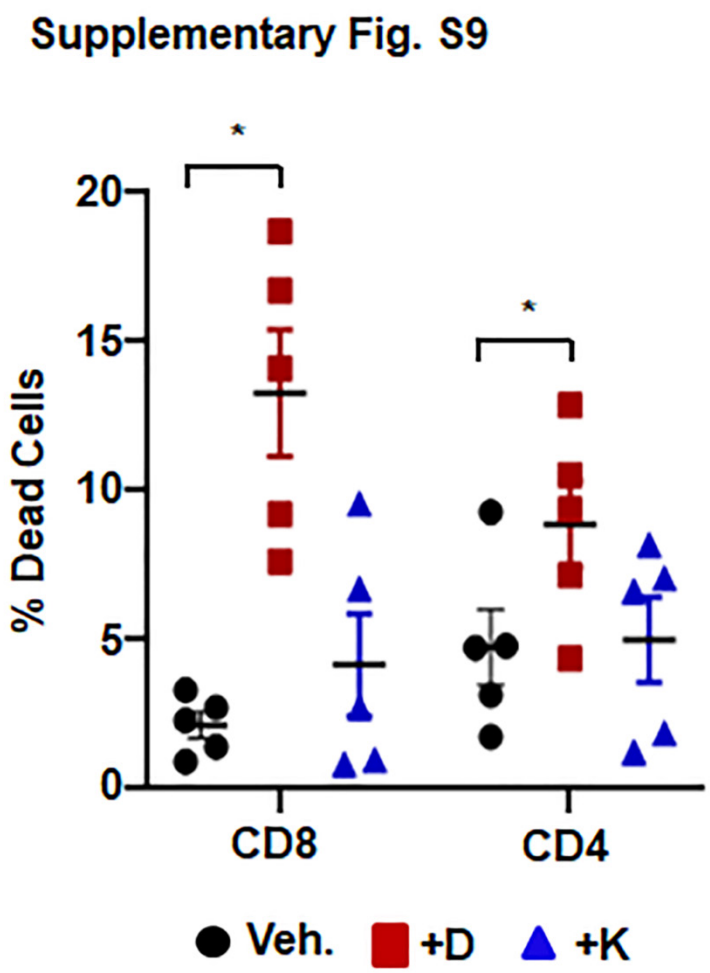
Daratumumab increases the proportion of IL-17+ and IFNγ+ CD4+ T cells: The effect of daratumumab (D, 1µg/mL, 72hr) on IL-17 and IFNγ producing CD4+T cell was measured. PBMCs from CLL patients (n=5) were treated with vehicle or daratumumab followed by quantification of IL-17 or IFNγ using flow cytometer. The % CD4+IL17+ or % CD4+IFNγ+ cells were represented as flow scatter plots as representative and data were represented as % positive cells ± SEM in dot plot. Each experiment was performed at least thrice in duplicate. *$p<0.05$.

Supplementary Fig. S8



Supplementary Fig. S8.

Daratumumab increases the proportion of IL-17+ and IFNγ+ CD8+ T cells: The effect of daratumumab (D, 1μg/mL, 72hr) on IL-17 and IFNγ producing CD8+T cell was measured. PBMCs from CLL patients (n=5) were treated with vehicle or daratumumab followed by quantification of IL-17 or IFNγ using flow cytometer. The % CD8+IL17+ or % CD8+IFNγ+ cells were represented as flow scatter plots as representative and data were represented as % positive cells ± SEM in dot plot. Each experiment was performed at least thrice in duplicate. *$p<0.05$.



Supplementary Fig. S9.

Daratumumab induces minimal death of CD4+ and CD8+ T cells: The effect of daratumumab (D, 1µg/mL, 24hr) or kuromanin (K, 30µM, 24hr) on CD8+T cell apoptosis was measured in magnetic-bead isolated CD8+T cells (n=5). Following isolation of CD8+ cells from CLL patients, cells were treated with daratumumab or kuromanin and analyzed for annexin-V and propidium (AV/PI) positivity. % total AV/PI and AV positive cells were analyzed and data were represented as % dead cells ± SEM in dot plot. Each experiment was performed at least thrice in duplicate. *$p<0.05$

Supplementary Materials & Methods

Isolation and culture of primary human cells

Heparinized blood was obtained from consenting patients with a confirmed diagnosis of CLL who consented to biospecimen donation under a Mayo Clinic Institutional Review Board approved protocol, in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient from CLL patients (n=22, with ≥ 90% tumor B-cells; clinical characteristics and biological data detailed in **Table 1**.) as well as from healthy human donors (n=6). CD19+ CLL cells were selected out by magnetic bead separation followed by CD5+ cell selection using Biotin conjugated anti human CD5 (EasySepTM Human CD19 Positive Selection kit and EasySep™ Release Human Biotin Positive Selection Kit, StemCell Technologies, Vancouver BC, Canada). Clinical annotation indicated that CLL cells were typically more >98% CD5+CD19+ B cells; with normal B cells generally being <1% of CD19+ cells and lacking CD5 positivity. All cells were cultured in AIM-V media, per previously reported conditions. In most experiments we additionally used flow-sorted, purified CD19+CD5+/CD38hi and CD19+CD5+/CD38lo cells from CLL patients. Briefly, CD19+CD5+ B cells from CLL patients were sorted using automatic magnetic sorter (RoboSep™, Stemcell Technologies, MA, USA) followed by staining with APC conjugated anti-CD38 antibody and flow sorted using FACS Aria II (BD Biosciences, San Jose, CA, USA) according to manufacturer's instructions. Post-sorted purity was validated using an Attune NxT flow cytometer (Invitrogen, MA, USA). CD38 expression in certain assays where cells CD38+ sorted/magnetically isolated B or T-cells were treated with CD38-targeting agents was again reassessed using a multi-epitope FITC conjugated anti-CD38 antibody (Cytognos CD38 multi-epitope-FITC antibody, Ref: CYT-38F2), as described by us previously.[1]

Reagents

78c, a highly potent inhibitor of CD38 enzymatic function[2,3] was gifted from Dr. Eduardo N. Chini, (Mayo Clinic, Rochester, MN, USA) and kuromanin (a flavonoid, small molecule inhibitor of CD38,[4] was

purchased from SelleckChem (Houston, TX, USA). Daratumumab was acquired through the Mayo Clinic pharmacy and came pre-dissolved/diluted.

ELISA assay

Cytokines in plasma isolated from patients with active CLL or healthy human donors were measured by the ELISA MAX™ delux set (Biolegends, San Diego, CA, USA) using 100µl samples from each. CD19+CD5+ cells were sorted from the peripheral blood of CLL patients using a flow cytometer (BD FACSARIA III, BD Biosciences, San Jose, CA, USA) and were plated ($2x10^6$ cells/well) in a 6 well plate for 72h at 37˚C with 5% CO_2 incubators. Cell supernatant were collected and assayed for IFN-γ, IL-2, IL-10 and TGF-β using ELISA MAX™ delux set using 100µl samples from each according to the manufacturer's protocol. Samples were measured in triplicate.

For measuring total TGF-β1 in plasma or cell culture supernatant, samples were activated via adding 10 µL of serum/plasma/40µL of culture supernatant to 5 µL/10µL of acidification solution respectively, mixed well, and incubated for 10 minutes at room temperature. Following that, neutralization solution (5µl/10µL) was added and mixed well. Plasma samples were diluted 10-fold and culture supernatant was diluted 2-fold with sample diluent according to estimated sample concentration.

Flow Cytometry

Isolation of CLL cells: $CD19^+$ cells were isolated from PBMC isolated from healthy donors as well as from patients with CLL by magnetic bead separation followed by CD5+ cell selection using Biotin conjugated anti human CD5 (EasySepTM Human CD19 Positive Selection kit and EasySep™ Release Human Biotin Positive Selection Kit, StemCell Technologies, Vancouver BC, Canada).

Cell gating strategy: B regulatory like cell were gated based on $CD19^+CD24^+CD38^{hi}$ phenotypic markers. To assess different T helper cell (Th) population using flow cytometer, we used following phenotypic

markers. $CD3^+CD8^-CD4^+CCR7^+CD45RO^-$ (**naïve**); $CD3^+CD8^-CD4^+CCR7^-CD45RO^-$ (**effector**); $CD3^+CD8^-CD4^+CCR7^+CD45RO^+$ (**CM; central memory**); $CD3^+CD8^-CD4^+CCR7^-CD45RO^+$ (**EM; effector memory**); $CD3^+CD8^-CD4^+CCR6^-CXCR3^+$ (**Th1**); CD3+CD8-$CD4^+CCR6^-CXCR3^-$ (**Th2**); CD3+CD8-$CD4^+CCR6^+CXCR3^-$(**Th17**). Regulatory T cells (**Treg**) were evaluated as $CD4^+CD25^+CD127^{dim}$ $FoxP3^+$ and cytotoxic T cells (**cTL**) were evaluated based on $CD3^+CD4^-CD8^+$ as described by Palma et al.[5] To assess cytotoxic T cells (cTL) activation, tumor necrosis factor receptor superfamily member 18 (TNFRSF18) or glucocorticoid-induced TNFR-related protein (GITR); CD69 and CD137 were evaluated on CD3+CD8+ gated CTL cells. In parallel, cTL exhaustion markers were also evaluated on CD3+CD8+ gated cTL named as T cell immunoreceptor with Ig and ITIM domains (TIGIT), Lymphocyte-activation gene 3 (Lag3, CD223), 2B4 (CD244), Programmed cell death protein 1 (PD1, CD279) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4, CD152).[5]

Intracellular cytokines assay: To assess intracellular IFNγ and IL-10 production, $CD19^+CD5^+$ cells were first labeled with anti-CD19 and anti-CD5 antibody, followed by fixation and permeabilization with fix/perm buffer (BD, 1ml) for 30 min in dark. Cells were washed with perm wash buffer (1X, 2ml) using centrifugations (600Xg, 5 min) at RT. With 100µl of perm wash buffer, anti-IL-10 antibody was added and incubated in dark for another 30 min. Following another washing, cells were analyzed using a flow cytometer.

CLL cell-induced Treg transformation assay: There are two types of Tregs: naturally occurring Tregs that are produced in the thymus and traverse into the periphery thereafter vs. induced Tregs (iTregs), which can be generated from ($CD4^+CD25^-$) naïve Th cells outside the thymus by means of cell-cell contact or cytokines.[6,7] To assess the role of CLL cells in differentiation of Naïve Th cell (CD4+CD25-) cells towards Treg cells *ex vivo*, co-stimulated naïve T cells with anti CD3 (5µg/ml)/CD28 (5µg/ml), were co-cultured with/without autologous Breg like CLL cells (CD19+CD5+CD38+) for 3 days in IL-2 containing medium (AIM-V) in a 1:1 ratio, followed by surface staining for CD4, CD25,CD127 and intracellular labelling for

FoxP3 using eBioscience FoxP3/Transcription factor staining buffer (Invitrogen, CA, USA); data were analyzed using a flow-cytometer.

Cytokine-induced Treg transformation assay: To assess the effect of anti CD38 immunotherapy on Tregs, a cytokine induced Treg (iTreg) model was utilized (Chatterjee et a., 2018). Naïve Th cells ($CD4^+CD25^-$) were co-stimulated with anti-CD3 (5µg/ml)/anti-CD28 (5µg/ml) antibodies for 2 h followed by incubation with a cytokine cocktail [(TGF-β (5ng/ml, rIL-2 (10U), anti-IL-4 (10µg/ml) and anti-IFN-γ (10µg/ml)] for 3 days in IL-2 containing medium (AIM-V). This was followed by surface staining for CD4, CD25, CD127 and intracellular labelling for FoxP3 using eBioscience FoxP3/Transcription factor staining buffer (Invitrogen, CA, USA); data were analyzed using a flow-cytometer.

Trans well assay

Naïve helper T cells ($CD4^+CD25^-$) and Breg like cells ($CD19^+CD5^+CD24^+CD38^+$, **B10**) were flow sorted from the PBMCs of patients with CLL. Naïve helper T cells T cells co stimulated anti-CD3 (5µg/ml)/anti-CD28 (5µg/ml) antibodies (1 h) were placed in the lower chamber of a trans well plate; with B10 cells placed in the upper chamber for +/- anti-IL-10 neutralizing antibody (10µg/ml) or/and anti-TGF-β (10µg/ml) for 3 days. Thereafter, cells from the lower chamber were harvested and analyzed for Tregs ($CD4^+CD25^+Cd127^{dim}$ and $FoxP3^+$), as stated in the previous section. Cytokine-induced Treg (iTreg) used as a positive control.[8]

Treg cell death assay/apoptosis

$CD4^+CD25^+CD127^{dim}$ Treg cells were isolated from patients with CLL or induced after co-stimulation with anti-CD3 [(5µg/ml)/anti-CD28 (5µg/ml) for 2 h] using a cytokine cocktail [(TGF-β (5ng/ml, rIL-2 (10U), anti-IL-4 (10µg/ml) and anti IFN-γ (10µg/ml) for 3 days]. Thereafter, % cell death was measured using Fixable Viable Stain 570 (FVS570). % apoptosis was measured using Annexin V-FITC/propidium iodide (PI) dual staining after treatment for 24h with IgG1-b12 control Ab (0.1µg/mL), kuromanin (10uM),

daratumumab (1.0 µg/mL) using flow cytometry. Heat killed cells were used as PI positive control and H_2O_2 (20µM, 30 min) was used as Annexin V-FITC positive control.

Cytotoxic T cell proliferation assay

Cytotoxic T cells (cTL, $CD8^+$) were isolated using a magnetic bead sorter (StemCell Technologies), labeled with CellTrace™ carboxyfluorescein succinimidyl ester (CellTrace™ CFSE, ThermoScientific, MA USA) at 5µM for 30 min and co-stimulation with anti-CD3 [(5µg/ml)/anti-CD28 (5µg/ml) for 2 h], and then treated with anti-CD38 therapeutic antibody (daratumumab) for 3 days. Cell proliferation was measured on a flow cytometer. Untreated control was used as a negative control and LPS (100ng/ml) was used as positive control.

Cytolytic activity of cytotoxic T cell (cTL)

Isolated cytotoxic T cells (cTL, $CD8^+$) using a magnetic bead sorter (StemCell Technologies) and cultured in AIM-V serum free medium supplemented with Human Serum Albumin (HSA), L-Glutamine, Gentamicin (50ug/mL) and Streptomycin Sulfate (10ug/mL) as antibiotic at a cell density of $1x10^6$ cells/mL. Subsequently, cells were co-stimulation with anti-CD3 [(5µg/ml)/anti CD28 (5µg/ml) for 2hr], were treated with daratumumab for 3 days; followed by co-culture with calcein-AM (1µM) labelled autologous/heterologous CLL cells ($CD19^+CD5^+$) at an effector:target (E:T) ratio of 50:1 for another 6hr in the dark at 37˚C with 5% CO_2. After centrifugation, supernatant was transferred to a glass bottom black plate and calcein fluorescence was measured in a fluorimeter equipped with an argon laser (Ext 488nm; Em 528nm±10). Results were analyzed as % specific lysis using the following formula:

$$\text{Specific lysis} = 100\times\frac{\text{Experimental release (RFU)} - \text{Spontaneous release (RFU)}}{\text{Maximal release (RFU)} - \text{Spontaneous release (RFU)}}$$

CLL Patient Derived Xenograft mouse model (CLL-PDX model)

A short term CLL-PDX model was established as described elsewhere,[9] using whole PBMCs isolated from a patient with Rai Stage III CLL (Pt. 22; aggressive disease [>60% tumor burden]; $CD38^{hi}$ [>30%]). On day 1, we injected (20 x 10^6 /mice) in NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice (The Jackson Laboratory) by I.V. tail vein route. After a 24h engraftment period, mice were randomly assigned to 3 groups (5 mice/group) and treated with either vehicle; Daratumumab (20µg/ml loading dose followed by 10µg/ml) or kuromanin (small molecule inhibitor of CD38; 20ug/ml) all via I.V. tail vein administration on post-implantation Days 2, 5, 8. On Day 9, mice were sacrificed; with the spleen and blood harvested. In splenocytes, B and T-cell subsets were characterized by flow cytometry. Absolute cell counts for CLL cells, Breg-like cells, Tregs, Th1, Th2, Th17 and cTL were determined as cell counts/spleen ± SEM (as shown in Supplementary Table 2).

Statistical analysis

All values are expressed as means ± SEM. Data were analyzed for significance in GraphPad Prism by paired t test, Mann-Whitney U test, or One-way ANOVA with Bonferroni adjustment as specified. Correlation coefficients and their significance were calculated by two-tailed Spearman's rank correlation. A confidence interval of 95% or a P value of <0.05 was considered statistically significant.

References

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Supplementary Table 1. Tregs present in CLL patients (n=19)

Patient number	% CD38+ B cells	CD38 (sAbc), B cells	% Tregs	Tregs count
1	38.01	3528	16.09	35840
2	32.31	3548	10.58	36547
3	36.46	3801	10.34	25358
5	24.55	2818	12.09	31782
6	98.00	12558	5.4	21552
7	37.43	3489	17.22	32785
8	6.3	776	5.66	18328
9	10.54	954	8.29	10777
10	46.71	8317	19.47	34582
11	13.39	2454	15.17	33684
12	13.12	2511	5.41	26325
14	18.11	3025	14.26	35214
15	36.1	3801	13.58	29659
16	38.54	4142	18.74	36366
17	45.95	5498	30.79	36584
18	7.58	1011	7.35	10558
19	15.98	2544	14.88	39885
21	13.99	2358	6.18	16328
22	37.49	NA	18.77	42547

Supplementary Table 2. Absolute cell numbers (±SEM) of human B and T-cells isolated from PDX mice spleens.

	Vehicle group *(count/spleen)*	**Daratumumab group** *(count/spleen)*	**Kuromanin group** *(count/spleen)*
B-cells	39086±993	28943±1474	25615±1667
Bregs	14071±357	2199±138	1844±120
Tregs	22136±1916	3326±1350	3316±778
Th1	728801±22677	489564±25489	502985±17515
Th2	627683±42018	586854±15637	766494±15607
Th17	152843±4825	254212±8275	176494±6135
cTL	152843±4825	254212±8275	176494±6135