SUPPLEMENTARY APPENDIX

Combining ibrutinib and checkpoint blockade improves CD8+ T-cell function and control of chronic lymphocytic leukemia in Eμ-TCL1 mice

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Supplementary Material and Methods

Flow cytometry and functional testing

After preparation of single cell suspensions as described before,(1) cells were incubated with recommended dilutions of antibodies against cell surface proteins in PBS containing 0.1% fixable viability dye (eBioscience, Frankfurt am Main, Germany) for 30 minutes at 4°C. Cells were fixed using IC fixation buffer (eBioscience), washed and stored at 4°C in the dark until analyzed by flow cytometry.

For labelling of cells in whole blood, 50-100 µl of PB were stained with antibodies specific for surface molecules for 30 minutes at 4°C, followed by incubation for 10 minutes with 2 ml of 1X BD FACS™ lysing solution (BD Biosciences, Heidelberg, Germany) to remove erythrocytes. After centrifugation, supernatants were carefully aspirated and pelleted cells were resuspended in 150 µl of 1X BD FACSLysing solution. 50 µl of 123count eBeads™ (eBioscience, Frankfurt am Main, Germany) were directly added before data acquisition. Absolute cell numbers in blood were calculated according to the formula: absolute count (cells/µL) = (cell count **x** bead volume **x** bead concentration) **/** (bead Count **x** cell Volume).

For transcription factor staining, cells were fixed after surface molecule staining with FoxP3 fixation/permeabilization buffer (eBioscience) for 30 minutes at room temperature, followed by permeabilization with 1X permeabilization buffer (eBioscience) and staining with antibodies against transcription factors or IL-10 in 1X permeabilization buffer for 30 minutes at room temperature. After washing twice with 1X permeabilization

buffer, cells were resuspended in a final volume of 200 µl 1X permeabilization buffer and stored at 4°C in the dark until analyzed by flow cytometry.

Cytokine release, granzyme B production and degranulation capacity of $CD8^+$ T-cells were assayed as previously described with minor modifications.(2) Cells were resuspended in complete medium (DMEM supplemented with 10% FSC, 10 mM HEPES, 1 mM sodium pyruvate, 0.1% β-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin), and seeded at a density of $3x10^6$ cells/200 µl. Cells were incubated in cell stimulation cocktail in the presence of protein transport inhibitor cocktail (both from eBioscience) for 6 hours at 37 °C/ 5% CO_2. Degranulation capacity of T-cells was measured by adding fluorochrome-conjugated anti-CD107a antibody (eBioscience) to the culture as previously described.(3) Afterwards, cells were washed, stained for surface proteins and fixed using IC fixation buffer (eBioscience). After washing with 1X permeabilization buffer, cells were stained with fluorescently labelled antibodies against intracellular proteins for 30 min at room temperature in the dark, washed and analyzed by flow cytometry.

For all flow cytometric measurement, data acquisition was done using BD LSRII or BD FACSCanto flow cytometer (BD Biosciences). Median Fluorescence Intensity (MFI) was recorded and normalized by subtracting the MFI of the respective fluorescence-minus-one (FMO) control. Data analysis was performed using FlowJo X 10.0.7 software (FlowJo, Ashland, OR, USA).

References

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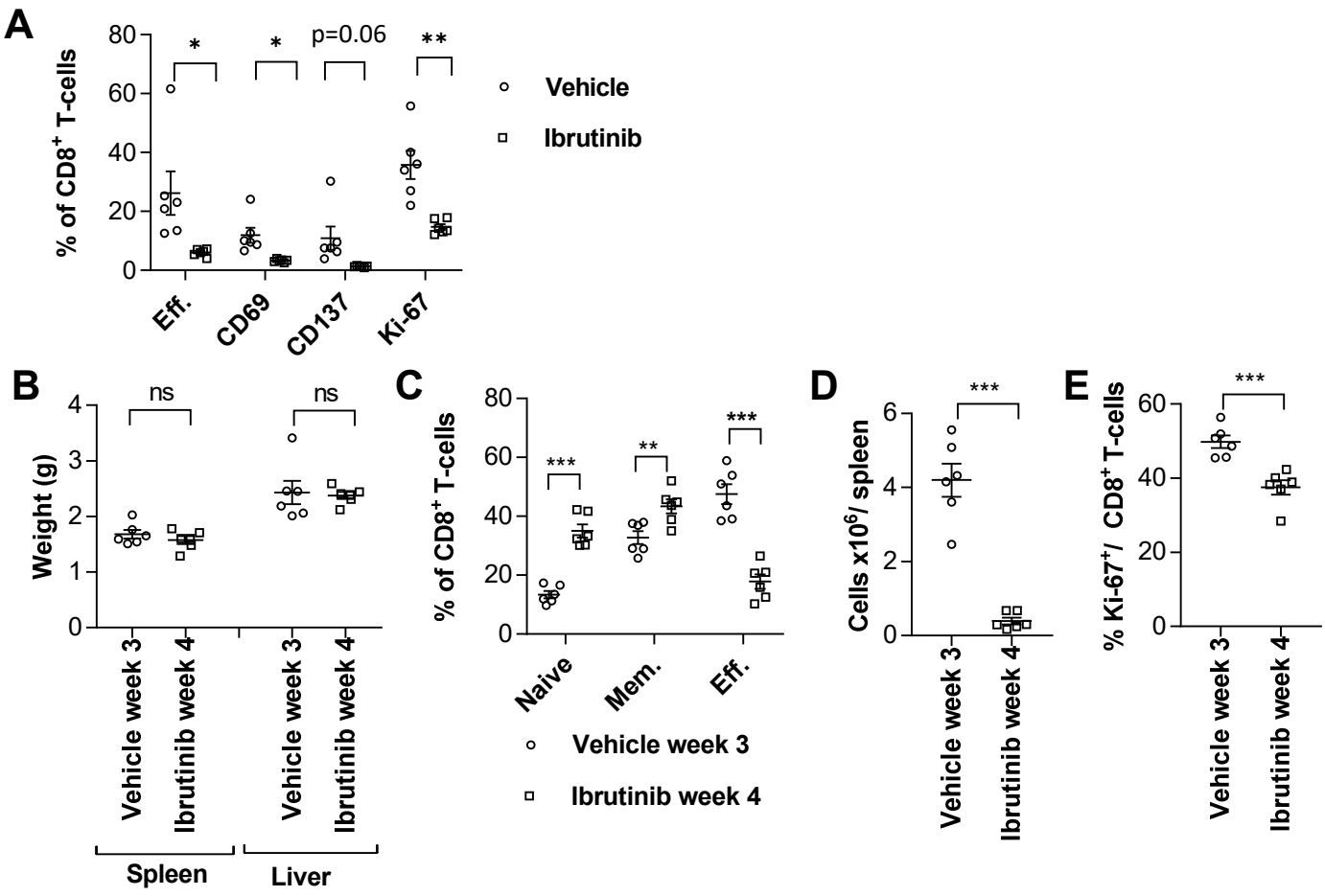
3. Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods. 2003 Oct 01;281(1-2):65-78.

Supplementary Table 1: List of flow cytometry antibodies

Mouse antibodies	Clone	Supplier
anti-mouse CD107a (LAMP-1)	eBio1D4B	eBioscience
anti-mouse CD19	eBio1D3	eBioscience
anti-mouse CD19	6D5	Biolegend
anti-mouse CD3e	145-2C11	eBioscience
anti-Mouse CD3e	500A2	BD
anti-mouse CD4	GK1.5	Biolegend
anti-mouse CD4	RM4-5	eBioscience
anti-mouse CD44	IM7	eBioscience
anti-mouse CD45	30F-11	Biolegend
anti-mouse CD5	53-7.3	BD
anti-mouse CD69	H1.2F3	Biolegend
anti-mouse CD8a	53-6.7	Biolegend
anti-mouse CD127	A7R34	Biolegend
Anti-mouse CD137	17B5	Biolegend
anti-mouse CD62L	MEL-14	eBioscience
anti-mouse CD25	PC-61	Biolegend
anti-mouse Granzyme B	NGZB	eBioscience
anti-mouse IFNg	XMG1.2	eBioscience
anti-mouse GITR	YGL 386	Biolegend
anti-mouse Lag3	eBioC9B7W	eBioscience
anti-mouse PD-1	RMP1-30	Biolegend
anti-mouse CD244	m2B4 (B6)458.1	Biolegend
anti-mouse Ki67	SolA15	eBioscience
anti-mouse Eomes	Dan11mag	eBioscience
anti-T-bet	4B10	Biolegend

Supplementary Figure 1

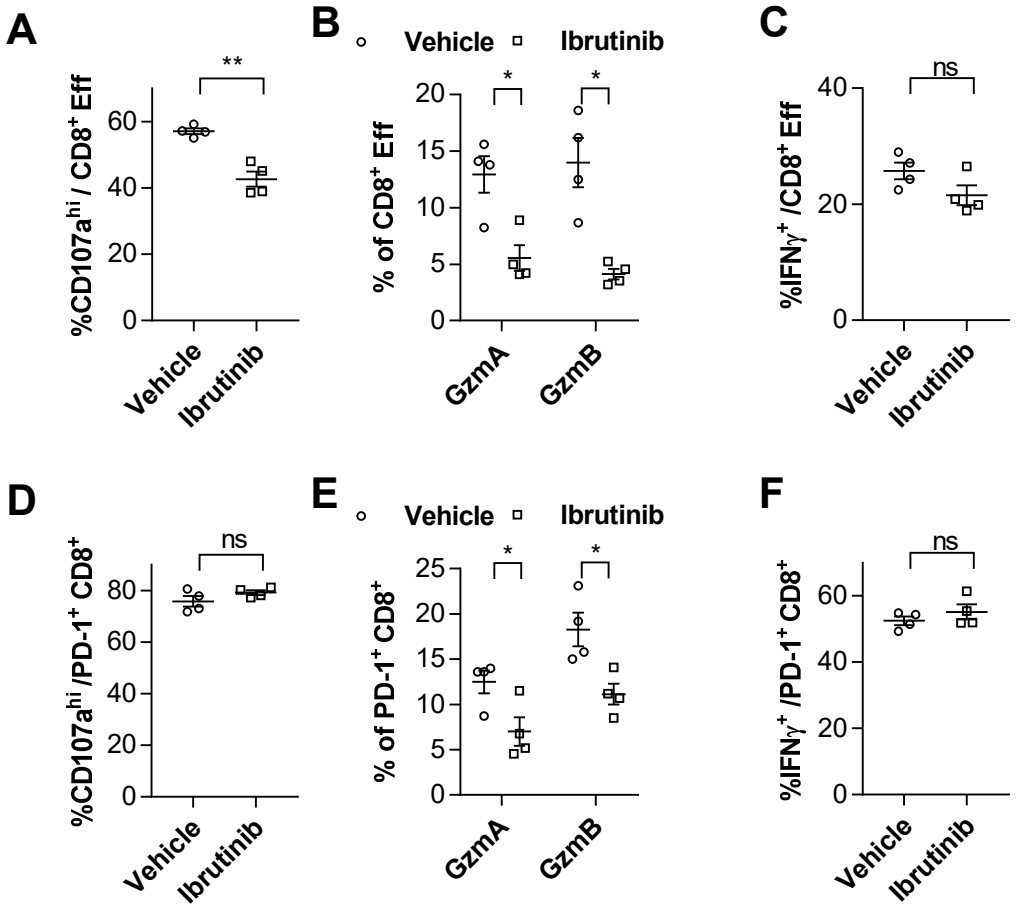


Supplementary Figure 1: Ibrutinib modulates effector CD8⁺ T-cell differentiation and proliferation in the TCL1 adoptive transfer model

C57BL/6 mice were transplanted with splenocytes from leukemic TCL1 mice, and later assigned to treatment with ibrutinib (0.16 mg/mL) or vehicle control in drinking water. **A)** Treatment started when all mice reached the leukemic threshold (5,000 CLL cells/µl of blood). Graph shows the impact of the treatment on CD8⁺ T-cell effector population, activation and proliferation. **B-E)** Vehicle- and ibrutinib-treated mice were sacrificed after 3 and 4 weeks of treatment, respectively, when mice reached disease end-stage. **B)** Spleen and liver weight of vehicle- or ibrutinib-treated mice (n=5). **C)** Flow cytometric analysis of splenic $CD3^+CD8^+$ T-cell subsets from vehicle- or ibrutinib-treated mice (n=5-6). **D)** Absolute numbers of splenic $CD8^+$ effector cells from vehicle- or ibrutinib-treated mice (n=5-6) were assessed by flow cytometry. **E)** Flow cytometric analysis of Ki-67 expression in $CD8^+$ T-cells from vehicle- or ibrutinib-treated mice (n=5-6).

Graphs show means ± SEM. *p<0.05 , **p<0.01, ***p<0.001, ns = not significant

Supplementary Figure 2

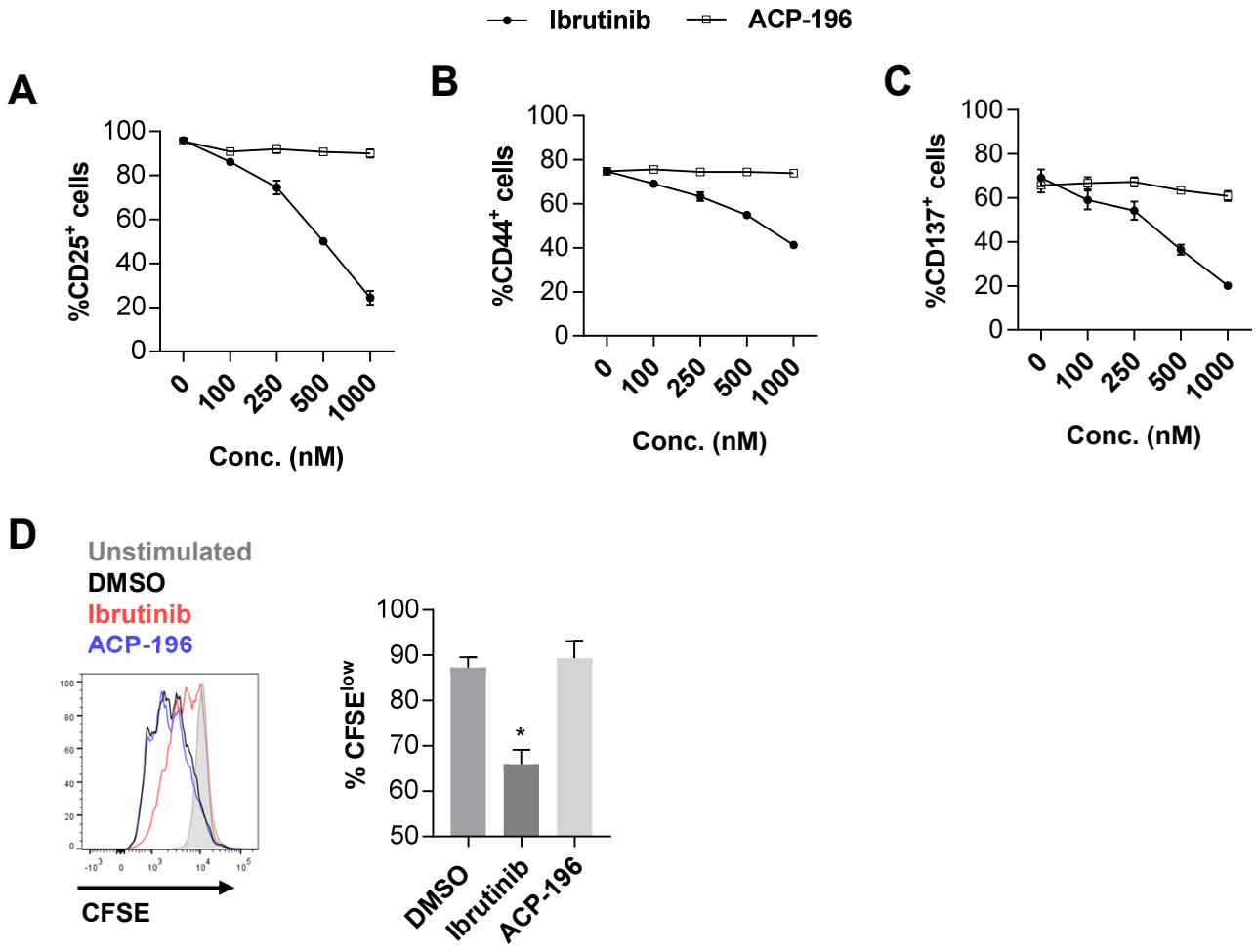


Supplementary Figure 2: Ibrutinib modulates CD8⁺ T-cell function in the TCL1 adoptive transfer model

C57BL/6 mice were transplanted with splenocytes from leukemic TCL1 mice, and after two weeks assigned to treatment with ibrutinib (0.16 mg/mL) or vehicle control in drinking water. Mice were sacrificed after 2 weeks of treatment. **A-C)** Cytotoxic function of CD8⁺ effector T-cells, and **D-F)** of PD-1⁺CD8⁺ T-cells was assessed by flow cytometric analysis of degranulation capacity, as measured by CD107a expression on the cell surface, GzmA and GzmB expression or IFNγ production (n=4).

Graphs show means ± SEM. *p<0.05, **p<0.01, ns = not significant; nMFI = normalized median fluorescence intensity

Supplementary Figure 3

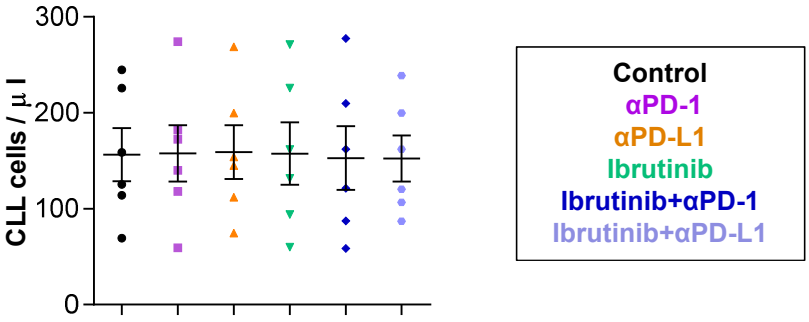


Supplementary Figure 3: Ibrutinib modulates T-cell receptor signaling in CD8⁺ T-cells

A-C) Splenocytes from C57BL/6 mice (n=4) were pretreated with different concentrations of ibrutinib, ACP-196 or DMSO for 30 minutes and then stimulated with anti-CD3 antibody. CD25, CD44 and CD137 expression were analyzed by flow cytometry in viable, 7-AAD-negative, single CD8⁺ T-cells after 24 hours. **D)** Splenocytes from C57BL/6 mice (n=4) were labeled with 5 µM CFSE, pretreated with 1 µM of ibrutinib, ACP-196 or DMSO for 30 minutes, and then stimulated with anti-CD3 antibody. Proliferation as measured by CFSE dilution after 48 hours was analyzed by flow cytometry in viable, 7-AAD-negative, single CD8⁺ T-cells.

Graphs show means ± SEM. *p<0.05; MFI= median fluorescence intensity

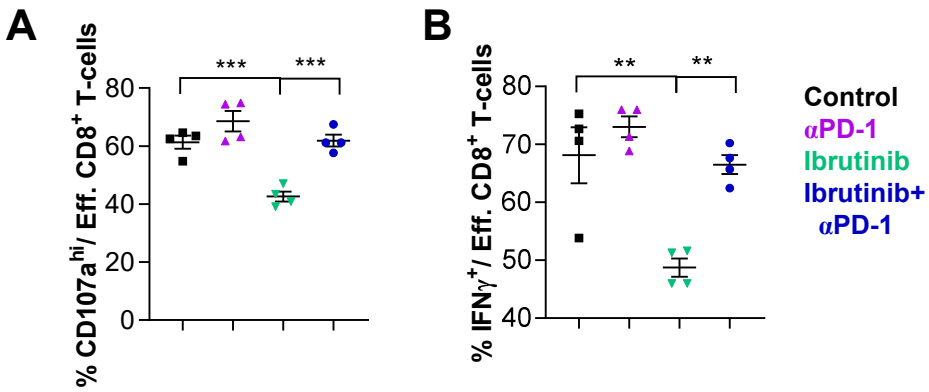
Supplementary Figure 4



Supplementary Figure 4: Blocking PD-1/PD-L1 axis enhances the anti-leukemic activity of ibrutinib

C57BL/6 mice were transplanted with splenocytes from leukemic TCL1 mice, and after two weeks assigned to treatment with isotype antibody plus vehicle (control), αPD-1, αPD-L1, ibrutinib, ibrutinib + αPD-1, or ibrutinib + αPD-L1. Graph shows the absolute numbers of $CD5^{+}CD19^{+}$ CLL cells in peripheral blood prior to treatment.

Supplementary Figure 5



Supplementary Figure 5: Blocking PD-1/PD-L1 axis expands the effector population and enhances the functional capacity of CD8⁺ T-cells

C57BL/6 mice were transplanted with splenocytes from leukemic TCL1 mice, and after two weeks assigned to treatment with isotype antibody plus vehicle (control), αPD-1, ibrutinib, or ibrutinib + αPD-1. Mice were sacrificed after 4 weeks of treatment. Cytotoxic function of CD8⁺ effector T-cells was assessed by flow cytometric analysis of **A)** degranulation capacity, as measured by CD107a expression on the cell surface, and **B)** IFNγ production (n=4).

Graphs show means ± SEM. **p<0.01, ***p<0.001