Supplementary Information For:

Combining nilotinib and PD-L1 blockade reverses $CD4^+$ T-cell dysfunction and prevents relapse in acute B-cell leukemia

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Supplementary Methods

Mice

CD45.1$^+$ and CD45.2$^+$ C57BL/6 mice were from Charles River Laboratories. CD90.1$^+$ *SM1xRag2*$^{-/-}$ mice, *Nur77-GFP, Cd4-Cre* and *Rag2*$^{-/-}$ mice were previously described[1-4]. All experiments were approved by the UMN Institutional Animal Care and Use Committee. Mice were 7–16 weeks old and randomly selected for experiments in age-matched cohorts.

T-cell isolation from leukemic mice

CD45.2$^+$ C57BL/6 mice were challenged with 2500 LM138 cells via tail vein injection. The mice were sacrificed at the indicated time-points post-leukemia challenge. The spleen and bone marrow were isolated. The spleen was processed into a single-cell suspension using frosted slides while the bone marrow was flushed using an insulin syringe. The single cell suspensions from the spleen and bone marrow were filtered using a 70μM filter (BD Biosciences). LM138 cells (or other leukemia cell lines) from the spleen were depleted by staining with biotinylated antibodies against CD19 and B220, followed by depletion using Streptavidin RapidSpheres (StemCell). T-cells from the LM138-depleted spleen and bone marrow were isolated using the EasySep CD90.2 positive-selection kit (StemCell).

Ex-vivo Restimulation with PMA and Ionomycin

The isolated enriched murine T-cells were incubated with PMA (20ng/mL) and ionomycin (1μg/mL), or DMSO, in a 96-well plate. One hour later, 1μg/mL GolgiPlug and 1.5μg/mL GolgiStop (BD BioSciences) were added. Five hours after the addition of GolgiPlug and GolgiStop, the cells were isolated for flow cytometry.

Adoptive Transfer Experiments with *SM1xRag2*$^{-/-}$ Mice

T-cells were enriched from the spleens of CD90.1$^+$ *SM1xRag2*$^{-/-}$ mice using biotinylated antibodies against CD11b (clone M1/70 Tonbo, 1:100), CD11c (clone M418, Tonbo, 1:100), CD19 (clone 1D3, Tonbo, 1:100), B220 (Clone RA3-6B2, Tonbo, 1:100), GR1 (Clone Rb6-8C5, Tonbo, 1:100), NK1.1 (Clone PK136, Biolegend, 1:100), and Ter119 (Clone: TER119, Biolegend, 1:100) followed by depletion using LD columns and Streptavidin Microbeads (Miltenyi Biotec). 5×10^5 T-cell-enriched splenocytes were transferred into CD45.2$^+$CD90.2$^+$ C57BL/6 mice via intravenous injection. At the same time, mice were either challenged with 2500 LM138 cells or PBS via intravenous injection. Six days later, the mice were immunized with 20μg FliC peptide (VQNRFNSAITNLGNT: Genscript) plus 10μg MPLA-SM (Invivogen) subcutaneously on the hind flank.

On day 16 post-leukemia challenge, LM138 cells from the spleen were depleted by staining with biotinylated antibodies against CD19 and B220, followed by depletion using Streptavidin RapidSpheres (StemCell). Single-cell suspensions of LM138-depleted spleen and bone marrow cells were stained with anti-mouse CD90.1 (OX-7) PE, followed by enrichment of CD90.1$^+$ SM1-transgenic T-cells using the EasySep PE

positive-selection kit (StemCell). The enriched SM1-transgenic T-cells and the WT T-cells were analyzed via flow cytometry.

Re-challenge experiments
CD45.2$^+$ C57BL/6 mice were challenged with 2500 LM138 cells via intra-venous tail vein injection and treated with nilotinib plus anti-PD-L1, as described before. Forty-six days after the start of treatment (i.e., 60 days post-leukemia challenge), the absence of leukemia in the blood was confirmed using flow cytometry. Mice with no detectable leukemia in the blood were challenged with 2500 LM138 cells via intra-venous tail vein injection. Naïve CD45.2$^+$ C57BL/6 mice were also challenged as controls. Sixteen days-post leukemia challenge, the leukemia burden in the spleen was determined using flow cytometry.

Generation of Retrovirus containing the dominant clonotype TCR
The pMigR flox-GFP TCRa IRES TCRb plasmid (pMigR^{TCR}) and the Platinum-E packaging cell line were obtained from Dr. Kris Hogquist at the University of Minnesota. Gene blocks containing the *TCRa* and *TCRb* genes for the dominant clonotype were obtained from IDT. The TCR for the dominant clonotype was cloned into the pMigR^{TCR} vector using standard molecular biology techniques. The Platinum-E packaging cell line was transfected with the pMigR^{TCR} vector containing the dominant clonotype TCR and the pCL-Eco retroviral packaging vector (Novus Biologicals, Littleton, CO). The next day, fresh media supplemented with 30μM water-soluble cholesterol (Sigma Aldrich) and 1:500 ViralBoost Reagent (AlStem Bio) was added. Forty-eight hours later, the supernatant containing the retrovirus was harvested and filtered using a 0.45-micron filter.

Generation of Retrogenic mice producing the dominant clonotype TCR
Retrogenic mice were generated as previously described[5,6], except bone marrow from *Cd4-CrexRag2*$^{-/-}$ donor mice was used to infect with retroviruses containing the dominant clonotype TCR. Transduced bone marrow cells were used to reconstitute sub-lethally irradiated CD45.1$^+$ WT recipients (5x10^6 transfected bone marrow cells per recipient mouse). Mice were bled to test for TCR reconstitution 8 weeks post-transplant.

In-vitro activation assay using retrogenic T-cells
Splenic B cells were isolated from a WT CD45.1$^+$ mouse using CD19 microbeads and LS columns (Miltenyi Biotec). LM138 leukemia cells and the isolated B cells were both pre-treated with 1ng/mL recombinant mouse IFNγ (R&D Systems, Cat#: 485-MI) for 48 hours. The dominant clonotype T-cells were isolated from 8-week-old retrogenic mice via enrichment for CD45.2 using the EasySep APC positive selection kit (StemCell) and cultured with IFNγ pre-treated LM138 cells or IFNγ pre-treated WT B cells at a 10:1 ratio in a 96-well plate. T-cells cultured with 20ng/mL PMA were used as a positive control while unstimulated T-cells were used as a negative control. The activation of the dominant clonotype T-cells was assayed via CD44 expression using flow cytometry.

Flow-cytometry

The flow cytometry antibodies for murine and human T-cells and LM138 cells are in the table below. Intracellular staining was done using the FOXP3-transcription factor staining kit (ThermoFisher), as per the manufacturer's instructions. Cells from enriched fractions were analyzed on LSRII or Fortessa cytometers (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo (TreeStar, Ashland, OR).

Mouse

Target	Clone	Fluorophore(s)	Manufacturer	Dilution
CD4	GK1.5	BUV496	BD Biosciences	1:200
CD8	53-6.7	BV650	BD Biosciences	1:200
CD19	1D3	BV421, BV786	BD Biosciences	1:200
CD44	IM7	PerCP-Cy5.5, BV786	Invitrogen, Biolegend	1:200
CD90.1	OX-7	PE	Biolegend	1:400
CD90.2	53-2.1	BUV395	BD Biosciences	1:400
NK1.1	PK136	Biotin	Biolegend	1:200
TCRb	H57-597	BUV395	BD Biosciences	1:400
TCR Vb7	TR310	PE, FITC	Biolegend	1:200
GhostDye™Red-780	N.A.	APC-eF780	Tonbo Biosciences	1:1000
PD1	J43	PE-Cy7	Biolegend	1:100
TIM3	RMT3-23	BV605	Biolegend	1:100
LAG3	C9B7W	APC, BV786	Biolegend	1:100
CD40L	MR1	APC	Biolegend	1:200
FOXP3	FJK-16s	AF488, eF450	ThermoFischer	1:100
IFNγ	XMG1.2	BV421	Biolegend	1:100
TNF	MP6-XT22	PE	Biolegend	1:100
GZMB	GB11	FITC, PB	Biolegend	1:100

Human

Target	Clone	Fluorophore(s)	Manufacturer	Dilution
CD3	UCHT1	Pacific Blue	Biolegend	1:200
CD4	SK3	BV711	Biolegend	1:200
CD8	SK1	FITC	Biolegend	1:200
IL7R	A019D5	PE/Fire 640	Biolegend	1:100
GhostDye™Red-780		APC-eF780	Tonbo Biosciences	1:1000
PD1	EH12.2H7	APC	Biolegend	1:100
TIM3	F38-2E2	PE/Cy7	Biolegend	1:100
GZMB	GB11	PE/Dazzle	Biolegend	1:100

Vaccination with heat-killed LM138 leukemia cells
Heat-killed leukemia cells were generated by heating LM138 leukemia cells in PBS at 70℃ for 30 minutes. Vaccination with heat-killed leukemia cells was done by creating an emulsion of the heat-killed LM138 cells and complete freund's adjuvant at a density of 10^7 dead leukemia cells/mL, followed by sub-cutaneous injection of 100μL of the emulsion per mouse. Ten days after the first challenge, the mice were boosted with 5 x 10^6 heat-killed leukemia cells plus 10μg MPLA in a volume of 100uL, subcutaneously. The mice were sacrificed for harvest 7 days later.

Single cell bioinformatics analyses
The mouse and human datasets were processed using similar methods, except where noted. Raw sequencing data was processed using the 10X Genomics cellranger software (ver. 5.0.1) mkfastq function to demultiplex the Illumina libraries into gene expression (GEX), CITEseq expression (HTO and/or ADT), and T-cell receptor (TCR) datasets. The cellranger count function was used to align reads to the mouse or human reference genomes (refdata-gex-mm10-2020-A, refdata-gex-GRCh38-2020-A, refdata-cellranger-vdj-GRCm38-alts-ensembl-5.0.0, refdata-cellranger-vdj-GRCh38-alts-ensembl-5.0.0; provided by 10X Genomics). Information on the HTO and ADT antibodies are in the tables below:

Hashtag (HTO) antibodies:

Dataset	BioLegend Product Number	Sample (tissue, expt arm or patient ID)	Barcoded Read	Barcode pattern	Barcode Sequence
Mouse	C0301	bone_marrow1	R2	5PNNNNNNNNNN(BC)	ACCCACCAGTAAGAC
Mouse	C0302	bone_marrow2	R2	5PNNNNNNNNNN(BC)	GGTCGAGAGCATTCA
Mouse	C0303	bone_marrow3	R2	5PNNNNNNNNNN(BC)	CTTGCCGCATGTCAT
Mouse	C0304	bone_marrow4	R2	5PNNNNNNNNNN(BC)	AAAGCATTCTTCACG
Mouse	C0305	bone_marrow5	R2	5PNNNNNNNNNN(BC)	CTTTGTCTTTGTGAG
Mouse	C0306	spleen1	R2	5PNNNNNNNNNN(BC)	TATGCTGCCACGGTA
Mouse	C0307	spleen2	R2	5PNNNNNNNNNN(BC)	GAGTCTGCCAGTATC
Mouse	C0308	spleen3	R2	5PNNNNNNNNNN(BC)	TATAGAACGCCAGGC
Mouse	C0309	spleen4	R2	5PNNNNNNNNNN(BC)	TGCCTATGAAACAAG
Mouse	C0310	spleen5	R2	5PNNNNNNNNNN(BC)	CCGATTGTAACAGAC
Human	C0251	patient1	R2	5PNNNNNNNNNN(BC)	GTCAACTCTTTAGCG

Human	C0252	patient2	R2	5PNNNNNNNNNN(BC)	TGATGGCCTATTGGG
Human	C0253	patient3	R2	5PNNNNNNNNNN(BC)	TTCCGCCTCTCTTTG
Human	C0254	patient4	R2	5PNNNNNNNNNN(BC)	AGTAAGTTCAGCGTA
Human	C0255	patient5	R2	5PNNNNNNNNNN(BC)	AAGTATCGTTTCGCA

CITEseq (ADT) antibodies

Dataset	BioLegend Product Number	Protein Target	Barcoded Read	Barcode Pattern	Barcode Sequence
Mouse	C0195	TNFRSF4	R2	5PNNNNNNNNNN(BC)	CTCACCTACCTATGG
Mouse	C0097	IL2RA	R2	5PNNNNNNNNNN(BC)	ACCATGAGACACAGT
Mouse	C0004	PDCD1	R2	5PNNNNNNNNNN(BC)	GAAAGTCAAAGCACT
Mouse	C0003	HAVCR2	R2	5PNNNNNNNNNN(BC)	ATTGGCACTCAGATG
Mouse	C0378	LAG3	R2	5PNNNNNNNNNN(BC)	ATTCCGTCCCTAAGG
Mouse	C0848	TIGIT	R2	5PNNNNNNNNNN(BC)	GAAAGTCGCCAACAG

Raw count tables were loaded into R (ver. 4.0.3) and analyzed with the Seurat (ver. 4.0.1) or tidyverse (1.3.1) R packages. The GEX dataset was filtered to include only gel beads in emulsion (GEMs, which are oil droplets containing uniquely barcoded beads that ideally contain one individual cell) expressing more than 300 genes (counts > 0) and genes expressed in more than 3 GEMs (counts > 0). The proportion of mitochondrial RNA in each GEM was calculated and GEMs with extreme levels (top 0.5% of all GEMs) were removed from the analysis. For the remaining GEMs, the raw HTO count table was supplied to GMM-demux software (ver. 0.2.1.3) to classify which HTO tags were detected in each GEM[7]. GEMs containing multiple HTOs (i.e. doublets or multiplets) were removed from downstream analysis.

Raw single cell surface protein expression data (ADT counts) were normalized according to the centered-log-ratio method using Seurat. Raw GEX counts were normalized and transformed using the Seurat SCTransform function including the percentage of mitochondria expression as a regression factor[8]. Each cell was classified according to its expression of canonical cell cycle genes using the SeuraT-cellCycleScoring function (S-phase and G2/M-phase gene sets provided by Seurat were originally developed by Tirosh et al[9]. Principal components analysis (PCA) was performed using the normalized, mean-centered, and scaled SCT dataset (RunPCA function). Two-dimensional projections were generated using the top 30 PCA vectors as input to RunUMAP function in Seurat. Cells were clustered using the FindNeighbors (top 30 PCA vectors) and FindClusters functions (testing a range of possible resolutions: 0.4, 0.5, 0.6, 0.7, 0.8, 0.9).

Both mouse and human datasets contained contaminating B cells (leukemic blasts) and myeloid populations. For the mouse studies, T-cells were identified using the distribution of normalized gene expression levels of selected marker genes. Cells with high levels of

Cd3e and *Cd3g* and low levels of *Cd79a* and *Ebf1* were classified as T-cells. For the human studies, after initial clustering of all cells, four distinct clusters that expressed high levels of *Cd3e* and *Cd3g* and low levels of *Cd79a* and *Ebf1* were classified as T-cells. For each dataset, PCA, clustering, and UMAP steps were repeated using only the T-cell subsets. Final clustering resolutions of 0.8 (mouse) and 0.7 (human) were selected for each dataset.

Pairwise differential expression (DE) testing (Wilcox rank-sum) using the Seurat FindMarkers function was performed between all clusters. DE genes were significant based on log2-fold-change (≥ 0.25) and BH adjusted p-value (≤ 0.01). Figures were generated using the ggplot2 R package (ver. 2.3.3.3)[10]. The distribution of normalized GEX and ADT expression levels were displayed for cells/clusters using color heatmaps on UMAPs, dot plots, and tile plots.

Raw and processed data have been deposited at the Gene Expression Omnibus (GEO) database (accession number: GSE195964).

Pseudotime Cell Trajectory Analysis

For the mouse studies, a pseudotime cell trajectory analysis was completed using the Singshot R package (ver. 1.8.0)[11]. The Seurat-based R object was converted for use with Slingshot using the Seurat function as.SingleCellExperiment. A trajectory was inferred with the slingshot function using cluster labels, UMAP coordinates, and the naïve/central-memory T-cell cluster (Tn/Tcm) as the root of the trajectory. Cell groups were compared (e.g. arm 1 vs. arms 2, 3, 4, 5) along each of the slingshot lineages using a differential topology approach. To determine statistical significance, a permutation test was performed. For each cell group, the weighted mean of pseudotime values was calculated using the slingshot curve weights. An initial test-statistic was calculated as the difference in weighted means between the two cell groups. A null distribution was generated by randomization of cell group labels and resampling 10,000 times. P-values were calculated as the number of test-statistics generated under the null distribution that were as or more extreme than the initial test-statistic (i.e. using real cell group labels).

Single cell differential composition analysis

The uncertainty associated with cell-type proportions was estimated for each cell group (experimental arm and cluster) using the R package scDC (ver. 0.1.0)[12]. A bias-corrected and accelerated (BCa) bootstrap confidence interval was generated from 50 bootstrap resampling iterations. For each iteration, the proportion of cells in the group was calculated. The median proportion across all resampling events was plotted with error bars representing the upper and lower 95% confidence interval.

Calculation of TCR Diversity

TCR Diversity for each cluster, subsetted by the treatment arm, was calculated using the inverse Simpson's index: $^1/_\lambda$, where

$$\lambda_{arm\ x,cluster\ y} = \sum_{All\ T\ cell\ clones\ in\ arm\ x,cluster\ y} \left(\frac{number\ of\ TCRs\ in\ the\ T\ cell\ clone}{total\ number\ of\ TCRs\ in\ arm\ x,cluster\ y}\right)^2$$

Statistical Analysis

Comparisons of two groups were done by either paired t-test (paired, normal data), Wilcoxon matched-pairs test (paired, non-normal data), t-test (non-paired, normal data) or Mann–Whitney (non-paired, non-normal data); tests were always two-sided. Comparison of three or more groups was done by one-way ANOVA (non-paired, normal data), two-way ANOVA (non-paired, normal data), or Kruskal–Wallis (non-paired, non-normal data). $P < 0.05$ was considered significant. Statistics were calculated using Prism (GraphPad Software). All data, except those specifically mentioned in figure legends, are displayed as median ± s.d.

References

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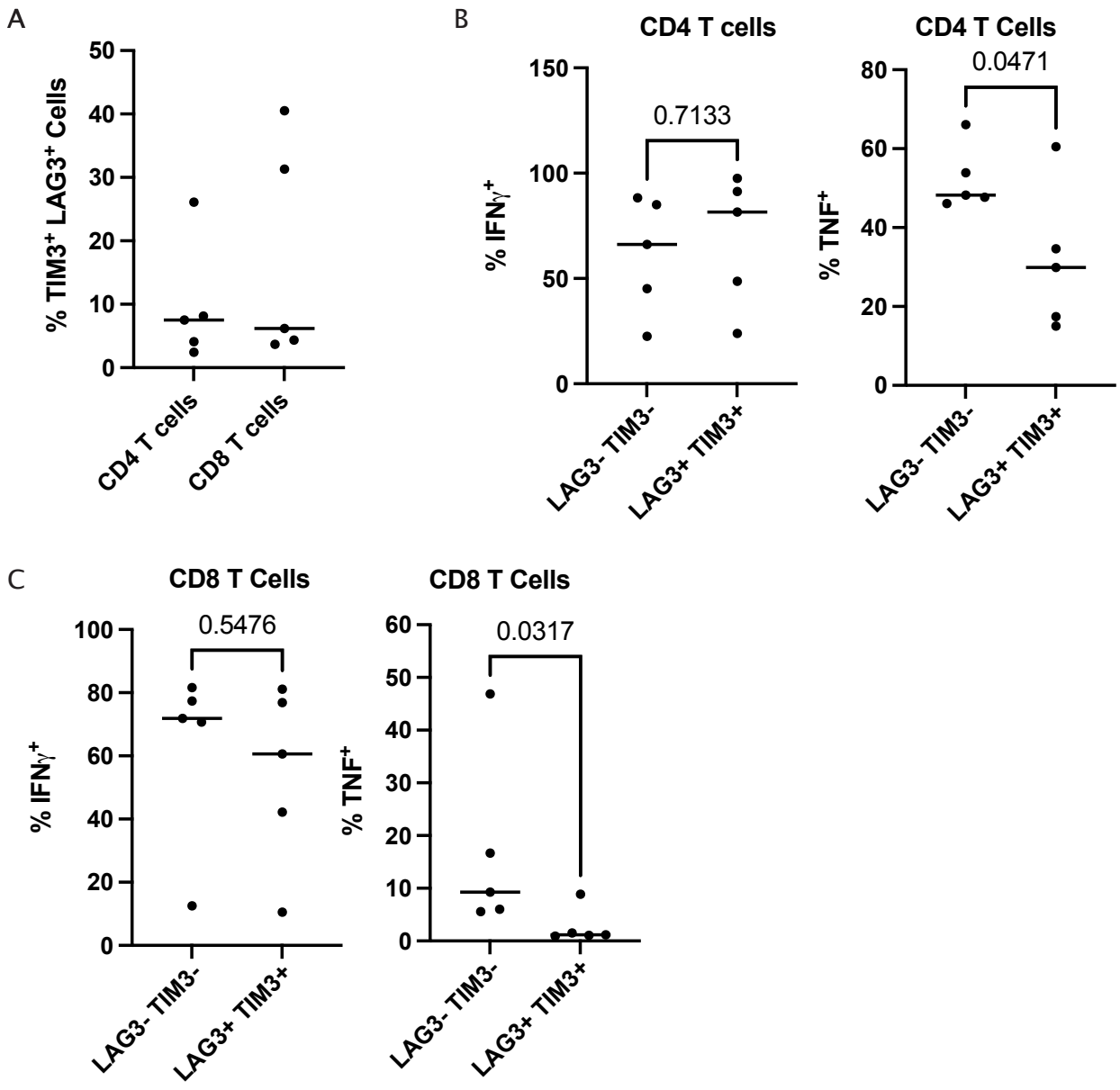
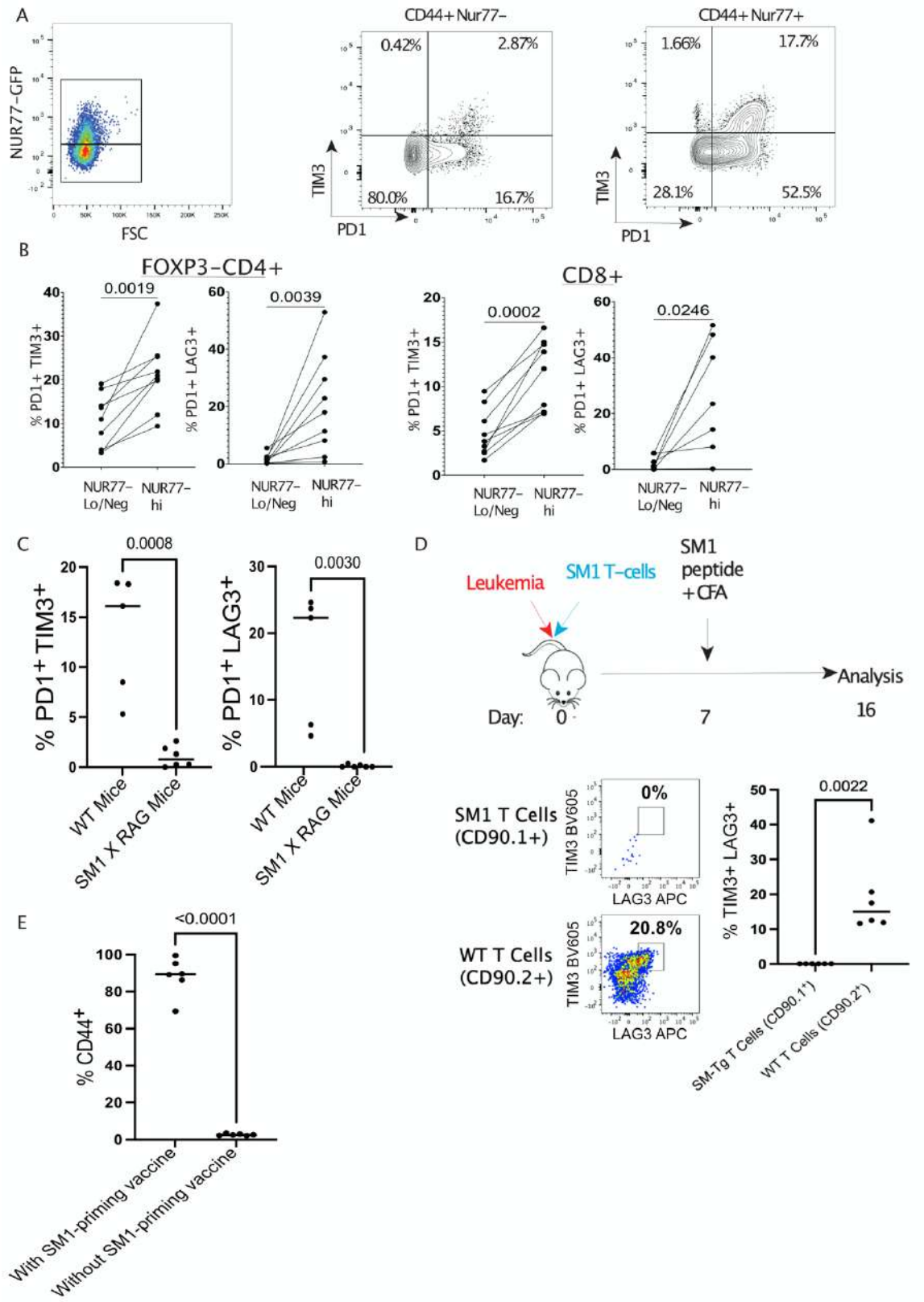
Supplementary Table 1 (Excel File): Excel file showing statistically significant differentially-expressed genes between CD4 T cells from either the bone marrow or spleen of the leukemic mice in Arms 2-5 (Figure 3A). Statistical significance was determined using a Wilcoxon Rank-Sum test. Refer to the Supplemental methods for details on analysis.

Supplementary Table 2 (Excel File): Pairwise DE analysis showing the top differentially-expressed genes in each cluster across the different treatment arms (Figure 3A, 3B, 5A). Each tab in the excel file contains a table of differentially-expressed genes from a particular cluster between 2 treatment arms. Statistical significance was determined using a Wilcoxon Rank-Sum test. Refer to the Supplemental methods for details on analysis.

Supplementary Table 3 (Excel File): Excel file showing raw cell counts for each T cell cluster in each treatment arm (Figure 5A, 5B).

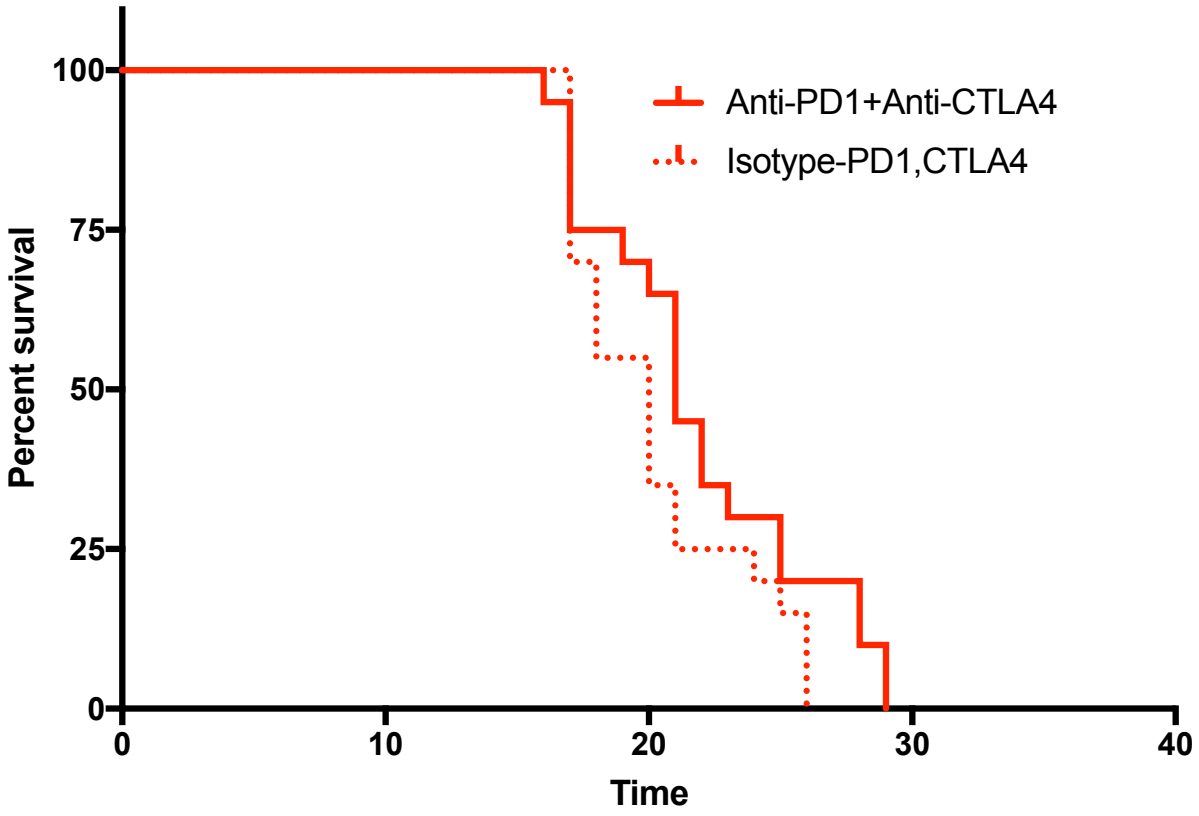


Supplementary figure 1: Phenotypic exhaustion of CD4⁺ and CD8⁺ T-cells occurs in *Pax5*$^{+/-}$ *x Ebf*$^{+/-}$ leukemias. A. T-cells from WT CD45.2⁺ mice challenged with 1 x 10^6 *Pax5*$^{+/-}$ *x Ebf*$^{+/-}$ leukemia cells were analyzed for expression of the indicated exhaustion markers. The expression of the exhaustion markers TIM3 and LAG3 in CD4⁺ and CD8⁺ T-cells is shown. The results are a summary of 2 experiments with n=5 mice.
B and C. T-cells from the *Pax5*$^{+/-}$ *x Ebf*$^{+/-}$ leukemia-challenged mice above were stimulated ex-vivo with PMA and ionomycin. The proportion of CD4⁺ (**B**) or CD8⁺ (**C**) T-cells producing IFNγ or TNF is shown. The data is shown as median, with normality of the data determined using a Shapiro-Wilk test and statistical significance determined using an unpaired t-test.

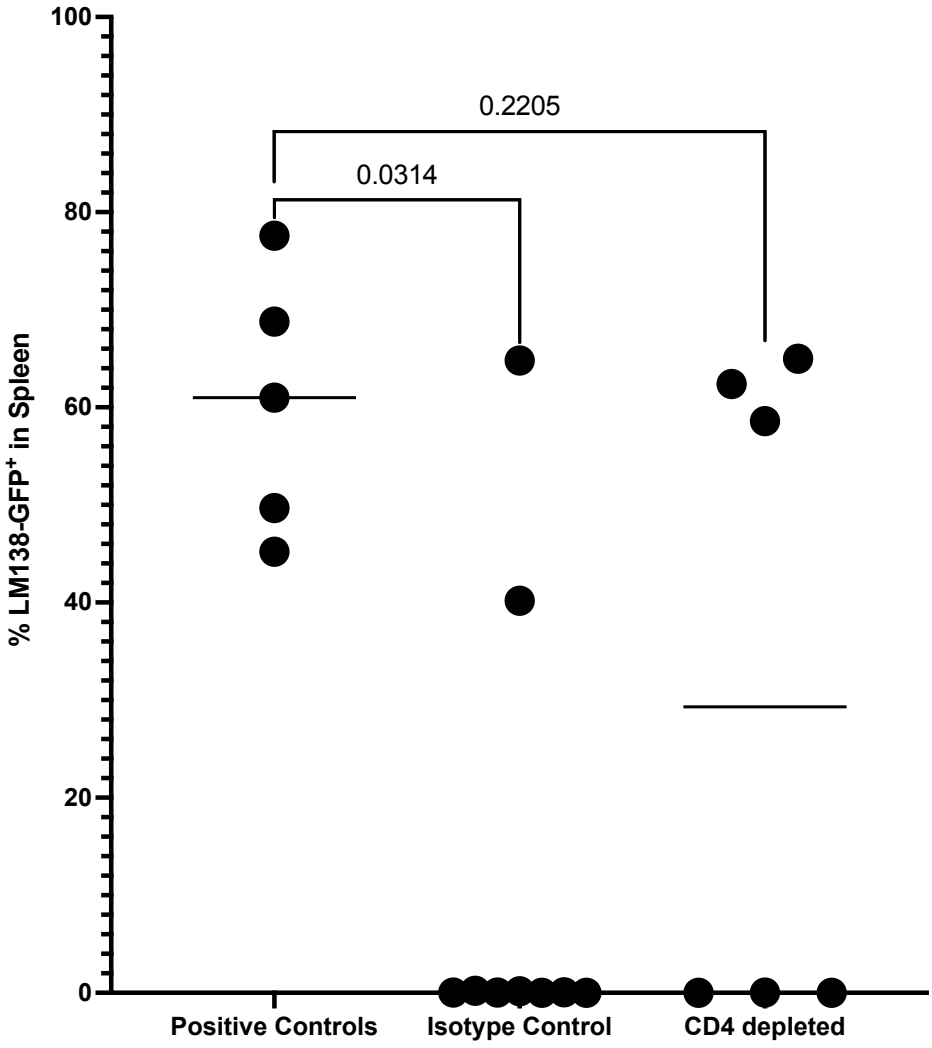


Supplementary Figure 2: Induction of exhaustion markers is dependent on TCR stimulation. A. NUR77-GFP mice were challenged with 2500 LM138 leukemia cells

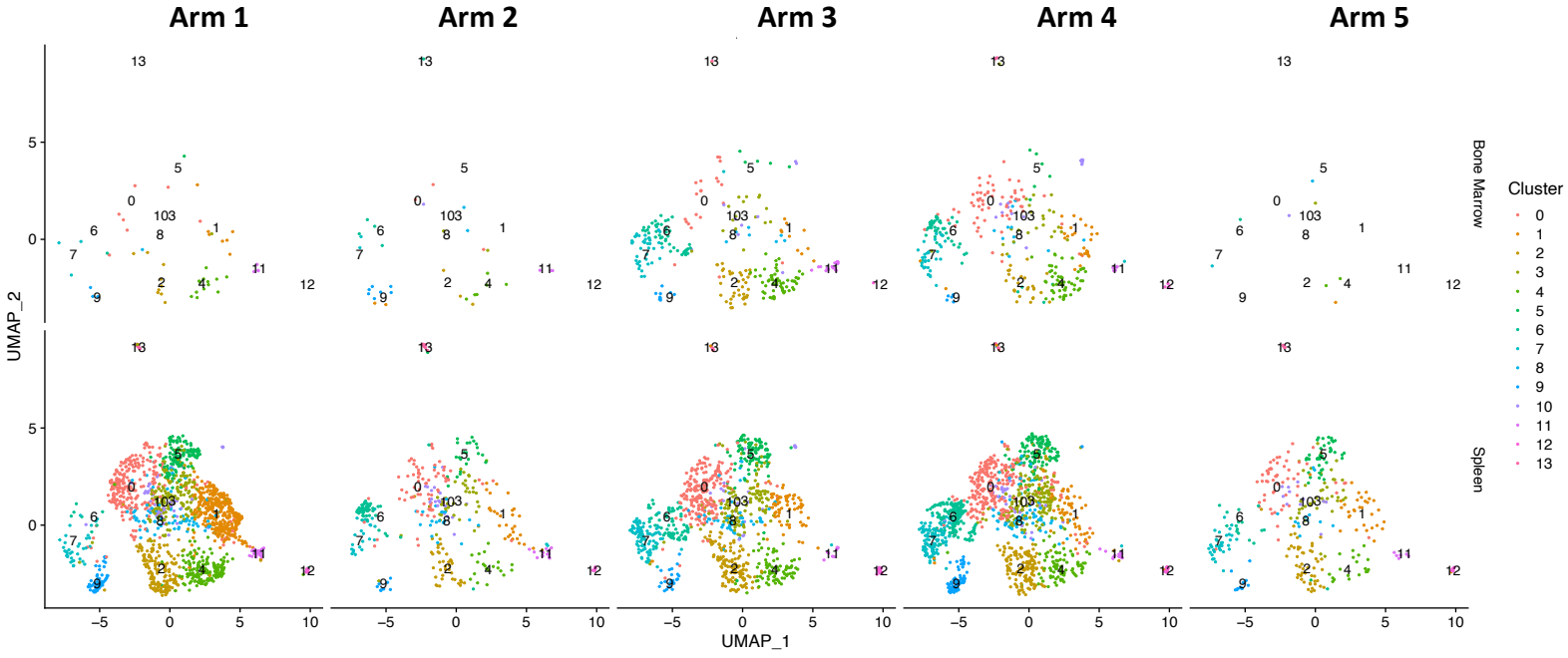
Representative flow plots showing expressing of exhaustion markers on NUR77-GFP^{hi} or NUR77-GFP$^{lo/-}$ cells at day 16 post-leukemia challenge. **B.** Plots showing the proportion of NUR77-GFP^{hi} or NUR77-GFP$^{lo/-}$ cells expressing the indicated exhaustion markers at day 16 post-leukemia. The data is shown as median, with statistical significance determined using a paired t-test. (n=8 mice, 3 experiments). **C.** WT CD45.2^{+} mice (n=5 mice) or *SM1xRag2*$^{-/-}$ mice (n = 6 mice) were challenged with 2500 LM138 leukemia cells. The proportion of CD4^{+} T-cells from each of the mice that express the indicated exhaustion markers is shown. The data from 2 independent experiments are shown as median, with statistical significance determined using an unpaired t-test. **D.** Flow plots and graph showing exhaustion marker expression in the transferred SM1-transgenic T-cells or endogenous T-cells (n=6 mice per group from 2 independent experiments). The median is shown, with statistical significance determined using an unpaired t-test. **E.** Expression of CD44 on the SM1-T cells from the *SM1xRag2*$^{-/-}$ mice in panel "C" (without SM1 priming vaccine) versus the transferred SM1-T cells in panel "D" (with SM1 priming vaccine). The data is shown as median, with statistical significance determined using an unpaired t-test.



Supplementary figure 3: Dual PD-1/CTLA4 mAb therapies do not prolong survival when administered at the time of leukemia establishment. WT CD45.2^{+} mice were challenged with 2500 LM138 leukemia cells and treated with anti-PD1 plus anti-CTLA4, or the indicated isotype controls, at the start of the leukemia challenge (n=10 mice per group, two independent experiments). The Kaplan-Meier survival curves for both treatment conditions are shown (p=0.12).



Supplementary figure 4: Rechallenge experiments show variable protection after CD4$^+$ T-cell depletion. WT mice were challenged with 2500 LM138 cells and treated with nilotinib and anti-PD-L1, as described before. At 60 days post-leukemia challenge, the mice were treated with isotype or a CD4-depleting antibody, as described in the methods, followed by re-challenge with 2500 LM138 leukemia cells. Naïve WT mice were concurrently challenged with 2500 LM138 cells as a control. The leukemia burden in the spleen at day 16 post-leukemia challenge is shown. The data is shown as median, with statistical significance determined using a Kruskal-Wallis test. The results of 2 experiments are summarized (positive control: n=5 mice, Isotype: n=9 mice, CD4-depleted: n=6 mice).



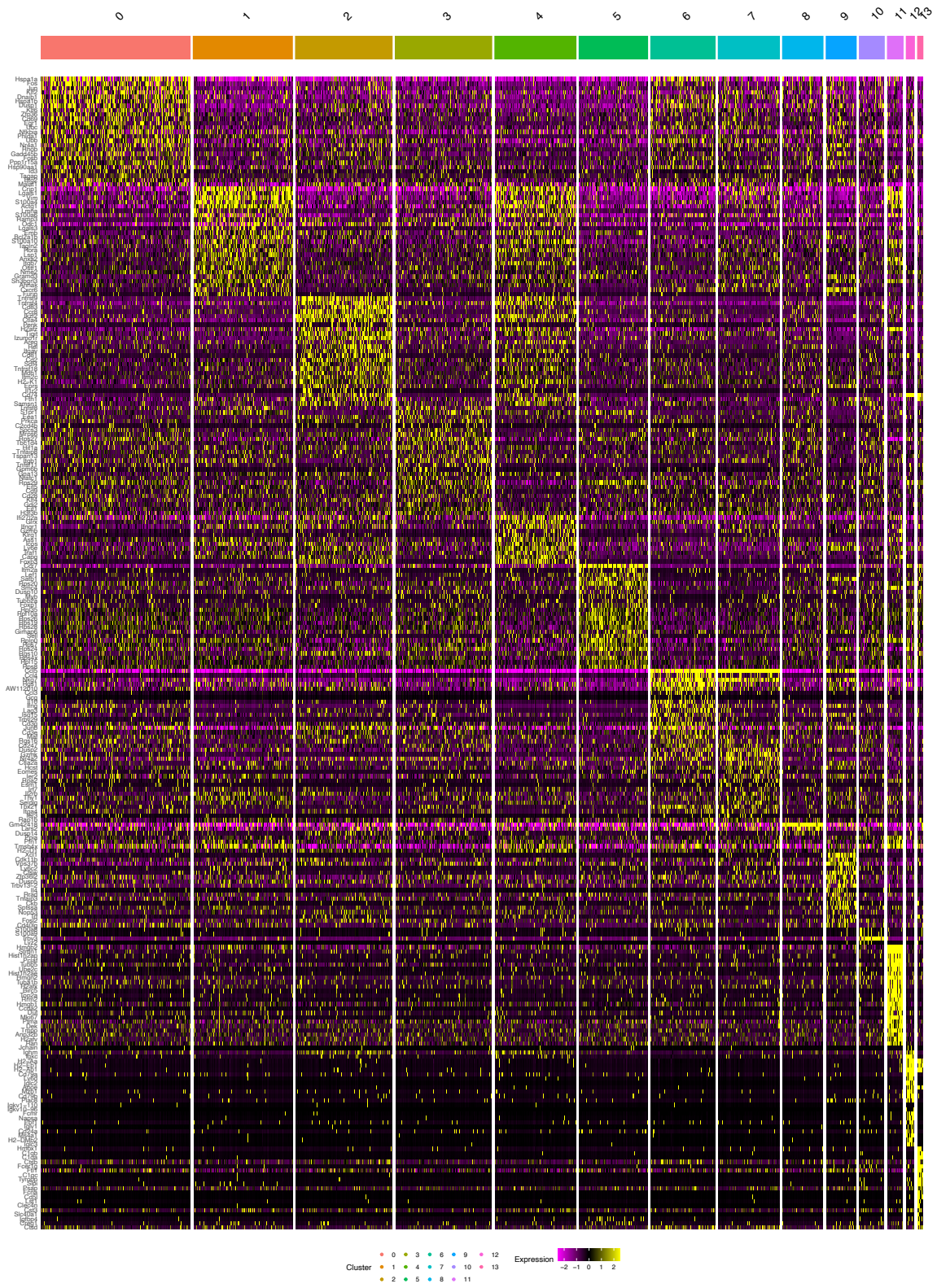
Bone Marrow

Treatment Arm	Cell Number
Arm 2	36
Arm 3	279
Arm 4	332
Arm 5	8

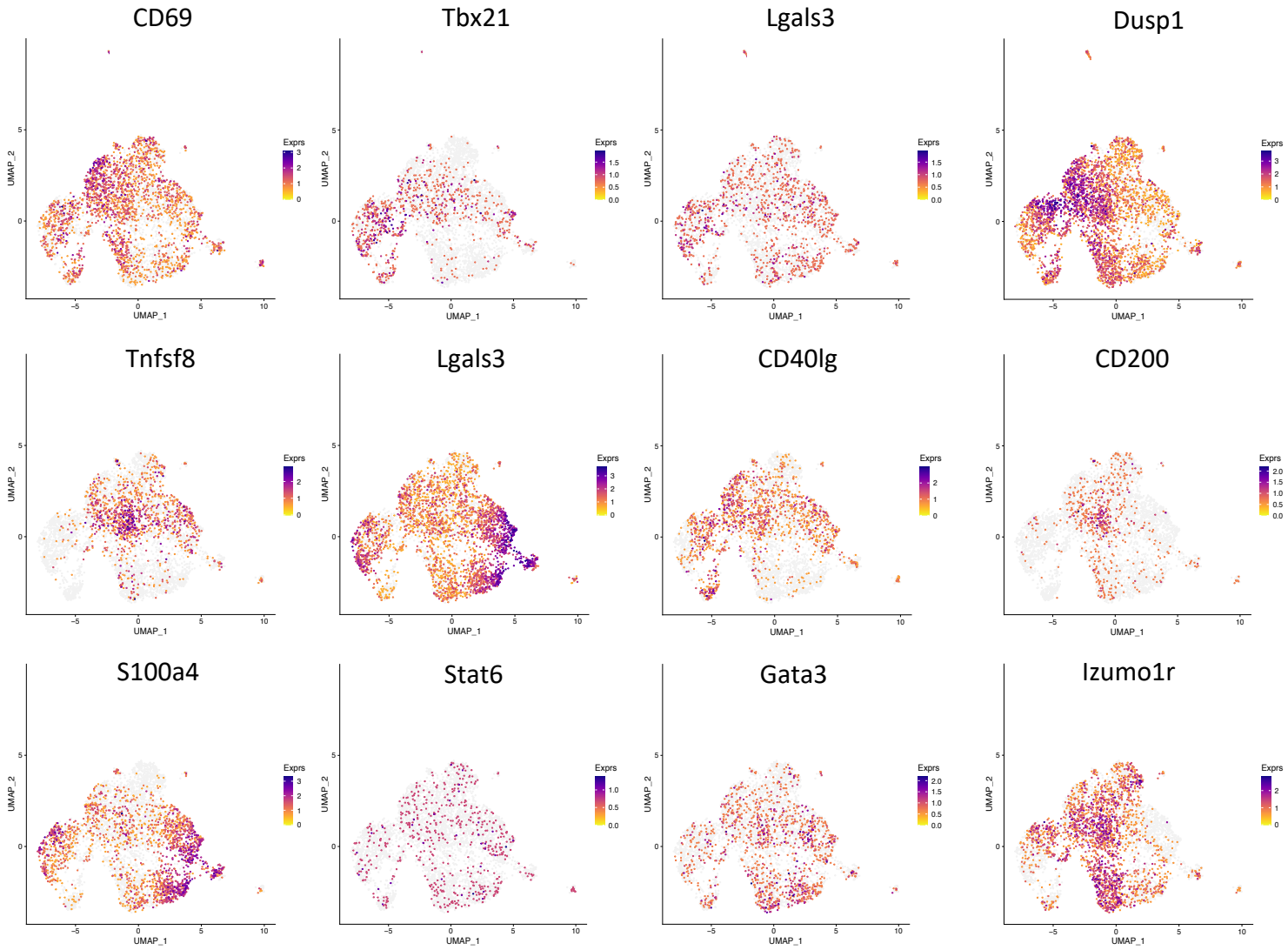
Spleen

Treatment Arm	Cell Number
Arm 2	381
Arm 3	1011
Arm 4	1360
Arm 5	351

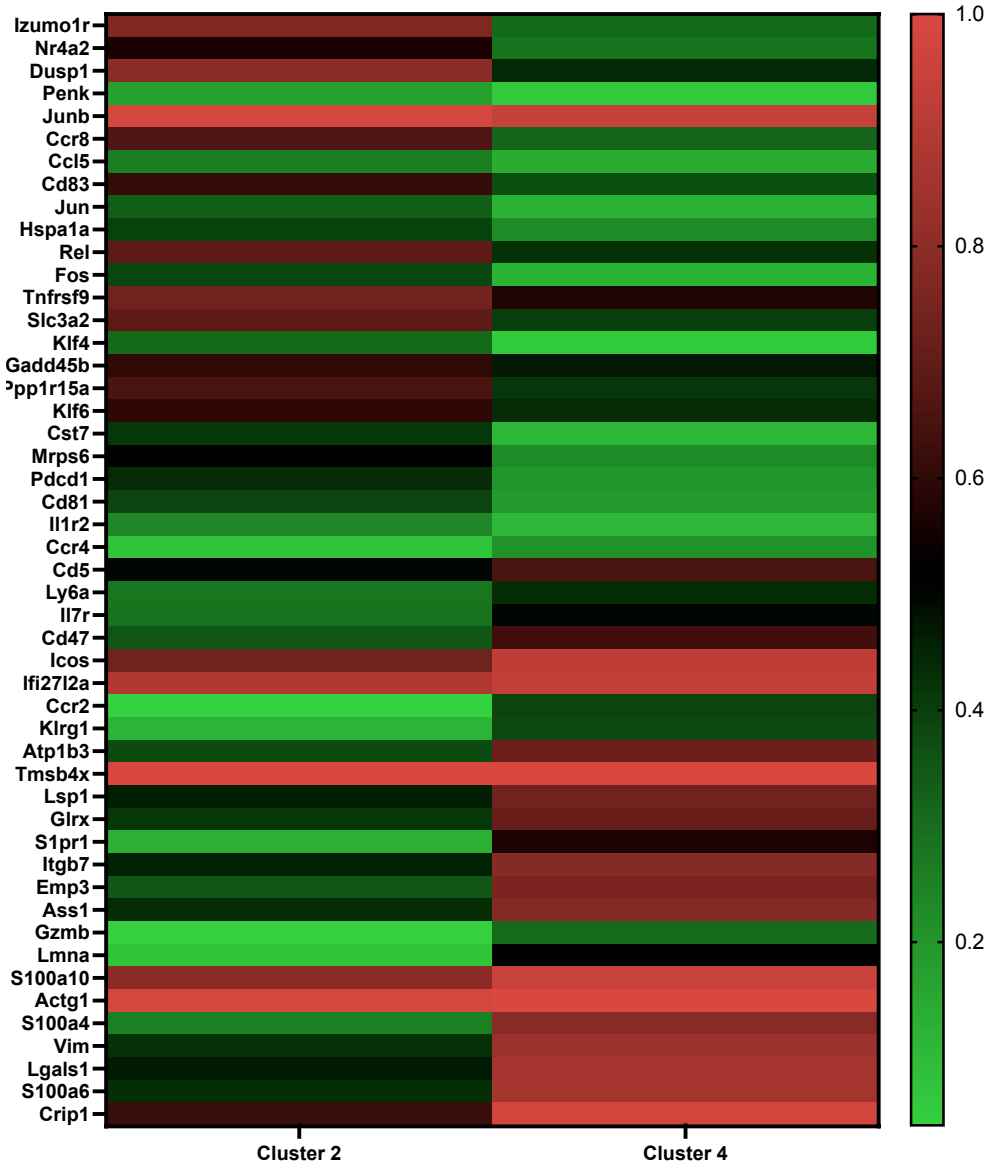
Supplementary figure 5: UMAP plot showing the different subsets of CD4⁺ T-cells from either the spleen (n=3103 cells) or the bone marrow (n=655 cells), stratified by the different treatment arms. Cell Numbers per treatment arm are in the tables.



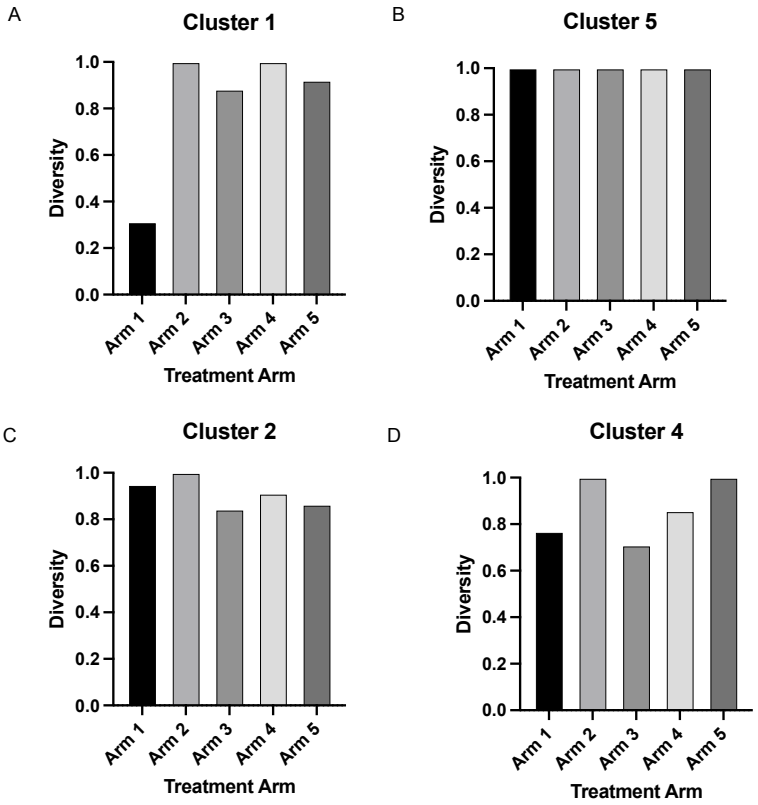
Supplementary figure 6: Heatmap showing the top 25 differentially-expressed transcripts among clusters from the murine scRNA-seq dataset.



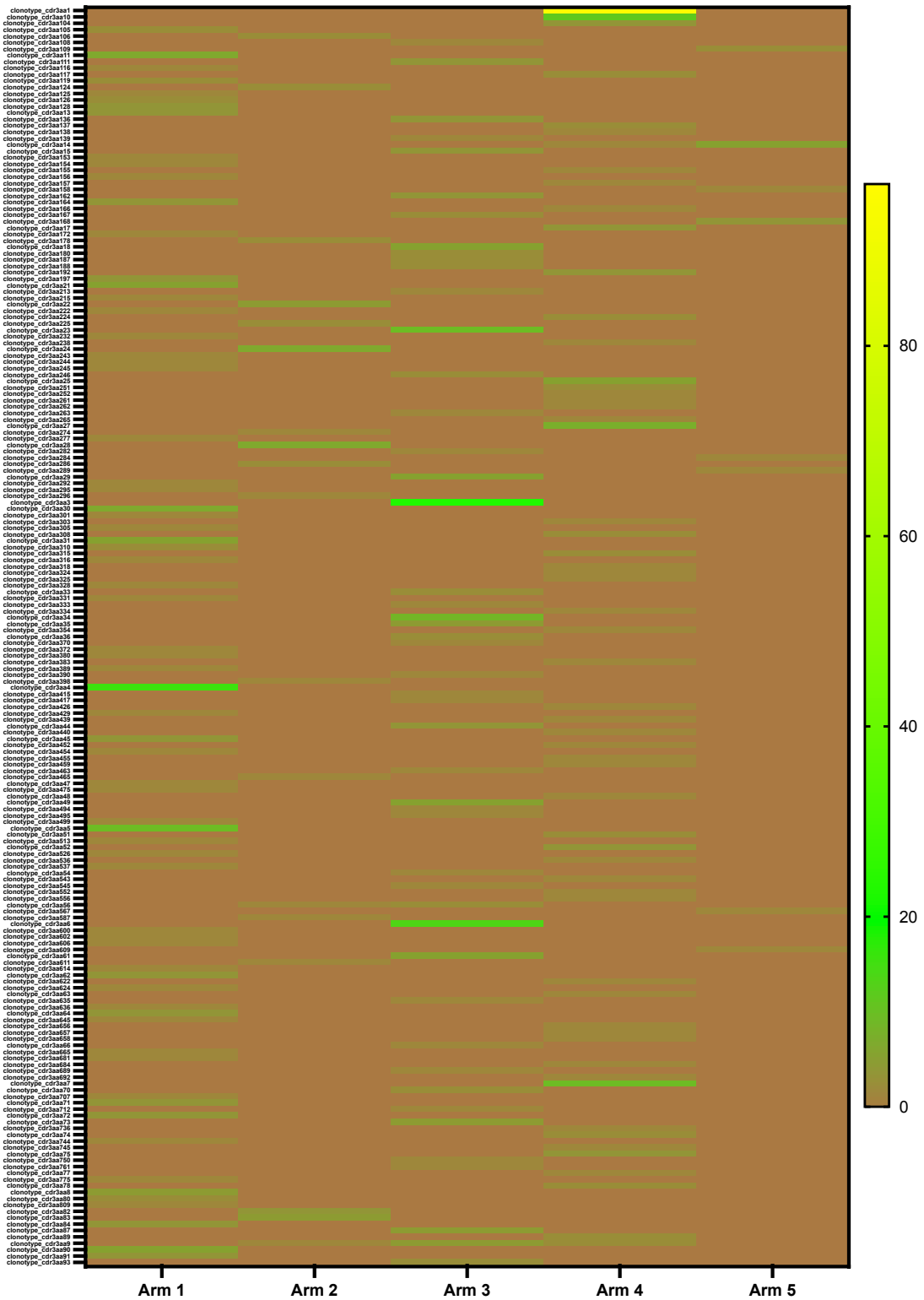
Supplementary figure 7: Feature plots of select transcripts from the murine scRNA-seq dataset.



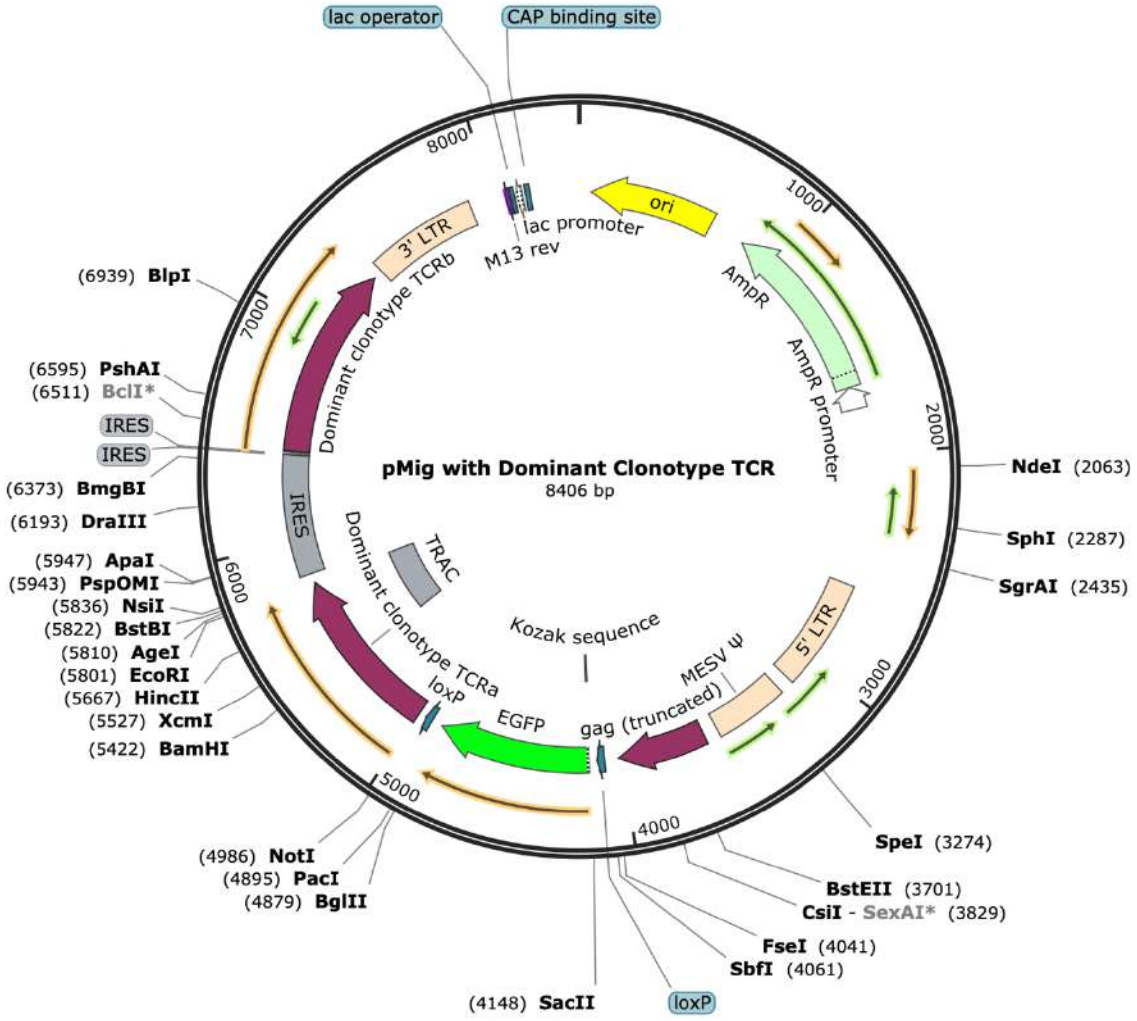
Supplementary figure 8: Differentially expressed transcripts between cluster 2 and cluster 4 Tregs from the murine scRNA-seq dataset.



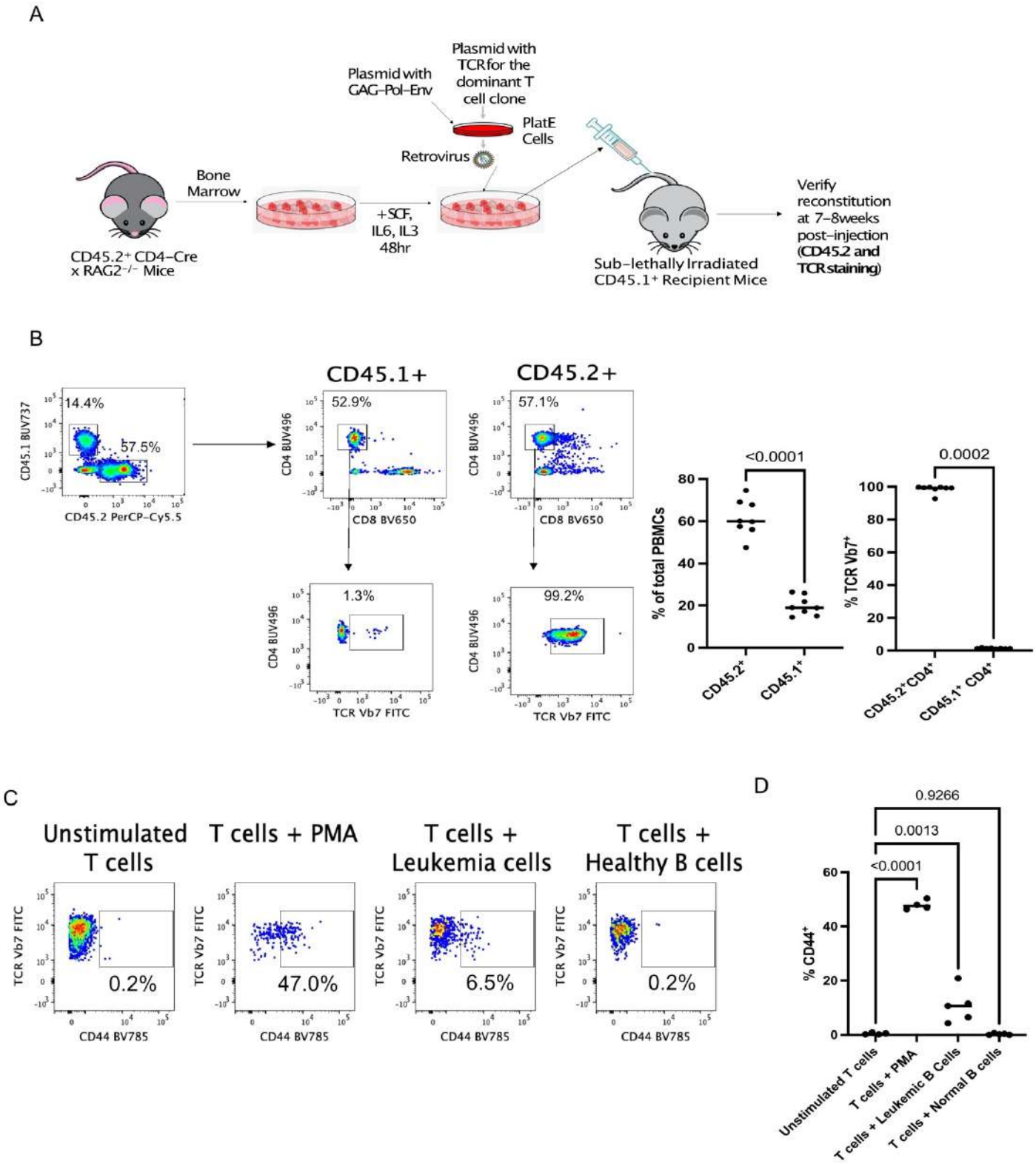
Supplementary figure 9: Inverse Simpson's diversity index across select clusters.



Supplementary figure 10: A heatmap of the scTCR-seq data from sorted murine CD4⁺ T-cells from the various treatment arms showing the expansion of a dominant clonotype in nilotinib plus anti-PD-L1 treated mice (Arm 4).



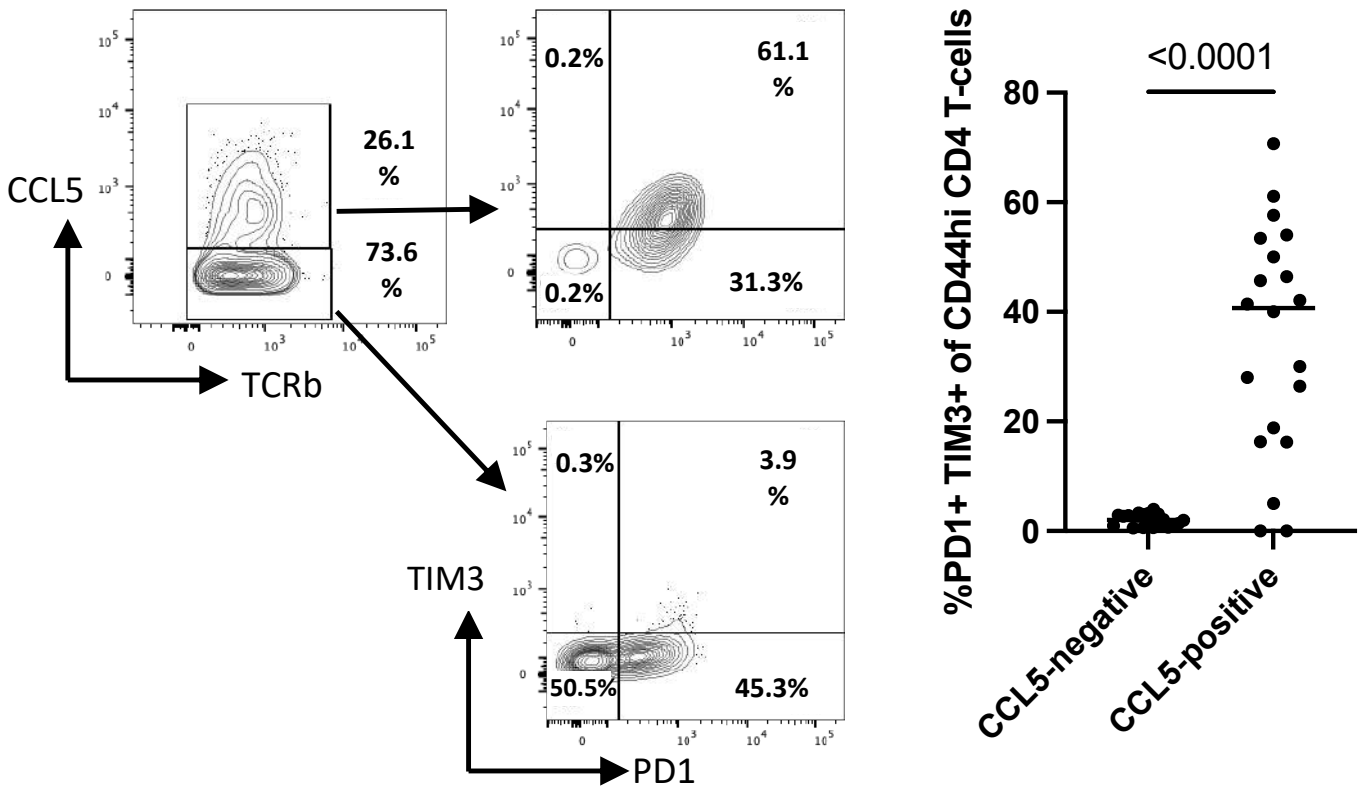
Supplementary figure 11: Plasmid map for generating retrogenic mice producing CD4⁺ T-cells with the dominant clonotype TCR.



Supplementary figure 12: The dominant clonotype T-cells from retrogenic mice are activated by LM138 leukemia cells, but not WT B cells, in-vitro. A. Schematic for generation of the retrogenic mice. **B**. Representative flow plots and graphs

demonstrating recovery of CD45.2^{+} TCRVb7^{+} T-cells from the PBMCs of retrogenic mice at 7 weeks post-reconstitution (n=8 mice, 2 experiments). TCR Vb7 is the TCR beta chain that the dominant T-cell clone uses. The median is displayed, and statistical significance was determined using an unpaired t-test

The dominant clonotype T-cells were enriched from retrogenic mice using CD45.2 enrichment. The enriched T-cells were cultured with either PMA, IFNγ pre-treated B cells or IFNγ pre-treated LM138 cells for 48 hours. **C**. Representative flow plots showing the expression of the activation marker CD44 among unstimulated retrogenic T-cells, or retrogenic T-cells cultured as indicated. **D**. Summary plots showing the expression of the activation marker CD44 among retrogenic T-cells under different culture conditions. The data is shown as median, and statistical significance was determined using a one-way ANOVA. The results of 2 experiments with 2 retrogenic mice are summarized.



Supplementary figure 13: Phenotypically exhausted murine CD4$^+$ T-cells are CCL5$^+$
Representative flow plots and graphs showing the proportion of PD1$^+$TIM3$^+$ cells among CCL5$^+$ or CCL5$^-$ CD44$^+$CD4$^+$ T-cells. The data is shown as a median, and statistical significance was determined using an unpaired t-test. The results of two independent experiments with n=20 mice are summarized.