

SUPPLEMENTARY FIGURES

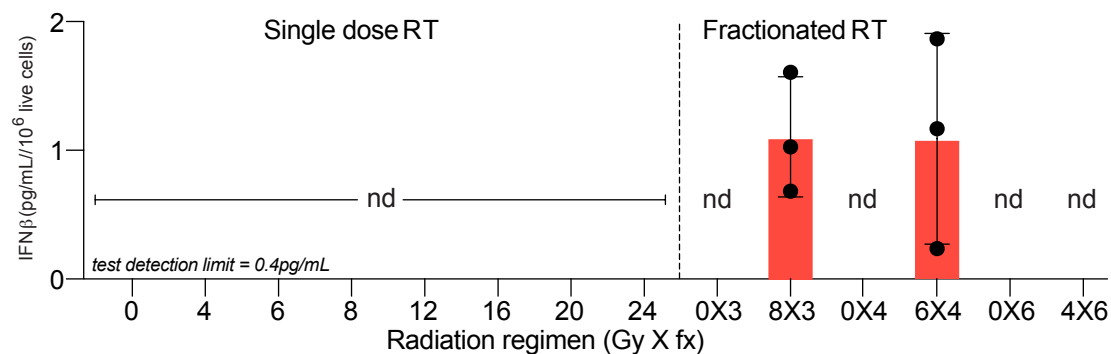


Figure: Supplementary Figure 1 (Figure S1)

Figure title: Interferon- β (IFN β) secretion by 4T1 cells following in vitro irradiation

Figure caption: IFN β concentration was measured by ELISA in cell supernatants collected 24 hours after the last radiation dose, each dot represents a biological triplicate.

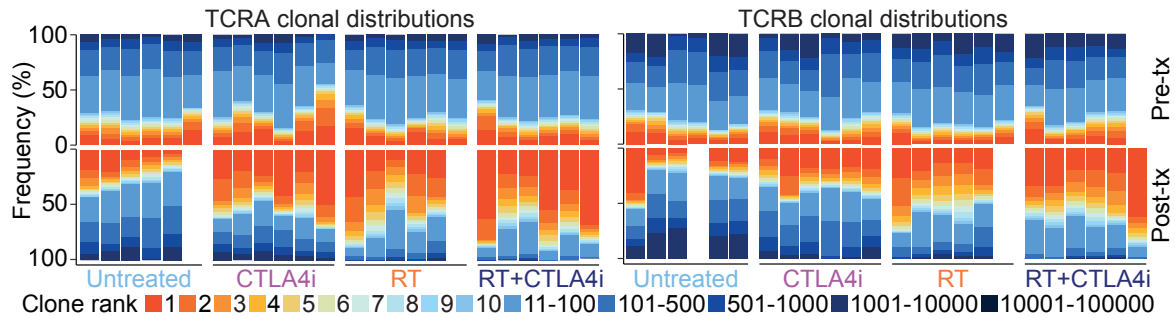


Figure: Supplementary Figure 2 (Figure S2)

Figure title: Clonal distributions of TCR repertoires.

Figure caption: Each column represents a mouse, and upper and bottom bars represent pre- (pre-tx) and post-treatment (post-tx) tumors, respectively. Color indicates clone rank. In some animals one of the samples did not meet the QC standards and is not included.

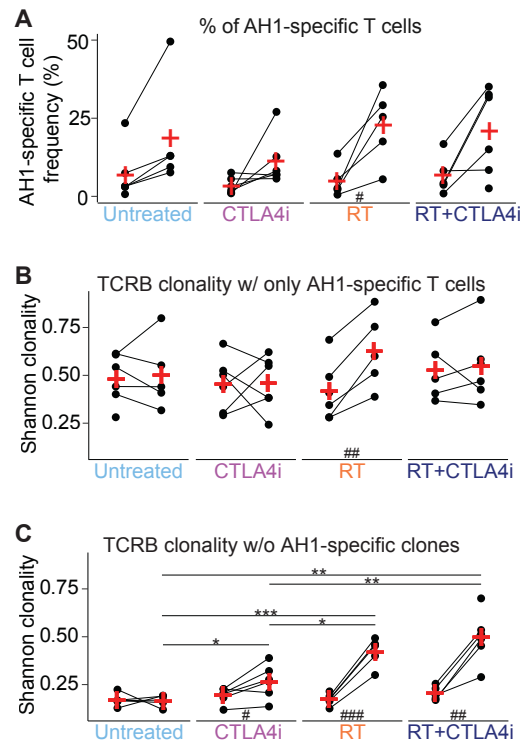


Figure: Supplementary Figure 3 (Figure S3)

Figure title: Clonality of TCRB repertoires segregated by AH1 reactivity

Figure caption: (A) Frequency of AH1-specific T cells in pre- and post-tx tumors calculated using previously published data on AH1-dextramer⁺ CD8⁺ T cells [25] and single cell VDJ and feature barcoding data from this paper. (B) Clonality of AH1-specific TCRB repertoires, and (C) clonality of AH1-unrelated TCRB repertoires*, **, ***, and ****, and #, ##, ###, and ####, indicate p-values < 0.05, 0.01, 0.001, and 0.0001 for pairwise and paired t-tests, respectively.

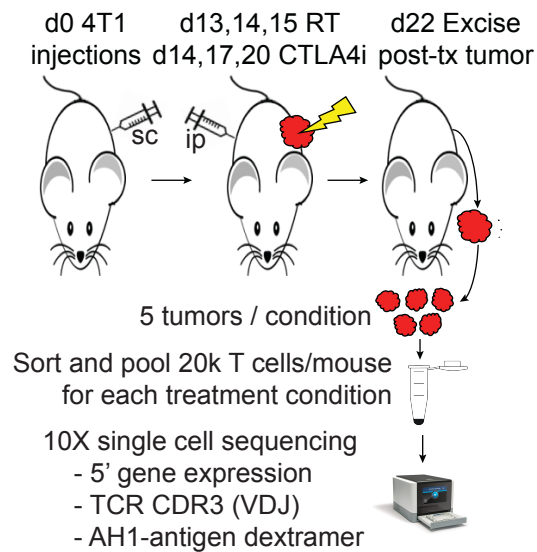
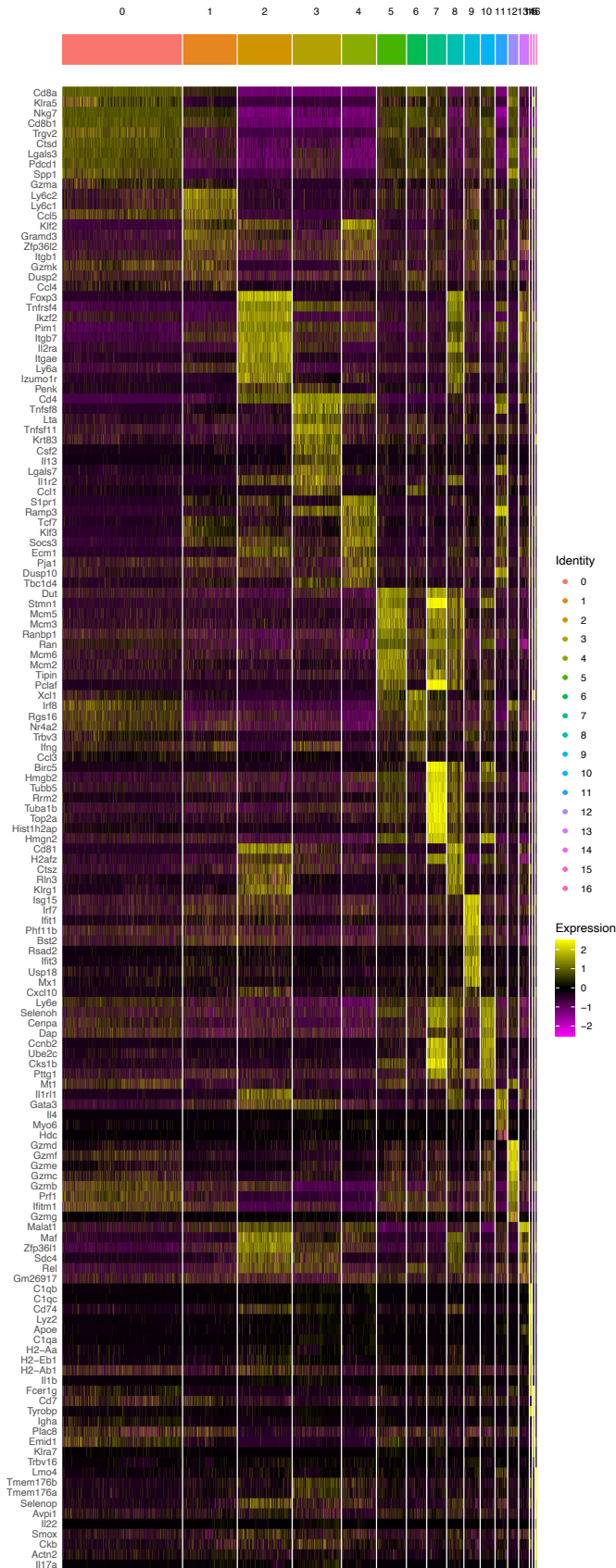


Figure: Supplementary Figure 4 (Figure S4)

Figure title: Diagram of single cell sequencing experiments

Figure caption: Single positive CD3⁺ cells from 4T1 tumors were processed using single cell sequencing (RNA/TCR/AH1-dCODE dextramer).



1 **Figure:** Supplementary Figure 5 (Figure S5)

2 **Figure title:** Heatmap of differentially expressed genes in each cluster from single cell
3 sequencing.

4 **Figure caption:** Heatmap showing differentially expressed genes in each cluster from the
5 experiment in **Figure 3**. Gene names are indicated on the y-axis and the level of differential
6 expression is indicated by the color.

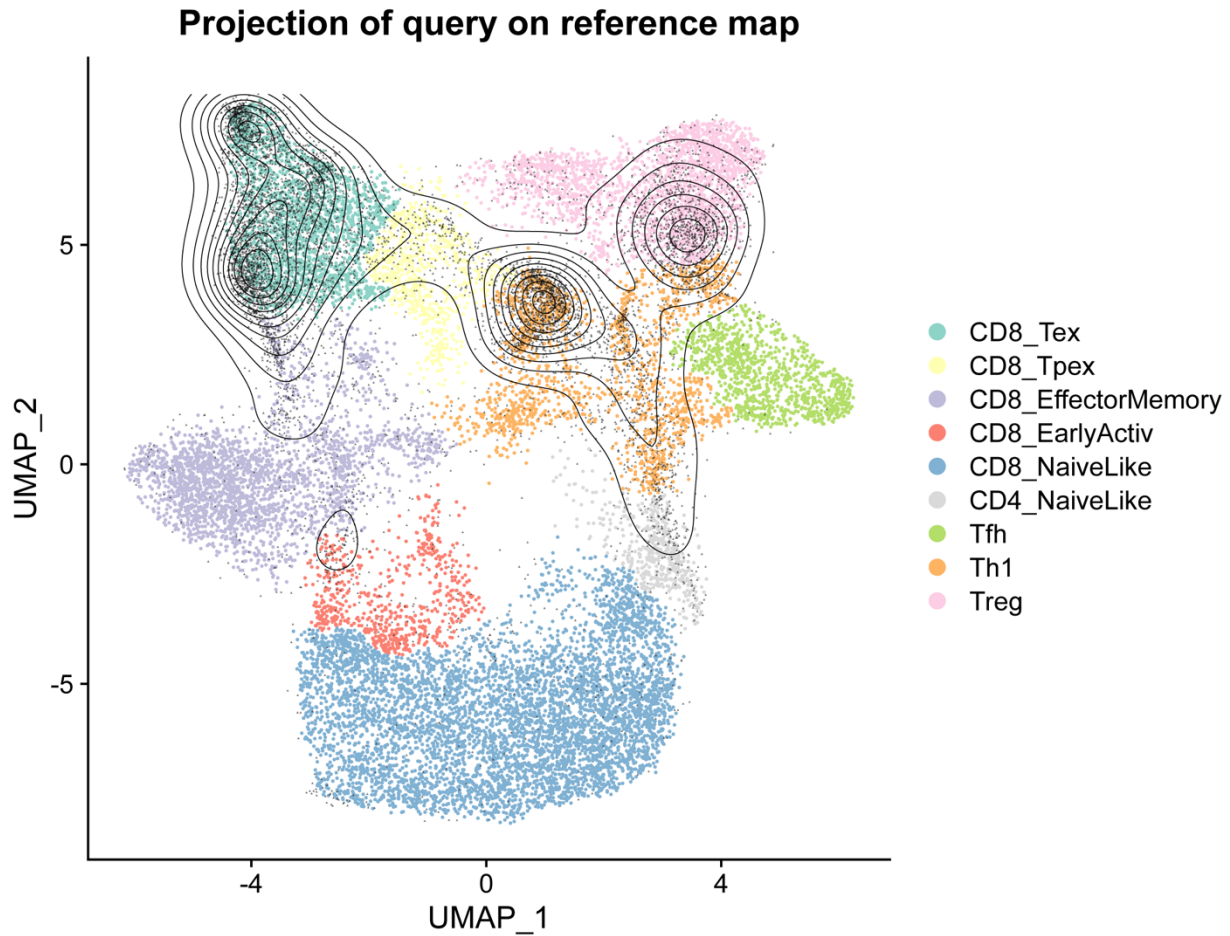
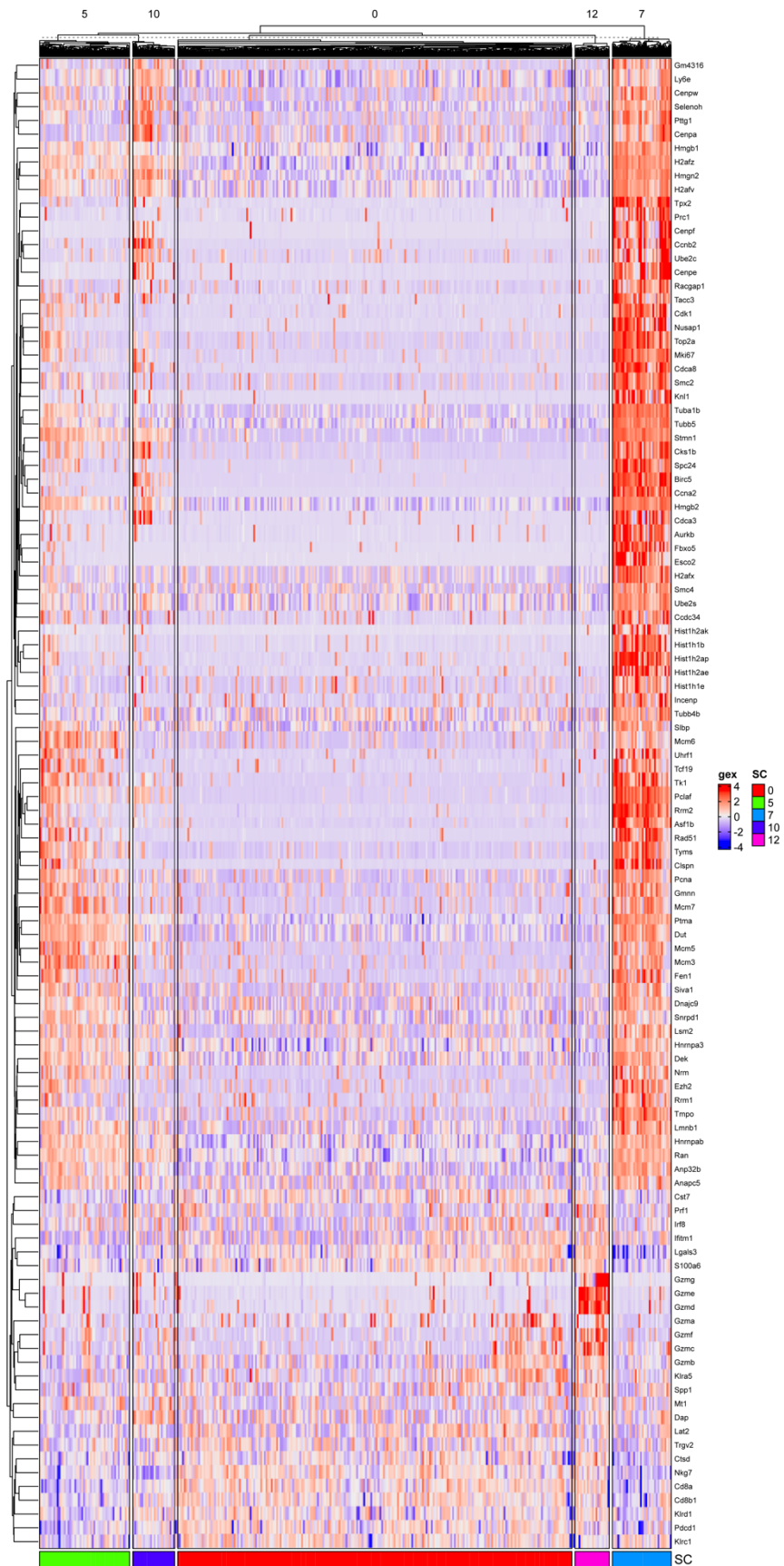


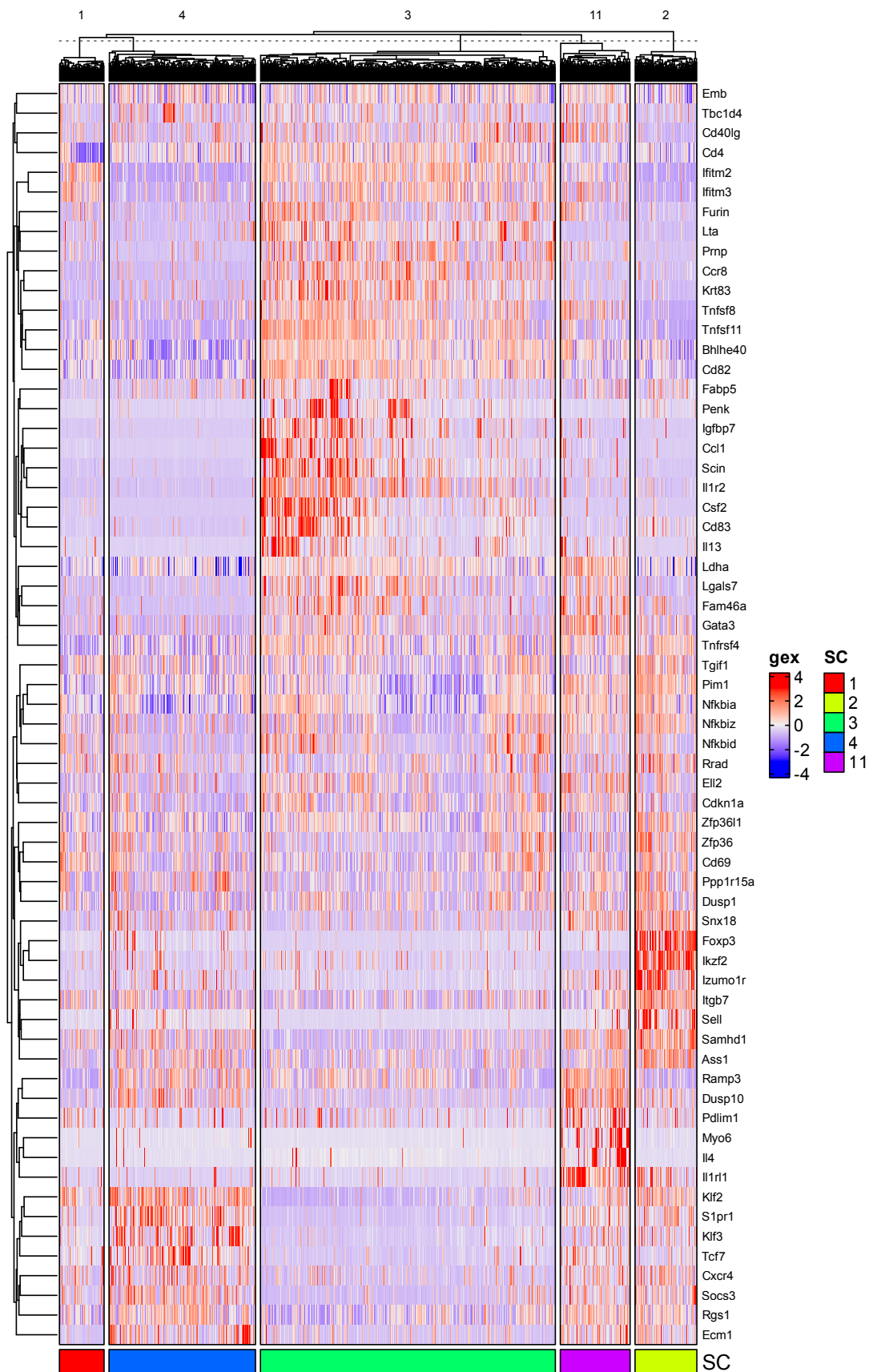
Figure: Supplementary Figure 6 (Figure S6)

Figure title: ProjectTILs map projection.

Figure caption: Functional states defined using ProjectTILs project were used to annotate the single cells from digested 4T1 tumors. Colors indicate ProjectTILs functional state. Black dots indicate single cells from 4T1 tumors in this study projected into the UMAP provided by ProjectTILs. Lines indicate density of single cells mapped to the UMAP.



1 **Figure:** Supplementary Figure 7 (Figure S7)
2 **Figure title:** Heatmap of differentially expressed genes for CD8 Tex population
3 **Figure caption:** Functional state defined using ProjecTILs project. SC indicate single cell
4 cluster defined using Seurat. Differentially expressed genes were determined for each combined
5 functional state + Seurat cluster, e.g., CD8_Tex in cluster 0 vs. all other cells.
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1 **Figure:** Supplementary Figure 8 (Figure S8)
2 **Figure title:** Heatmap of differentially expressed genes for Th1 population
3 **Figure caption:** Functional state defined using ProjecTILs project. SC indicate single cell
4 cluster defined using Seurat. Differentially expressed genes were determined for each combined
5 functional state + Seurat cluster, e.g., Th1 in cluster 1 vs. all other cells.
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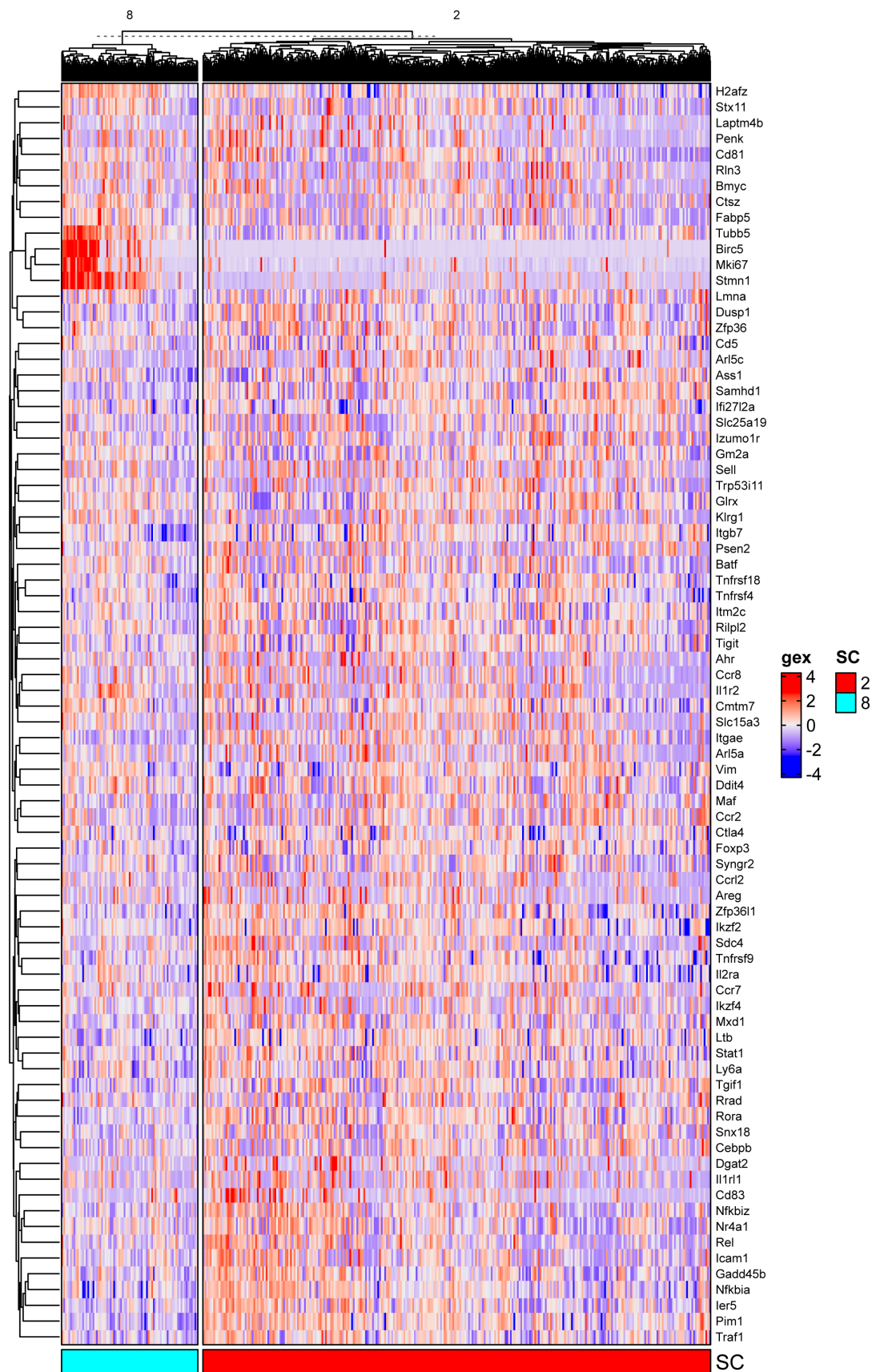
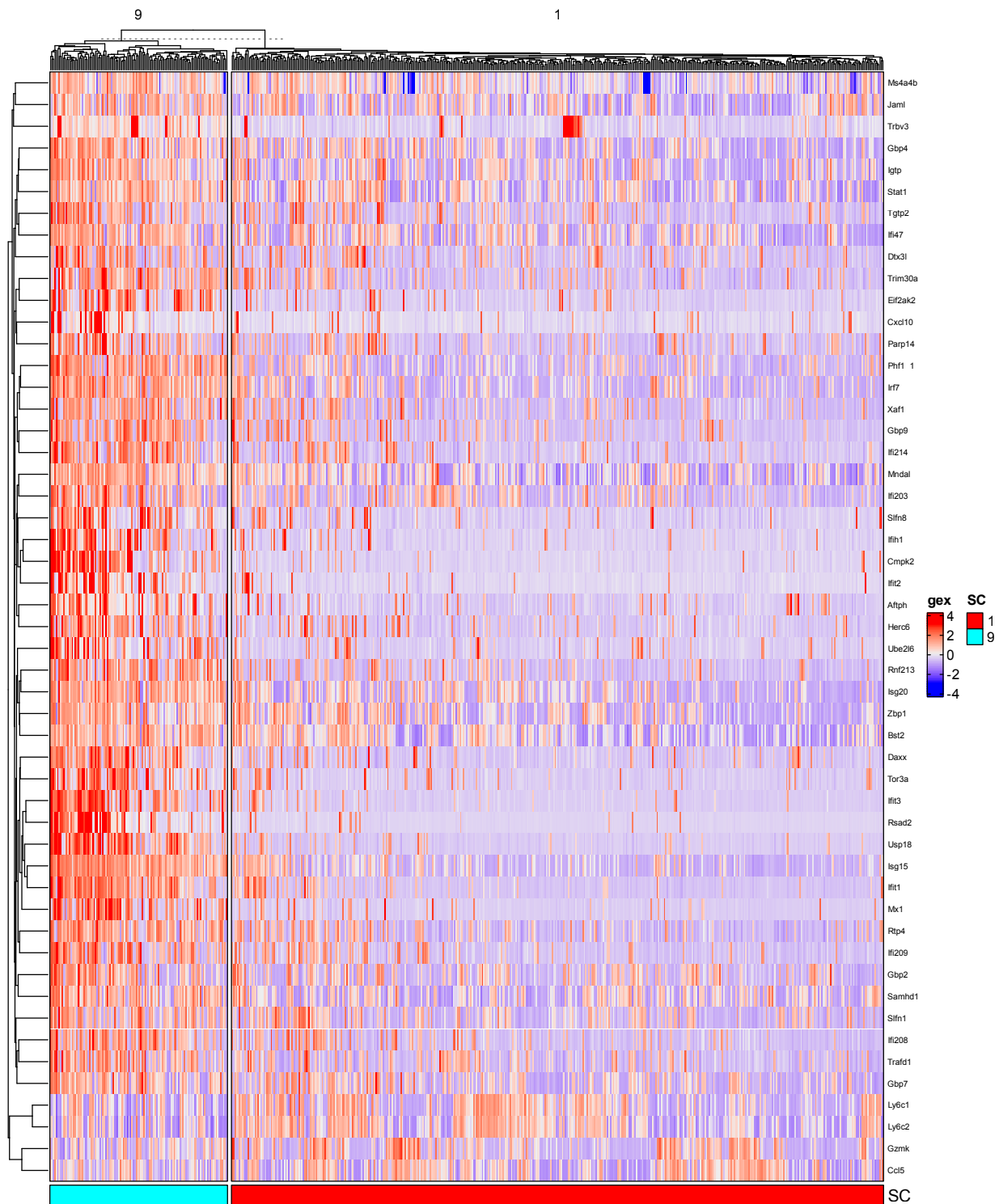


Figure: Supplementary Figure 9 (Figure S9)

Figure title: Heatmap of differentially expressed genes for Treg population

Figure caption: Functional state defined using ProjecTILs project. SC indicate single cell cluster defined using Seurat. Differentially expressed genes were determined for each combined functional state + Seurat cluster, e.g., Treg in cluster 2 vs. all other cells.



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Figure: Supplementary Figure 10 (Figure S10)

Figure title: Heatmap of differentially expressed genes for CD8 EM population

Figure caption: Functional state defined using ProjecTILs project. SC indicate single cell cluster defined using Seurat. Differentially expressed genes were determined for each combined functional state + Seurat cluster, e.g., CD8 EM in cluster 1 vs. all other cells.

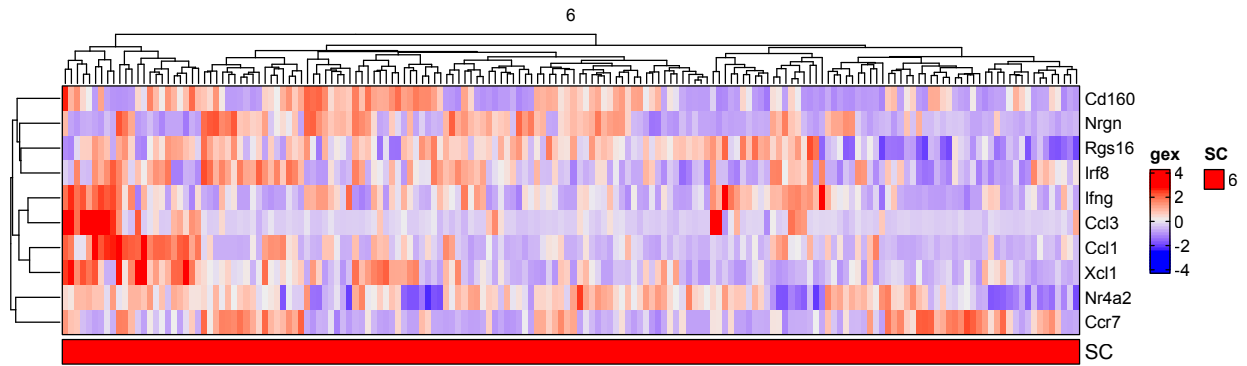


Figure: Supplementary Figure 11 (Figure S11)

Figure title: Heatmap of differentially expressed genes for CD8 Tpex population

Figure caption: Functional state defined using ProjectTILs project. SC indicate single cell cluster defined using Seurat. Differentially expressed genes were determined for each combined functional state + Seurat cluster, e.g., CD8 Tpex in cluster 6 vs. all other cells.

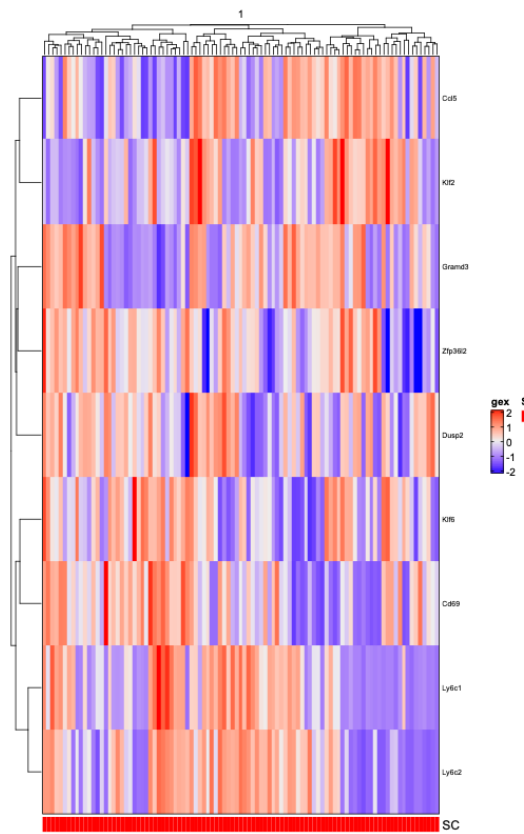


Figure: Supplementary Figure 12 (Figure S12)

Figure title: Heatmap of differentially expressed genes for CD8 EA population

Figure caption: Functional state defined using ProjectTILs project. SC indicate single cell cluster defined using Seurat. Differentially expressed genes were determined for each combined functional state + Seurat cluster, e.g., CD8 EA in cluster 1 vs. all other cells.

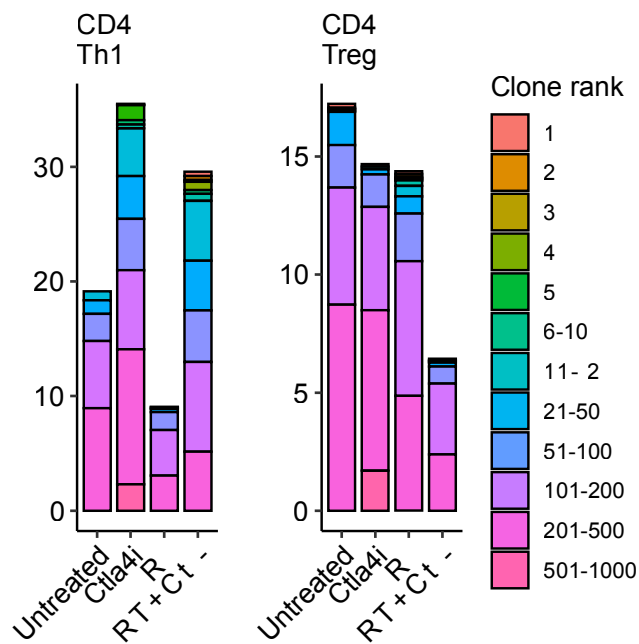


Figure: Supplementary Figure 13 (Figure S13)

Figure title: Clonal distribution of T cells in CD4 Th1 and Treg subsets

Figure caption: T cells were ranked based on frequency and grouped into bins based on rank for each treatment group. Color indicates the rank bin. The total size of bar for each treatment group indicates the proportion (%) of cells associated with each subset.

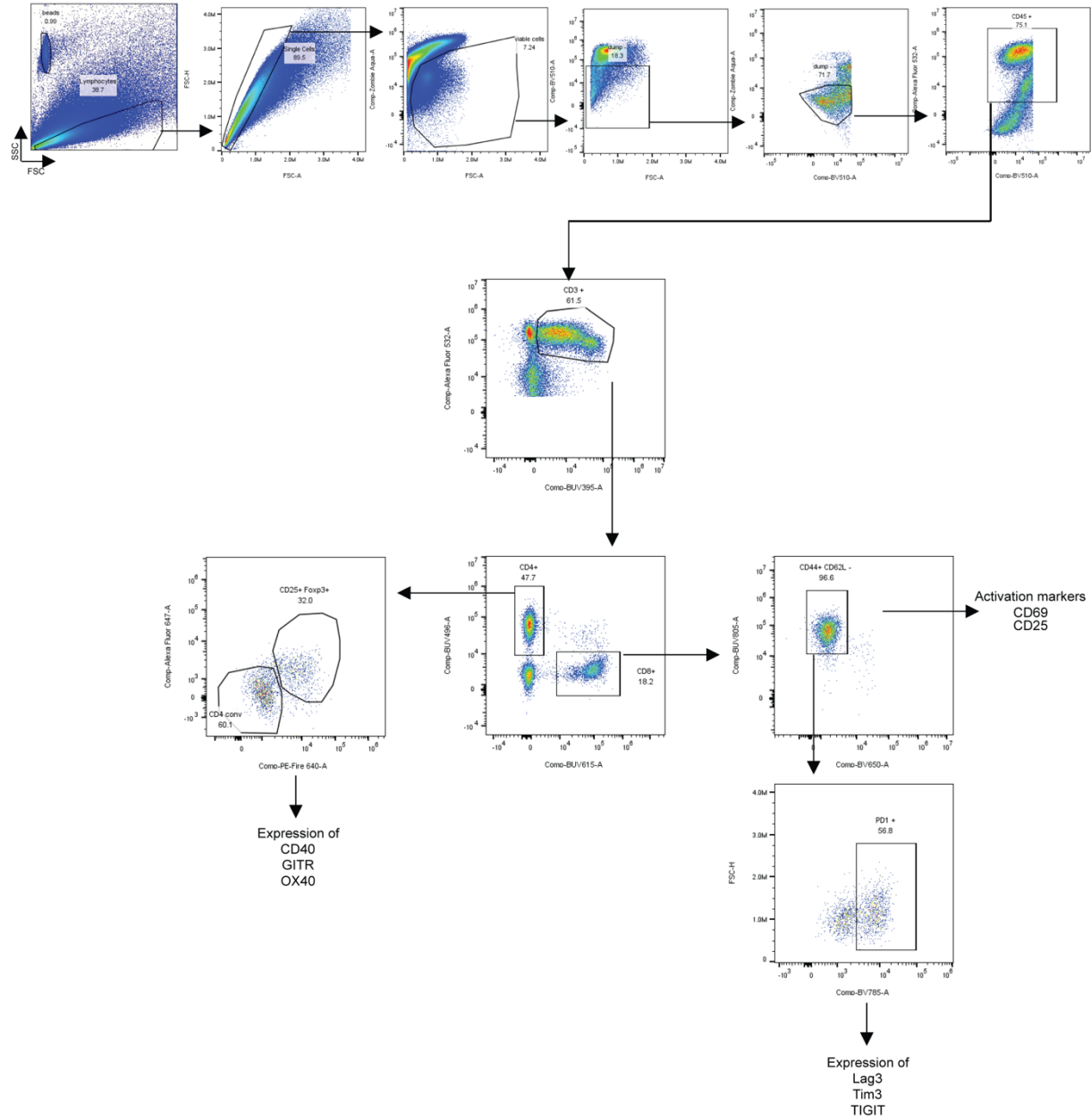


Figure: Supplementary Figure 14 (Figure S14)

Figure title: Gating strategy applied to tumor immune infiltrate

Figure caption: 4T1 tumors collected at day 22 and stained using a 25 biomarkers panel detailed in supplementary table X. Manual gating was performed as described on a FlowJo v.10 software.

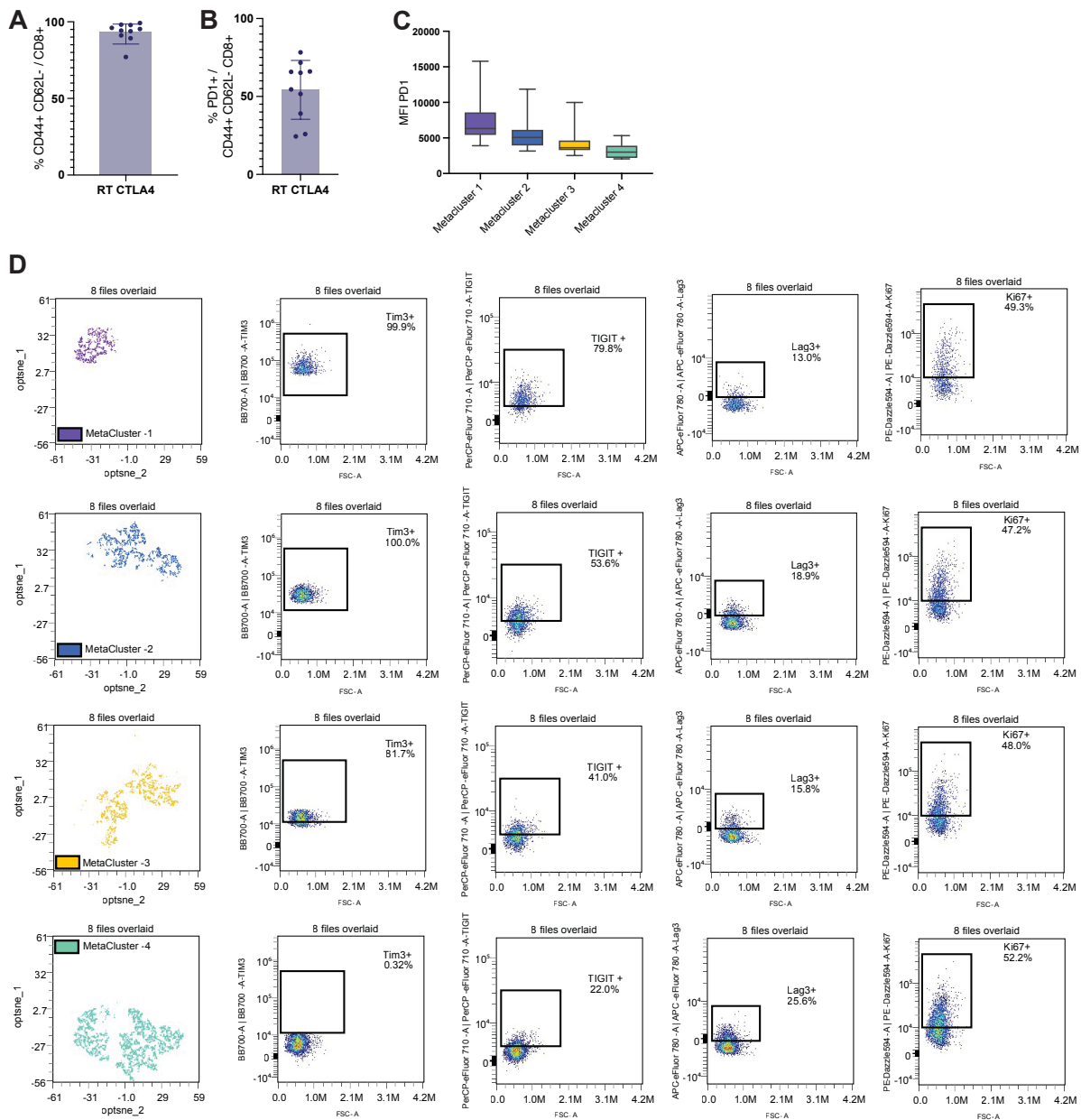


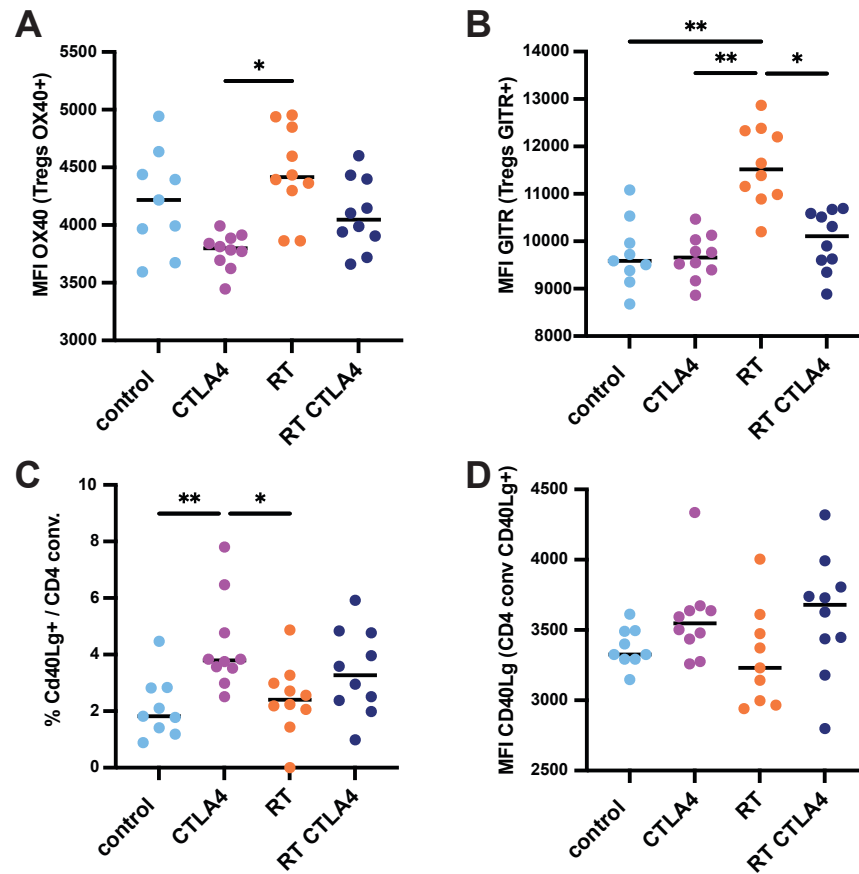
Figure: Supplementary Figure 15 (Figure S15)

Figure title: CD8 + T cell infiltration of 4T1 tumors

Figure caption: 4T1 tumors were collected at day 22 after implantation, dissociated and analyzed by flow cytometry. **(A)** Percentage of antigen experienced T cells (CD44+ CD62L-) among CD8 +. **(B)** Percentage of PD1+ among antigen experienced CD8+ T cells. **(C)** PD1 Fluorescence intensity of the 4 metaclusters resolved using Opt-sne and FlowSOM analysis

1 pipelines on PD1+ antigen experienced CD8+ T cells from RT+CTLA4i treated tumors **(D)**
2 Flowcytometry stainings for Tim3, TIGIT, Lag3 and Ki67 on individual metaclusters.

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5 **Figure: Supplementary Figure 16 (Figure S16)**

6 **Figure title:** Target expression by intra-tumoral CD4⁺ T cells

7 **Figure caption:** (A) Mean fluorescence intensity of OX40⁺ and (B) GITR⁺ regulatory CD4⁺ T

8 cells. (C) Percentage and (D) Intensity of CD40Lg expression on conventional CD4⁺ T cells.

9 Lines illustrate median and each dot represent an animal. Statistical significance was assessed by

10 Kruskal-Wallis test and post-hoc Dunn's test on each panel * and ** , indicate p-values < 0.05

11 and ≤ 0.01 respectively.

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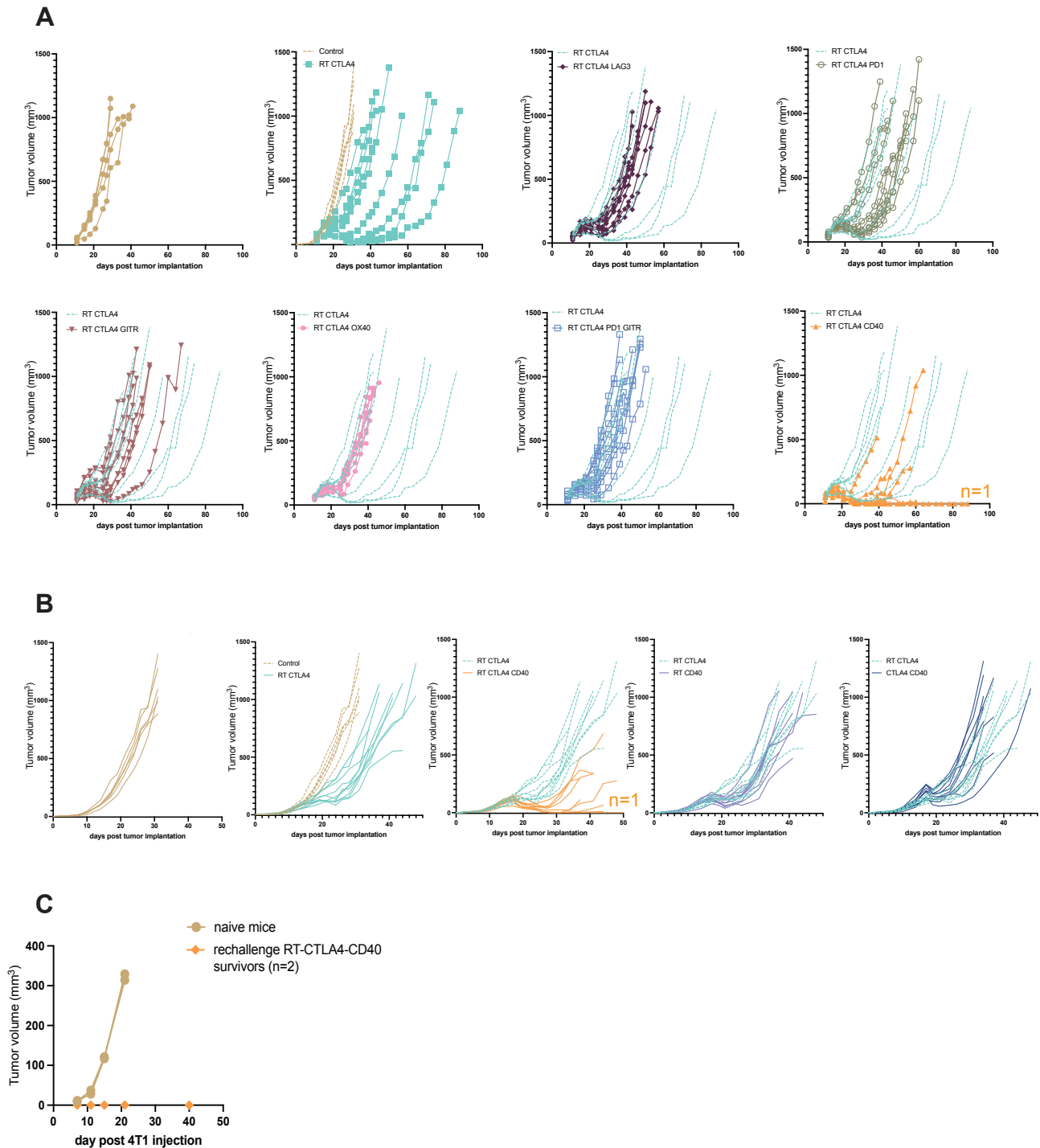


Figure: Supplementary Figure 17 (Figure S17)

Figure title: Individual tumor growth curves

Figure caption: (A) Individual tumor growth curves for mice treated with combination therapies as presented in figure 6A. Control (brown) or RT CTLA4i (light green) are provided in dotted

lines on all panels as a comparison. **(B)** Individual tumor growth curves for mice treated with combination therapies as presented in figure 6D. Control (brown) or RT CTLA4i (light green) are provided in dotted lines in all graphs as a comparison. Number mice experiencing complete tumor regression are indicated on the graphs. **(C)** Individual tumor growth curves long term survival mice from the experiments presented in Figure 6 B and E rechallenged with 4T1 tumors on the opposite flank (n=2) as compared to naïve mice.

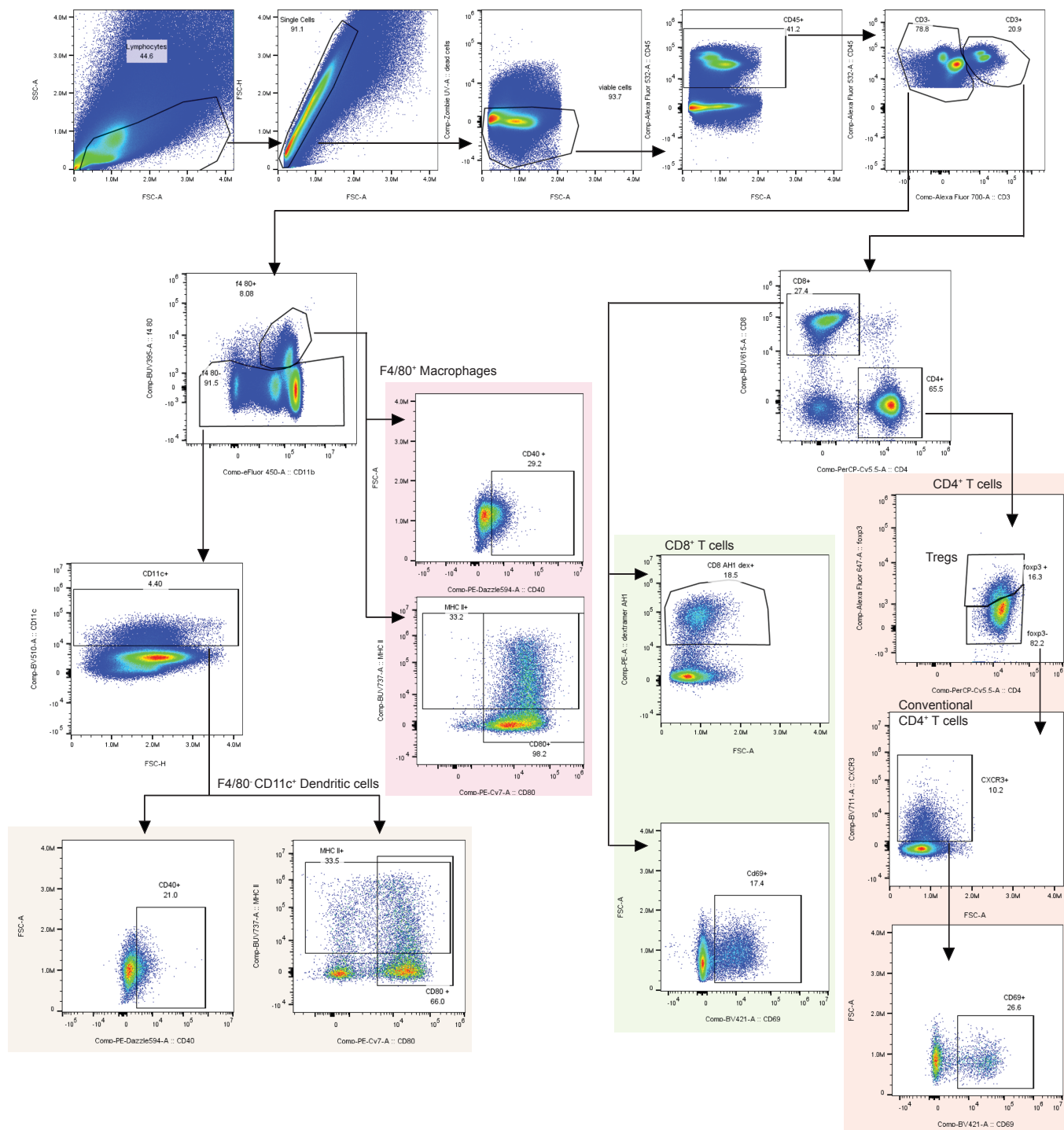


Figure: Supplementary Figure 18 (Figure S18)

Figure title: Gating strategy applied to lung immune infiltrate

Figure caption: Lungs from 4T1 tumors bearing mice collected at day 22 and stained using a 19 biomarkers panel detailed in supplementary table X. Manual gating was performed as described on a FlowJo v.10 software.

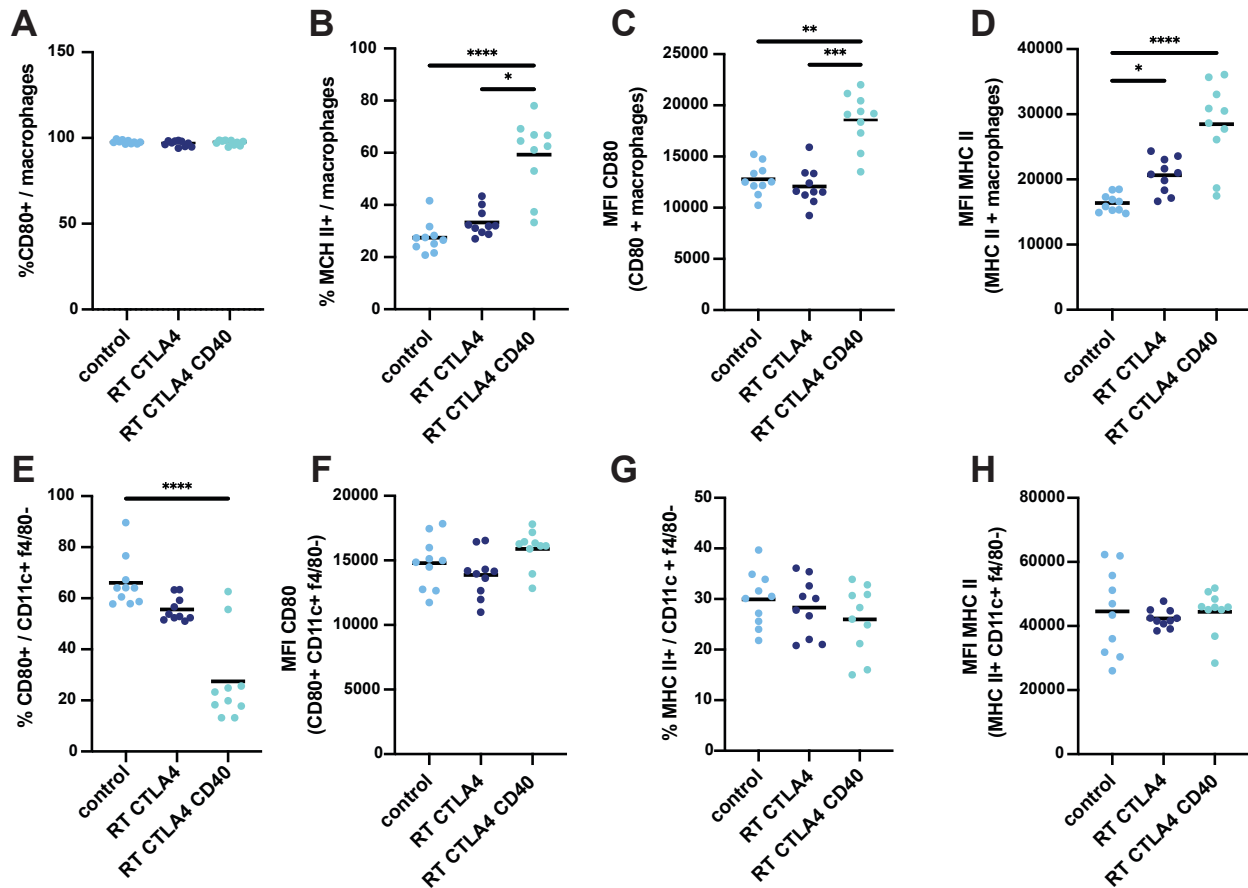


Figure: Supplementary Figure 19 (Figure S19)

Figure title: Expression of activation markers by lung infiltrating myeloid cells

Figure caption: Percentage (A-B) and Fluorescence intensity (C-D) of CD80 (A and C) and MHC II (H2Kb) (B and D) on lung macrophages. Percentage (E-F) and Fluorescence intensity (G-H) of CD80 (E and G) and MHC II (H2Kb) (F and H) on lung dendritic cells. Lines illustrate median and each dot represent an animal. Statistical significance was assessed by Kruskal-Wallis test and post-hoc Dunn's test on each panel * and ** , indicate p-values < 0.05 and ≤ 0.01 respectively.

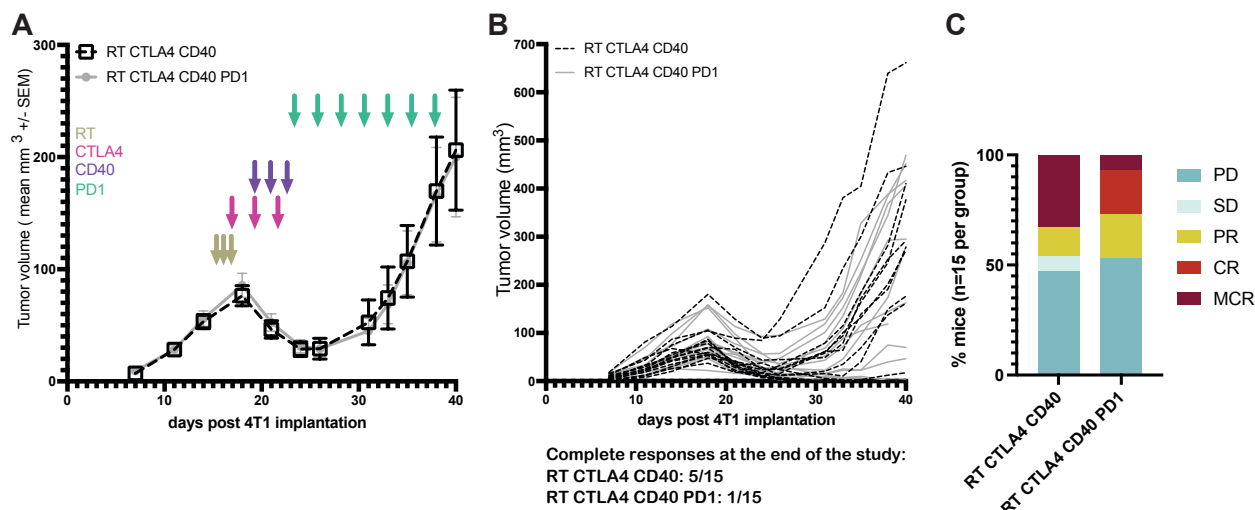


Figure: Supplementary Figure 20 (Figure S20)

Figure title: PD1 blockade does not improve the anti-tumor effect or RT + CTLA4i + CD40a

Figure caption: (A) mean (+/- SEM) of tumor growth for mice treated with RT + CTLA4i + CD40a with or without addition of anti-PD1 (RMP1-14) (n=15 mice per group). Treatment schedule is indicated by colored arrows: RT: brown, CTLA4i: pink, CD40a: purple, PD1:green (B) Individual tumor growth curves for mice treated with RT CTLA4 CD40 (black dotted line) or RT CTLA4 CD40 PD1 (grey plain line) combinations. (C) Response to treatment of each mouse was measured using the 5-category method that classifies responses into maintained CR (MCR), CR, PR, SD, and PD.

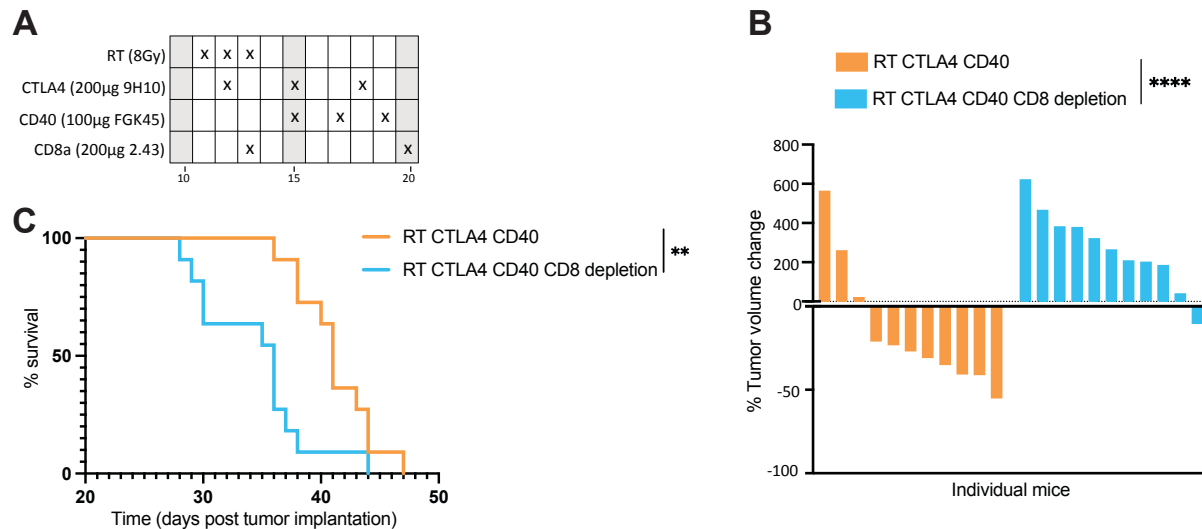


Figure: Supplementary Figure 21 (Figure S21)

Figure title: Depletion of CD8 T cells abrogates the effect of CD40 agonist in combination with RT and CTLA4 inhibition on tumor growth

Figure caption: (A) Treatment schedule for CD8⁺ T cells depletion on 4T1 implanted mice treated with RT + CTLA4i + CD40a combination. (B) Differential tumor volume between day 10 and day 24 (following tumor inoculation). Data shown to illustrate maximum therapeutic effect of the different mono or combination therapies. Treatment groups were compared by Mann Whitney tests on Log-transformed values for statistical significance. (C) Survival analysis of mice treated with RT + CTLA4i + CD40a with or without CD8⁺ T cells depletion (n=11 per group). p=0.0037, Log-rank test.

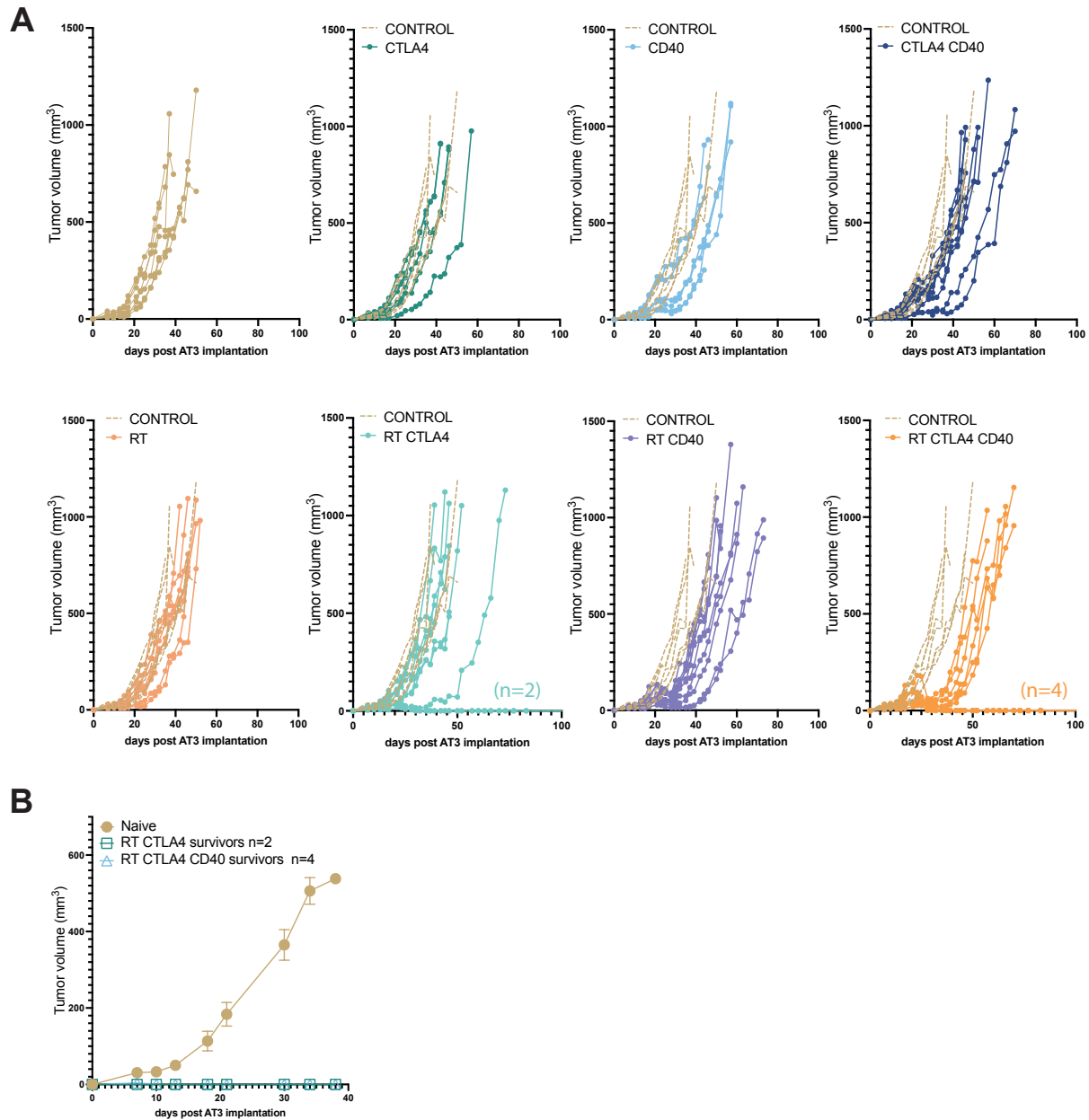


Figure: Supplementary Figure 22 (Figure S22)

Figure title: AT3 Individual tumor growth curves

Figure caption: (A) Individual tumor growth curves for mice treated with combination therapies as presented in figure 8A-D. Control (brown) or RT CTLA4i (light green) are provided in dotted lines on all panels as a comparison. The number of mice completely rejecting their tumor are

1 indicated on each plot. **(B)** Tumor growth on long term survivors rechallenged with AT3 tumors
2 on the opposite flank (n=2 for RT+CTLA4i and n=4 for RT+CTLA4i+CD40a group) as
3 compared to naïve mice shown as mean +/- SEM.

Supplementary Tables

Cell subset	Change in CTLA4i treated tumorsa	Change in RT treated tumorsa	Change in RT+CTLA4i treated tumorsa
CD4 Naïve	(1.2%; log2(OR) = -1.0; p = 0.022)	(1.2%; log2(OR) = -0.9; p = 0.029)	(0.3%; log2(OR) = -3.2; p = 1.9e-08)
CD8 EA	(1.6%; log2(OR) = 0.5; p = 0.35)	(0.7%; log2(OR) = -0.8; p = 0.2)	(2.8%; log2(OR) = 1.3; p = 0.00053)
CD8 EM	(8.0%; log2(OR) = 0.4; p = 0.033)	(9.1%; log2(OR) = 0.6; p = 0.0011)	(16.5%; log2(OR) = 1.6; p = 5.6e-24)
CD8 Naïve	(3.3%; log2(OR) = -0.5; p = 0.036)	(2.0%; log2(OR) = -1.3; p = 4e-06)	(1.1%; log2(OR) = -2.1; p = 4.6e-11)
CD8 Tex	(30.4%; log2(OR) = -0.8; p = 7.4e-16)	(59.8%; log2(OR) = 1.0; p = 7.2e-24)	(35.5%; log2(OR) = -0.5; p = 2.7e-06)
CD8 Tpex	(3.6%; log2(OR) = 0.4; p = 0.17)	(1.7%; log2(OR) = -0.7; p = 0.036)	(5.9%; log2(OR) = 1.1; p = 5.1e-06)
Tfh	(1.0%; log2(OR) = 0.0; p = 1)	(0.1%; log2(OR) = -3.3; p = 0.00044)	(0.7%; log2(OR) = -0.4; p = 0.5)
Th1	(36.1%; log2(OR) = 1.1; p = 3.1e-24)	(9.8%; log2(OR) = -1.3; p = 3.9e-21)	(30.5%; log2(OR) = 0.7; p = 5.7e-11)
Treg	(14.7%; log2(OR) = -0.3; p = 0.022)	(15.6%; log2(OR) = -0.2; p = 0.11)	(6.6%; log2(OR) = -1.6; p = 8.4e-25)

Table S1: Supplementary Table S1

Frequency of T cell subset in indicated group; log2 odds ratio of frequency compared to untreated tumors; FDR adjusted Fisher Exact test p-value of change

Cell subset	Change in CTLA4i treated tumors ^a	Change in RT treated tumors ^a	Change in RT+CTLA4i treated tumors ^a
CD8 EA in C1	(1.5%; log2(OR) = 0.8; p = 0.49)	(0.6%; log2(OR) = -0.4; p = 1)	(2.1%; log2(OR) = 1.4; p = 0.012)
CD8 EM in C1	(5.1%; log2(OR) = 0.7; p = 0.028)	(3.6%; log2(OR) = 0.2; p = 1)	(11.6%; log2(OR) = 2.0; p = 3.7e-22)
CD8 EM in C9	(0.9%; log2(OR) = -0.1; p = 1)	(2.8%; log2(OR) = 1.5; p = 0.00094)	(1.7%; log2(OR) = 0.8; p = 0.47)
CD8 Tex in C0	(18.1%; log2(OR) = -0.9; p = 1.7e-14)	(31.4%; log2(OR) = 0.1; p = 0.69)	(17.7%; log2(OR) = -0.9; p = 1.2e-15)
CD8 Tex in C1	(0.5%; log2(OR) = 0.0; p = 1)	(2.0%; log2(OR) = 2.1; p = 0.00023)	(2.4%; log2(OR) = 2.4; p = 6.6e-06)
CD8 Tex in C5	(4.1%; log2(OR) = -0.1; p = 1)	(8.3%; log2(OR) = 0.9; p = 3.4e-05)	(5.2%; log2(OR) = 0.2; p = 1)
CD8 Tex in C7	(2.8%; log2(OR) = 0.2; p = 1)	(5.6%; log2(OR) = 1.2; p = 3.4e-05)	(3.5%; log2(OR) = 0.5; p = 0.4)
CD8 Tex in C10	(2.1%; log2(OR) = -0.1; p = 1)	(4.1%; log2(OR) = 0.9; p = 0.011)	(1.9%; log2(OR) = -0.2; p = 1)
CD8 Tex in C12	(1.4%; log2(OR) = -0.3; p = 1)	(3.3%; log2(OR) = 1.0; p = 0.011)	(2.1%; log2(OR) = 0.4; p = 0.95)
CD8 Tpex in C6	(2.2%; log2(OR) = 0.4; p = 1)	(1.1%; log2(OR) = -0.6; p = 0.73)	(3.7%; log2(OR) = 1.1; p = 0.0025)
Th1 in C1	(1.7%; log2(OR) = 0.7; p = 0.59)	(0.5%; log2(OR) = -1.2; p = 0.26)	(2.8%; log2(OR) = 1.4; p = 0.002)
Th1 in C2	(3.6%; log2(OR) = 1.0; p = 0.014)	(1.6%; log2(OR) = -0.3; p = 1)	(1.4%; log2(OR) = -0.4; p = 1)
Th1 in C3	(16.2%; log2(OR) = 1.6; p = 6.4e-22)	(2.8%; log2(OR) = -1.2; p = 3.4e-05)	(15.2%; log2(OR) = 1.5; p = 1.4e-18)
Th1 in C4	(6.3%; log2(OR) = -0.2; p = 1)	(2.9%; log2(OR) = -1.3; p = 1.5e-07)	(3.9%; log2(OR) = -0.9; p = 0.00037)
Th1 in C11	(4.0%; log2(OR) = 1.1; p = 0.0016)	(1.0%; log2(OR) = -0.9; p = 0.26)	(2.6%; log2(OR) = 0.5; p = 0.68)
Treg in C2	(10.3%; log2(OR) = -0.3; p = 0.34)	(10.9%; log2(OR) = -0.2; p = 0.79)	(4.5%; log2(OR) = -1.6; p = 1.5e-16)
Treg in C8	(2.8%; log2(OR) = -0.5; p = 0.26)	(2.0%; log2(OR) = -1.1; p = 0.0025)	(1.4%; log2(OR) = -1.6; p = 6.6e-06)

Table S2: Supplementary Table S2

Frequency of T cell subset in indicated group; log2 odds ratio of frequency compared to untreated tumors; FDR adjusted Fisher Exact test p-value of change

Cytex Aurora		Antibodies					Titration
Laser	Detector	Marker	Clone	Fluorochrome	Vendor	Reference	µg/ 100µl
355 nm	UV2	CD3	17A2	BUV395	BD	563565	0.5
355 nm	UV7	CD4	RM4-4	BUV496	BD	741051	0.3
355 nm	UV10	CD8	53-6.7	BUV615	BD	613004	0.1
355 nm	UV14	CD69	H1.2F3	BUV737	BD	612793	0.03
355 nm	UV16	CD44	IM7	BUV805	BD	741921	0.1
405 nm	V1	ILR12	4. E2	BV421	BD	562926	0.5
405 nm	V2	GITR	DTA-1	super bright 436	ebioscience	62-5874-82	0.03
405 nm	V7	Viability		Zombie Aqua	Biolegend	423101	
405 nm	V7	CD19	6D5	BV510	Biolegend	115545	0.2
405 nm	V7	Epcam	G8.8	BV510	Biolegend	118231	0.2
405 nm	V7	CD11b	M1/70	BV510	Biolegend	101245	0.2
405 nm	V7	CD11c	N418	BV510	Biolegend	117337	0.2
405 nm	V11	CD62L	MEL-14	BV650	Biolegend	104453	0.03
405 nm	V13	RANKL	IK22-5	BV711	BD	740820	0.3
405 nm	V15	PD1	29F.1A12	BV785	Biolegend	135225	0.03
561 nm	YG4	CD25	PC61	PE fire 640	Biolegend	102071	0.03
488 nm	B3	CD45	30-F11	alexa fluor 532	eBioscience	58-0451-82	0.1
488 nm	B9	TIM3	5D12/TIM-3	BB700	BD	747619	0.3
561 nm	YG1	CD40Lg	SA047C3	PE	Biolegend	157003	2
561 nm	YG3	KI67	16A8	PE dazzle 594	Biolegend	652427	0.03
561 nm	YG9	OX40	OX-86	PE-Cy7	Biolegend	119415	0.03
640 nm	R2	FOXP3	MF23	alexa fluor 647	Biolegend	126407	0.1
640 nm	R4	CTLA4	UC10-4F10-11	APCR700	BD	565778	0.1
640 nm	R7	LAG3	eBioC9B7W (C9B7W)	APC eFluor780	ebioscience	47-223-180	0.1
488 nm	B10	TIGIT	GIGD7	PerCP eFluor 710	ebioscience	46-9501-80	0.3

Table S3: Supplementary Table 3

Flow cytometry staining panel for intra-tumoral immune infiltrate staining. Detector V7 was used as a dump channel for dead and non-lymphoid cells.

Cytex Aurora		Antibodies					Titration
Laser	Detector	Marker	Clone	Fluorochrome	Vendor	Reference	µg/ 100µl
640 nm	R4	CD3	17A2	alexa Fluor 700	eBioscience	56-0032-82	0.25
488 nm	B3	CD45	30-F11	alexa fluor 532	eBioscience	58-0451-82	0.1
488 nm	B9	CD4	GK1.5	Percp Cy5.5	Biolegend	100434	0.25
355 nm	UV10	CD8	53-6.7	BUV615	BD	613004	0.1
561 nm	YG4	CD25	PC61	PE fire 640	Biolegend	102071	0.03
405 nm	V1	CD69	H1.2F3	BV421	Biolegend	104545	0.4
640 nm	R2	Foxp3	MF23	alexa fluor 647	Biolegend	126407	0.1
405 nm	V15	PD1	29F.1A12	BV785	Biolegend	135225	0.03
561 nm	YG3	CXCR3	S18001A	BV711	Biolegend	155917	0.2
405 nm	V13	CD40	3 / 23	PE Dazzle 594	Biolegend	124629	0.4
405 nm	V7	CD11c	N418	BV510	Biolegend	117337	0.2
405 nm	V3	CD11b	M1/70	E fluor 450	eBioscience	48-0112-82	0.2
405 nm	V11	XCR1	ZET	BV650	Biolegend	148205	0.25
640 nm	R7	SIRPa	P84	APC-Cy7	Biolegend	144018	0.2
355 nm	UV2	F4/80	T45-2342	BUV395	BD	565614	0.5
355 nm	UV14	MHC II	M5/114.15.2	BUV737	BD	748845	0.2
561 nm	YG9	CD80	16-10A1	PE Cy7	Biolegend	104734	0.5
561 nm	YG1	dextramer AH1	-	PE	IMMUDEX	JG3294-PE	10µL
355 nm	UV7	Viability	-	Zombie UV	Biolegend	423107	

Table S4: Supplementary Table S4

Flow cytometry staining panel for staining of lung infiltrating immune cells.

Other supplementary information

Figure: Supplementary Data File 1

File: Supplementary Data File 1.xlsx

Figure caption: Differentially expressed genes for ProjecTILs-defined subsets within each Seurat cluster (all genes)

Figure: Supplementary Data File 2

File: Supplementary Data File 2.xlsx

File description: Differentially expressed genes for ProjecTILs-defined subsets within each Seurat cluster (genes passing differential expression threshold of log2 fold change > 1, adjusted p-value > 0.01, and percent in target single cell population > 60%)