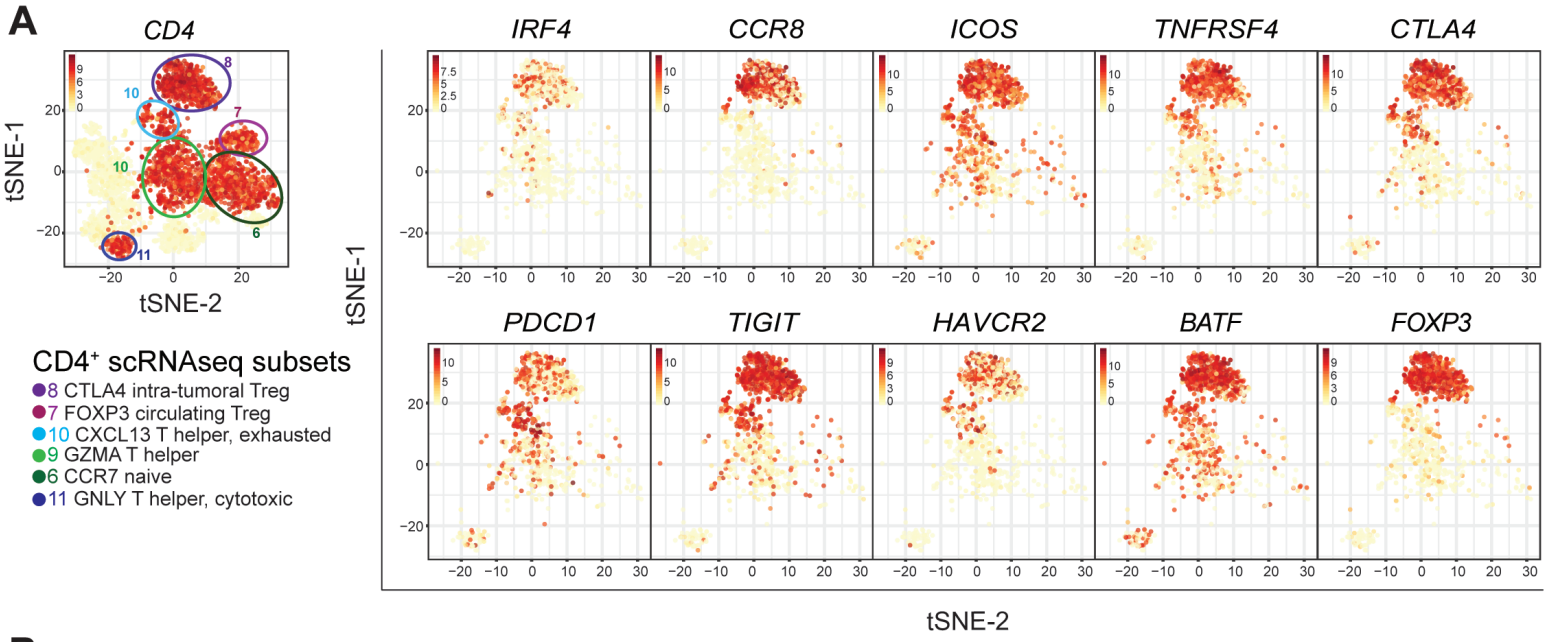
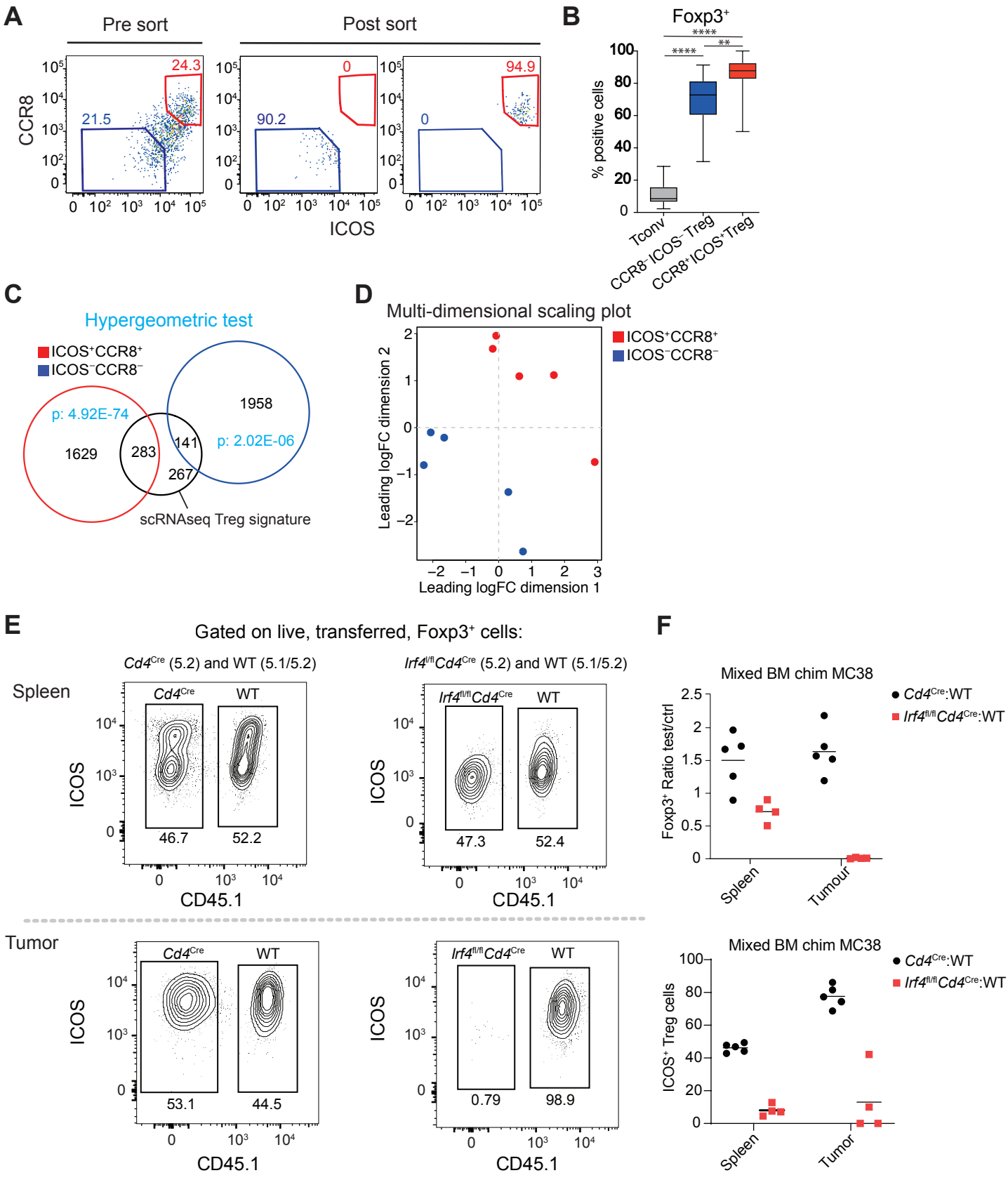
A



B

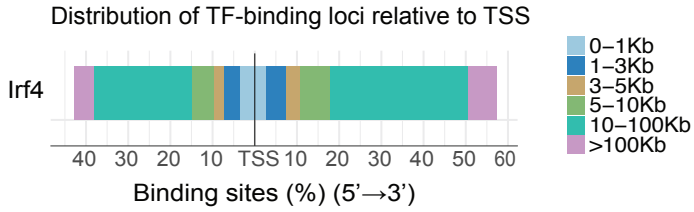
Rank	Gene	Score	P-Value
1	*IRF4*	1	--
2	*BATF*	0.251	<0.0001
4	*PRDM1*	0.231	<0.0001
8	*CTLA4*	0.216	<0.0001
9	*HLA-DRB5*	0.215	<0.0001
23	*IL1R2*	0.202	<0.0001
39	*GATA1*	0.192	<0.0001
56	*FOXP3*	0.187	<0.0001
90	*CCR8*	0.169	0.0002
123	*CD59*	0.161	0.0004
187	*IL2RA*	0.152	0.001
358	*CXCR6*	0.140	0.003
383	*CD177*	0.13	0.003
402	*ICOS*	0.13	0.004
991	*RORC*	0.104	0.025
3099	*GATA3*	0.059	0.204
3445	*TBX21*	0.054	0.247

Supplementary Figure 1. IRF4^{+} Treg signature conserved among different cancer types. **A.** t-SNE plots illustrating the expression level of selected genes across single CD4^{+} T cells from hepatocellular carcinoma lesions (Zheng et al., 2017). The 6 CD4^{+} subsets identified in the study are labelled in the plot on the left. **B.** Pearson analysis of IRF4^{-}correlated genes in melanoma (Tirosh. et al. Science, 2016).

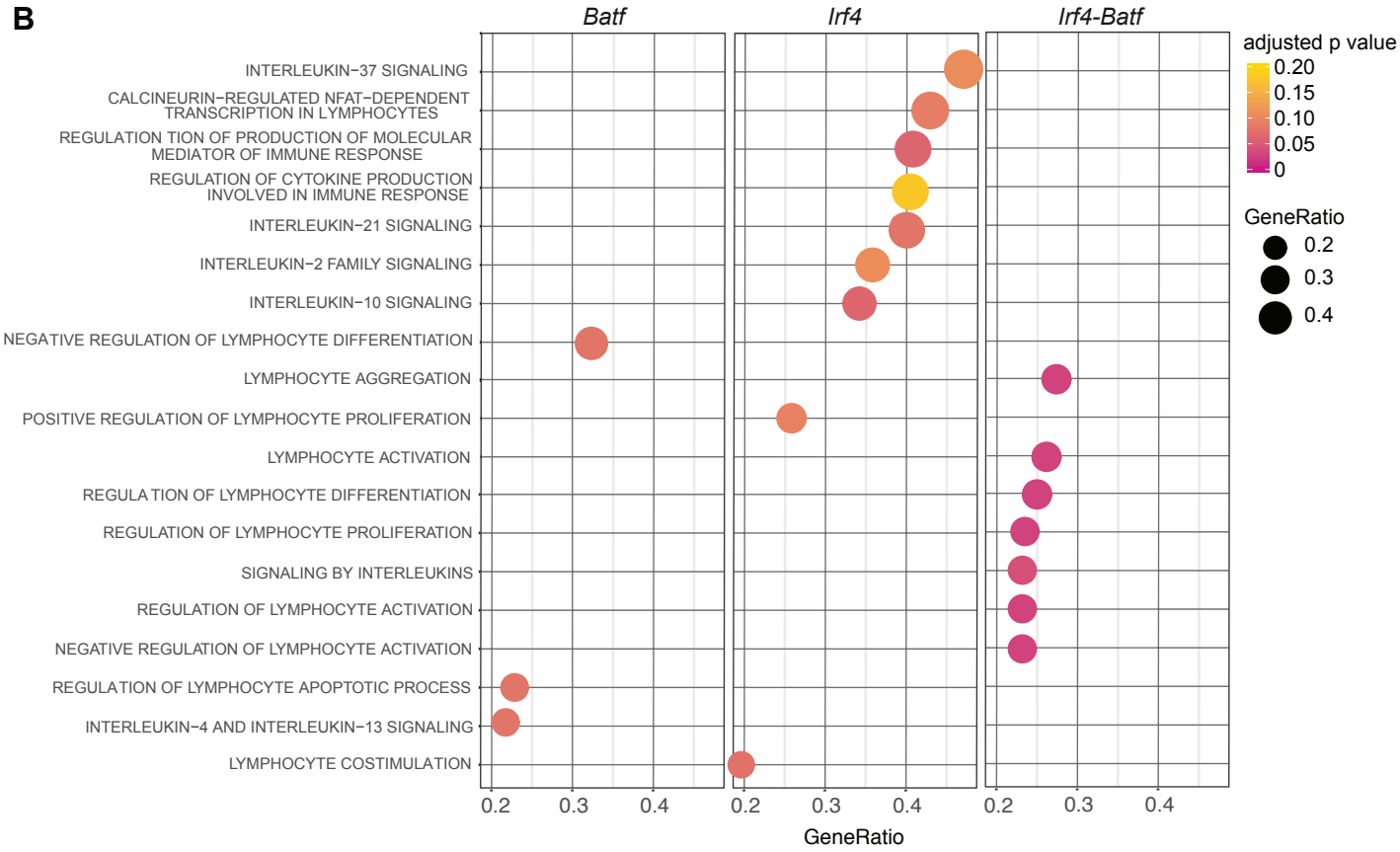

A
Pre sort
Post sort
CCR8
ICOS
24.3
21.5
0
90.2
94.9
0
B
Foxp3+
% positive cells
Tconv
CCR8−ICOS−Treg
CCR8+ICOS+Treg
C
Hypergeometric test
ICOS+CCR8+
ICOS−CCR8−
p: 4.92E-74
1629
283
141
267
1958
p: 2.02E-06
scRNAseq Treg signature
D
Multi-dimensional scaling plot
ICOS+CCR8+
ICOS−CCR8−
Leading logFC dimension 2
Leading logFC dimension 1
E
Gated on live, transferred, Foxp3+ cells:
Cd4Cre (5.2) and WT (5.1/5.2)
Irf4fl/flCd4Cre (5.2) and WT (5.1/5.2)
Spleen
Tumor
Cd4Cre
WT
Irf4fl/flCd4Cre
ICOS
CD45.1
46.7
52.2
47.3
52.4
53.1
44.5
0.79
98.9
F
Mixed BM chim MC38
Cd4Cre:WT
Irf4fl/flCd4Cre:WT
Foxp3+ Ratio test/ctrl
ICOS+ Treg cells
Spleen
Tumour
Tumor

Supplementary Figure 2. Bulk RNAseq of CCR8^{+}ICOS^{+} and CCR8^{-}ICOS^{-} tumor-infiltrating Treg subsets. A. Representative dot plots depicting the purity after FACS purification. Numbers are percentages of positive cells. **B.** Summary box-and-whisker (min to max) plot representing the frequency of FoxP3 expression in CD4^{+} Tregs that are CCR8^{+}ICOS^{+} and CCR8^{-} ICOS^{-}, and in conventional T (Tconv) cells from 48 NSCLC patients (non parametric Friedman test, **** = p<0.0001). **C.** Venn diagram depicting the genes that are overlapping between FACS-sorted CCR8^{+}ICOS^{+} (red) or CCR8^{-}ICOS^{-} (blue) Tregs (isolated as in Figure 2A) and a gene expression of bulk Tregs isolated from lung tumors (Guo et al., Nat. Med., 2018). **D.** Multidimensional scaling plot of bulk RNAseq data depicted in Figure 2B. **E.** ICOS expression in the spleen and tumor by congenically marked CD4^{+} T cells originated from *Irf4*$^{fl/fl}$*Cd4*^{Cre}:WT or *Cd4*^{Cre}(control):WT mixed bone marrow chimeras. Numbers indicate the percentage of cells identified by the gate. **F.** Summary of the data as in **E.** *Irf4*$^{fl/fl}$*Cd4*^{Cre}:WT (n=5) or *Cd4*^{Cre}(control):WT (n=4).

A


Distribution of TF-binding loci relative to TSS
Irf4
40 30 20 10 TSS 10 20 30 40 50 60
Binding sites (%) (5'→3')
0–1Kb
1–3Kb
3–5Kb
5–10Kb
10–100Kb
>100Kb

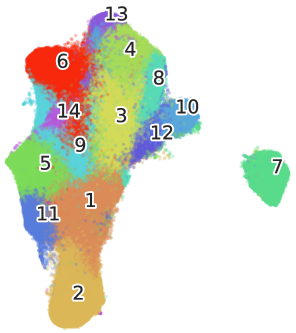
B


Batf
Irf4
Irf4-Batf
INTERLEUKIN-37 SIGNALING
CALCINEURIN-REGULATED NFAT-DEPENDENT TRANSCRIPTION IN LYMPHOCYTES
REGULATION TION OF PRODUCTION OF MOLECULAR MEDIATOR OF IMMUNE RESPONSE
REGULATION OF CYTOKINE PRODUCTION INVOLVED IN IMMUNE RESPONSE
INTERLEUKIN-21 SIGNALING
INTERLEUKIN-2 FAMILY SIGNALING
INTERLEUKIN-10 SIGNALING
NEGATIVE REGULATION OF LYMPHOCYTE DIFFERENTIATION
LYMPHOCYTE AGGREGATION
POSITIVE REGULATION OF LYMPHOCYTE PROLIFERATION
LYMPHOCYTE ACTIVATION
REGULATION OF LYMPHOCYTE DIFFERENTIATION
REGULATION OF LYMPHOCYTE PROLIFERATION
SIGNALING BY INTERLEUKINS
REGULATION OF LYMPHOCYTE ACTIVATION
NEGATIVE REGULATION OF LYMPHOCYTE ACTIVATION
REGULATION OF LYMPHOCYTE APOPTOTIC PROCESS
INTERLEUKIN-4 AND INTERLEUKIN-13 SIGNALING
LYMPHOCYTE COSTIMULATION
0.2 0.3 0.4
GeneRatio
adjusted p value
0.20
0.15
0.10
0.05
0
GeneRatio
0.2
0.3
0.4

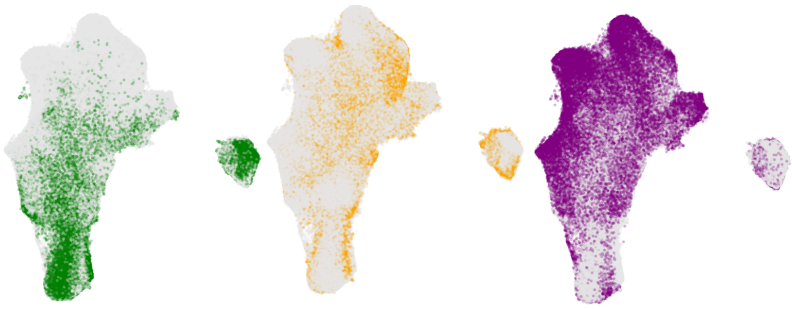
Supplementary Figure 3. *Batf* and *Irf4*-dependent gene regulation. A. Color-coded distribution of *Irf4* binding sites as relative to the TSS (indicated in Kb distance) in the mouse genome. **B.** Comparison of Immunologic signatures (C7) enrichment between *Batf*$^{-/-}$ vs WT and *Irf4*$^{-/-}$ vs WT DEGs. The color of the dots indicates statistical significance of the enrichment (adjusted p value) while the size indicates the fraction of genes annotated to each immunological process (Gene Ratio).

A

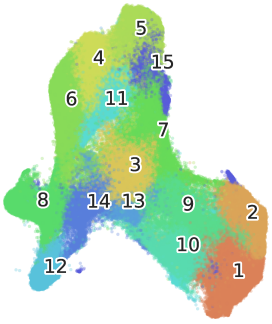
CD4+ PhenoGraph clusters



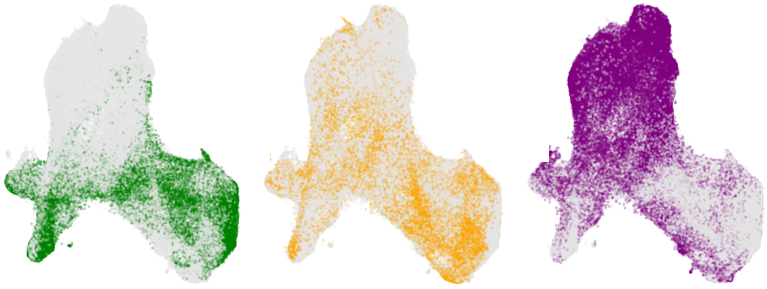
1 T_{CM}
2 T_N
3 T_M CCR5+
4 T_M CCR5+
5 T_M PD1+
6 Treg CCR8+ICOS+
7 T CTL
8 T_M CCR5+
9 T_M CCR8+
10 T_M GZMK+
11 T_{CM}
12 T_M GZMK+
13 Exh
14 Treg Ki-67+



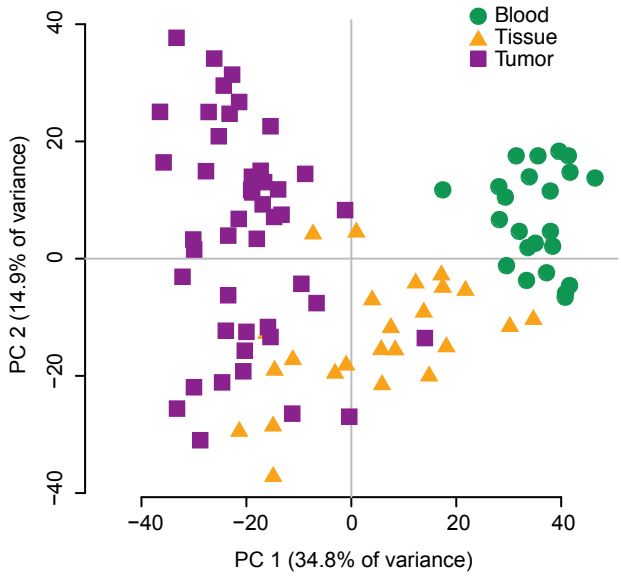
CD8+ PhenoGraph clusters

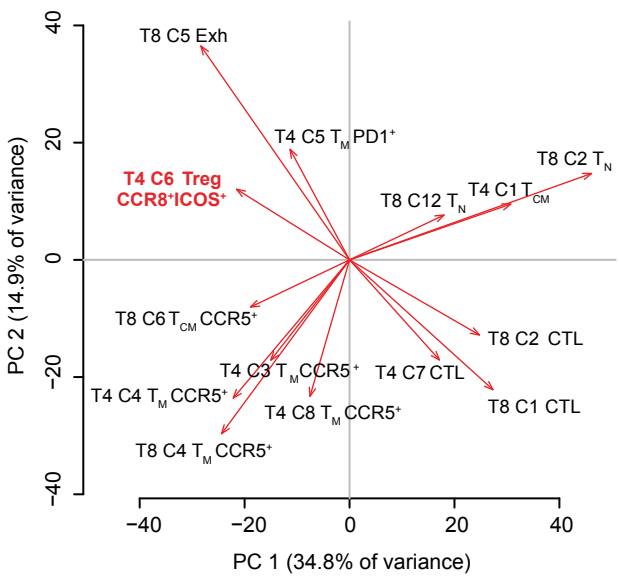


1 CTL
2 CTL
3 T_M GZMK+
4 T_M CCR5+
5 Exh
6 T_{CM} CCR5+
7 T_M GZMK+
8 Act
9 CTL
10 T_{TE} GZMK+
11 T_M CCR5+
12 T_N
13 T_M CXCL13+
14 T_{CM}
15 Exh



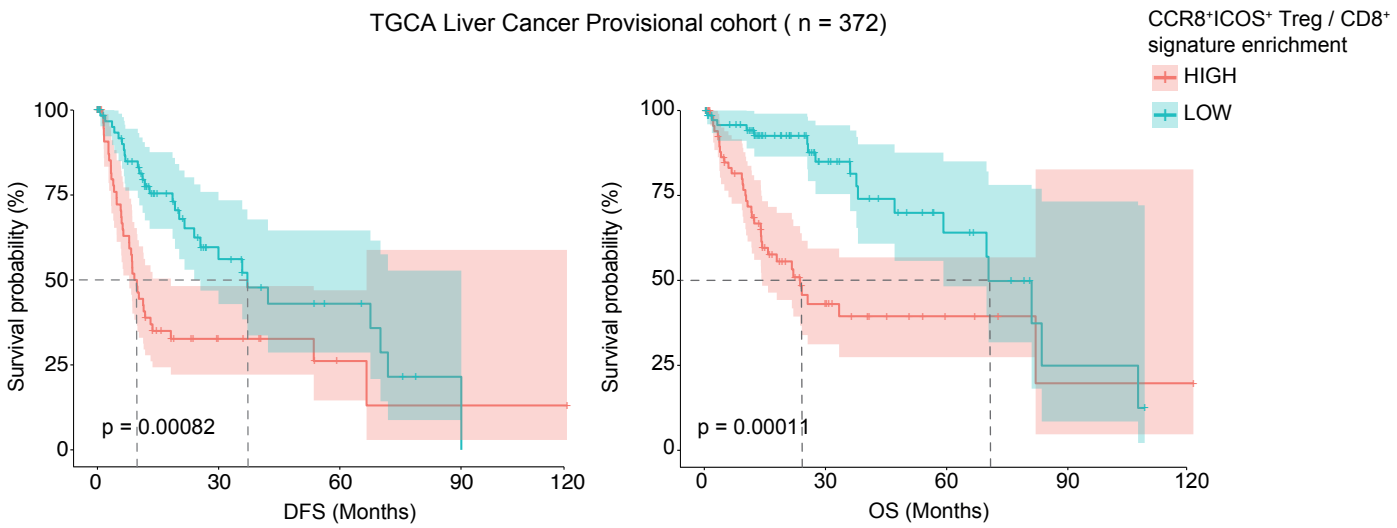
B



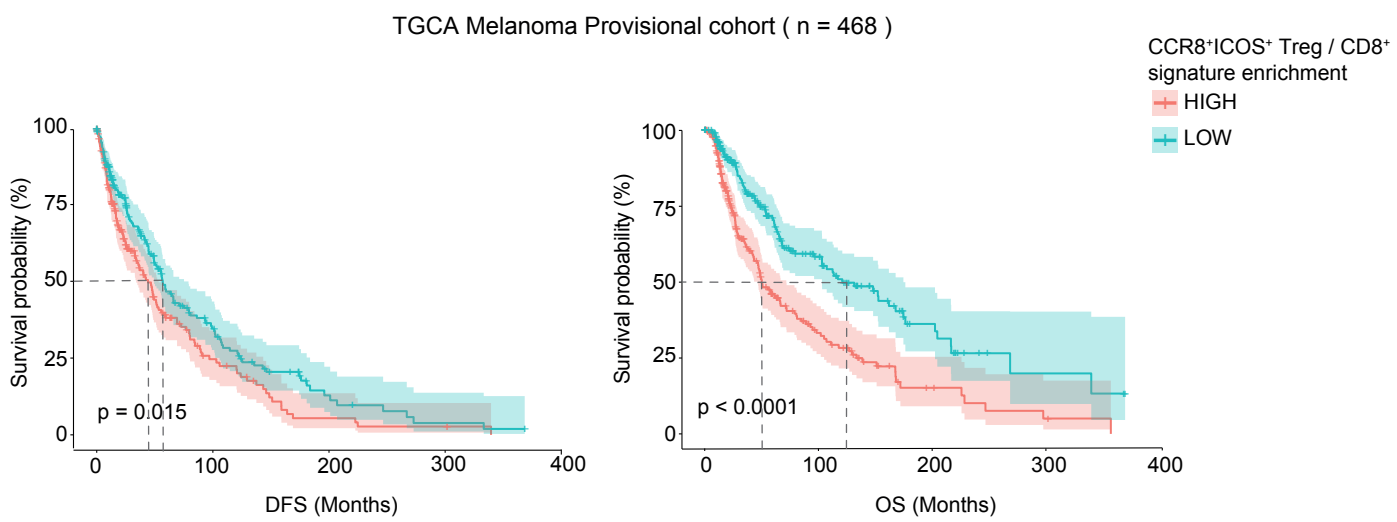


Supplementary Figure 4. Transcriptomic guided FACS panel design identifies tumor-specific T cell subpopulations A. UMAP analysis of concatenated $CD4^+$ (top) and $CD8^+$ (bottom) PhenoGraph clusters from peripheral blood (n = 23, green), normal lung tissue (n = 23, orange), and tumor (n = 45, purple) samples from NSCLC patients. **B.** Left: PCA plot showing the distribution of samples according to the frequency of $CD4^+$ and $CD8^+$ PhenoGraph clusters in each sample. The cohort was subdivided in three groups according to sample origin: Blood (green), Lung Tissue (orange), Tumor (purple). Right: PCA of PhenoGraph clusters. Those clusters most contributing to the PCA output in B are indicated.

A


TGCA Liver Cancer Provisional cohort (n = 372)
CCR8+ICOS+ Treg / CD8+ signature enrichment
HIGH
LOW
Survival probability (%)
100
75
50
25
0
p = 0.00082
0
30
60
90
120
DFS (Months)
p = 0.00011
OS (Months)

B


TGCA Melanoma Provisional cohort (n = 468)
CCR8+ICOS+ Treg / CD8+ signature enrichment
HIGH
LOW
Survival probability (%)
100
75
50
25
0
p = 0.015
0
100
200
300
400
DFS (Months)
p < 0.0001
OS (Months)

Supplementary Figure 5. $CCR8^{+}ICOS^{+}$ effector Treg infiltration relative to $CD8^{+}$ defines a signature of disease progression in different cancer types A. Kaplan–Meier disease free survival (DFS; left) and overall survival (OS; right) curves of TCGA LIHC liver cancer patients (n=372) grouped by percentile rank (0.8) according to the enrichment of the $CCR8^{+}ICOS^{+}$ bulk Treg signature (as obtained in Figure 2B) as relative to the $CD8^{+}$ T cell signature. + represents censored observations, and p value was calculated by multivariate Cox regression. Left: Disease Free Survival (DFS) analysis. Right: Overall Survival (OS) analysis **B.** Kaplan–Meier survival curves of TCGA melanoma patients (n=468) grouped by percentile rank (0.8) according to the $CCR8^{+}ICOS^{+}$ Treg bulk/$CD8^{+}$ T cell signature enrichment.+ represents censored observations, and p value was calculated by multivariate Cox regression. Left: Disease Free Survival (DFS) analysis. Right: Overall Survival (OS) analysis.