Supplementary Information

Lactate Increases Stemness of CD8+ T Cells to Augment Anti-Tumor Immunity

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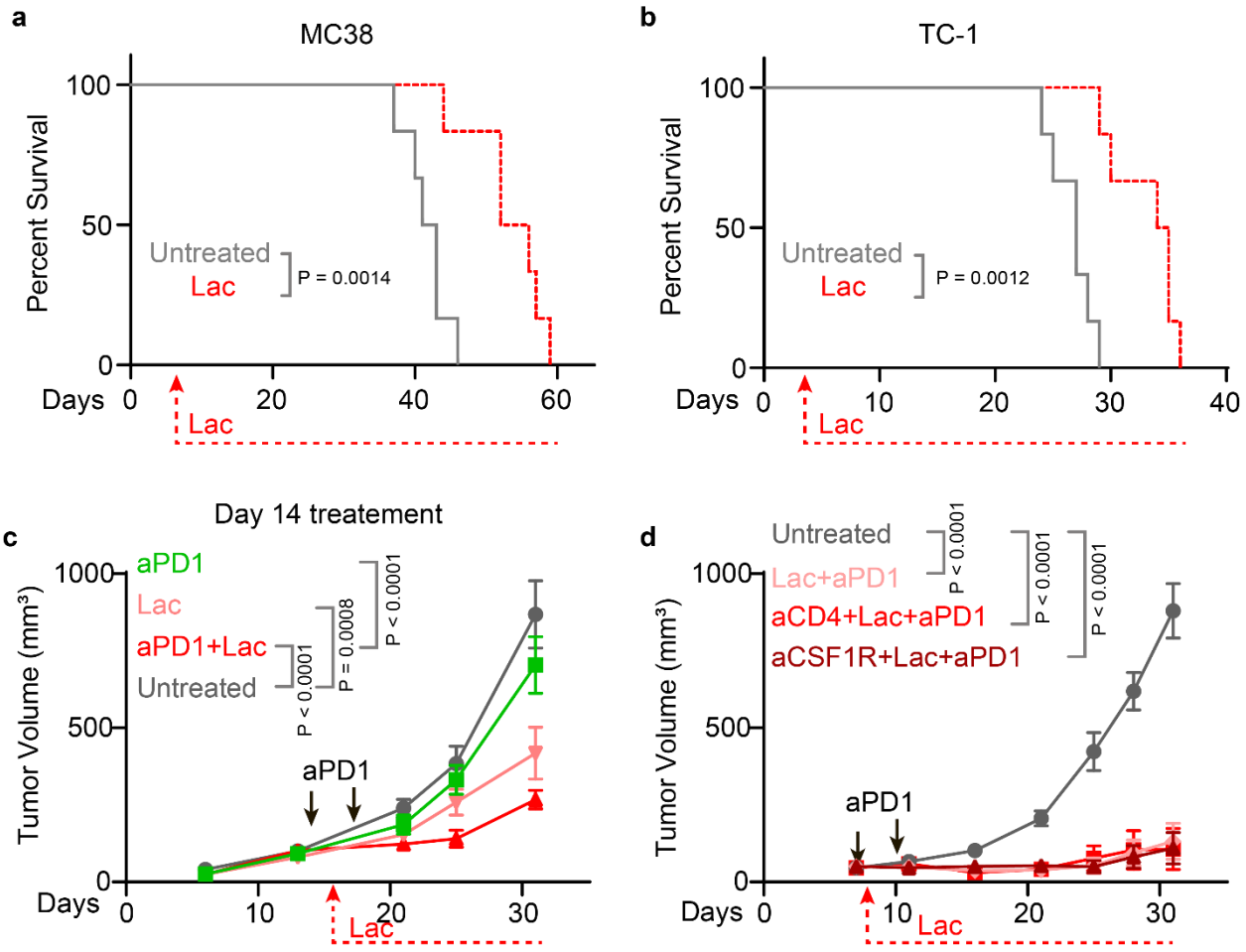
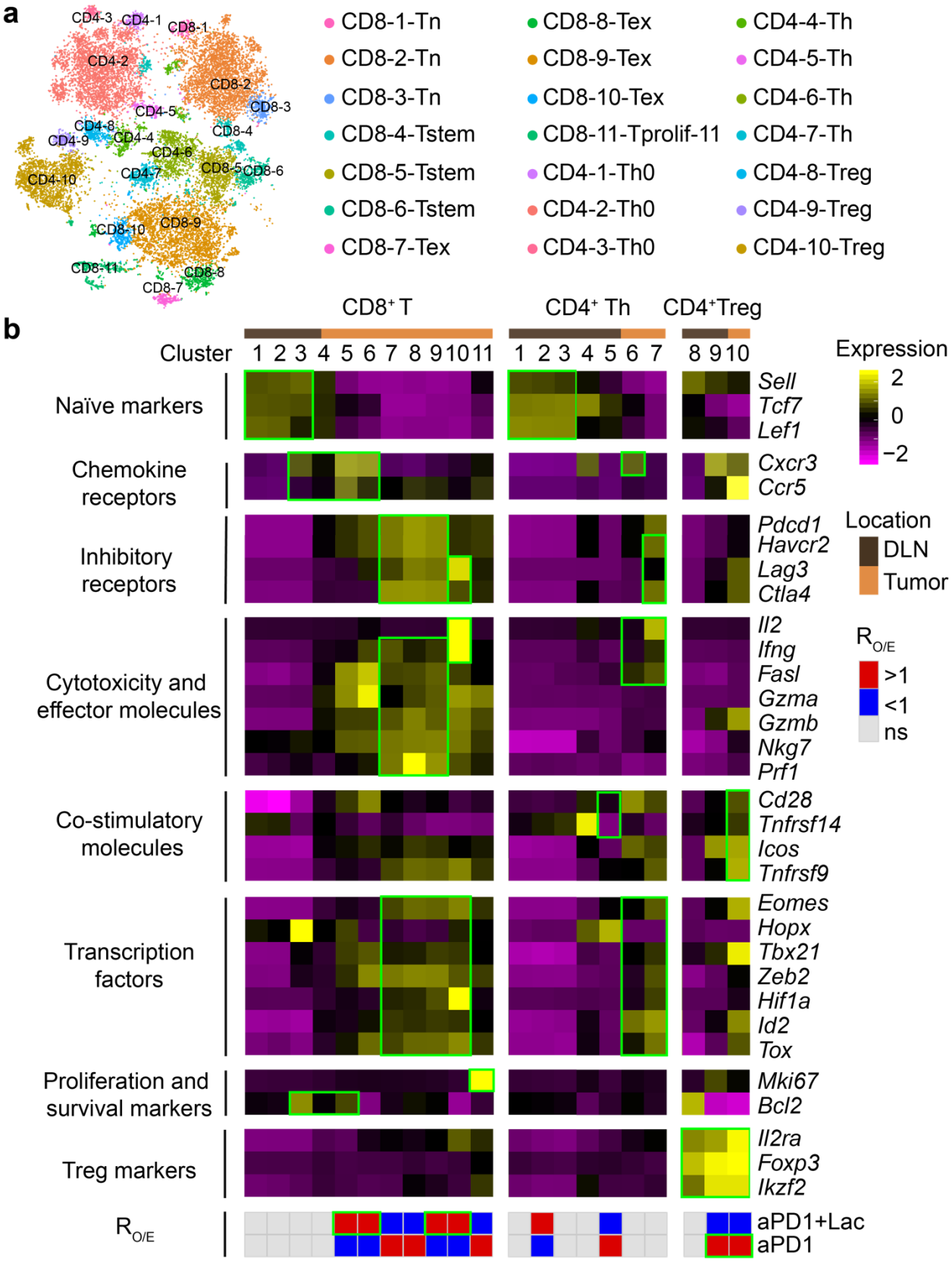


Supplementary Fig. 1. Tumor growth curves of lactate monotherapy and combination therapy with immune cell depletion.

Survival curves of lactate treatment in the MC38 (**a**) and TC-1 (**b**) tumor models. C57BL/6 mice (n = 6) were inoculated with 1×10^6 MC38 tumor cells and treated by lactate. The dose and treatment regimen of lactate is the same as **Fig. 2b**. The untreated group used the same data in **Fig. 2b** for a side-by-side comparison. C57BL/6 mice (n = 6) were inoculated with 1.5×10^5 TC-1 tumor cells and treated with lactate. The dose and treatment regimen of lactate is the same as **Fig. 2c**. **c,** Tumor growth curve of MC38 tumor model treated with lactate and anti-PD1 from day 14. C57BL/6 mice (n = 5) were inoculated with 1×10^6 MC38 tumor cells and treated by lactate. The dose of lactate is the same as **Fig. 2b**. The initial dose of anti-PD1 was on day 14. **d,** $CD4^+$ T and macrophage cell depletion assays in the MC38 tumor model. C57BL/6 mice (n = 6) were inoculated with 1×10^6 MC38 tumor cells and treated with anti-PD-1 (10 mg/kg, day 7 and 10) in combination with glucose or lactate. Anti-CD4 (10 mg/kg) or anti-CSF1R (20 mg/kg) was

administered from day 3 and then every three days until the end of the experiment. The dose and treatment regimen of lactate and anti-PD-1 are the same as **Fig. 2e**. The untreated and aPD1+Lac group used the same data in **Fig. 1e** for a side-by-side comparison. Data are shown as means ± SEM. P value was determined by one-tail two-way ANOVA with correction using Geisser-Greenhouse method. Source data and summary of all P values are provided in Source Data file.


a
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Tcf7
Lef1
Chemokine receptors
Cxcr3
Ccr5
Inhibitory receptors
Pdcd1
Havcr2
Lag3
Ctla4
Cytotoxicity and effector molecules
Il2
Ifng
Fasl
Gzma
Gzmb
Nkg7
Prf1
Co-stimulatory molecules
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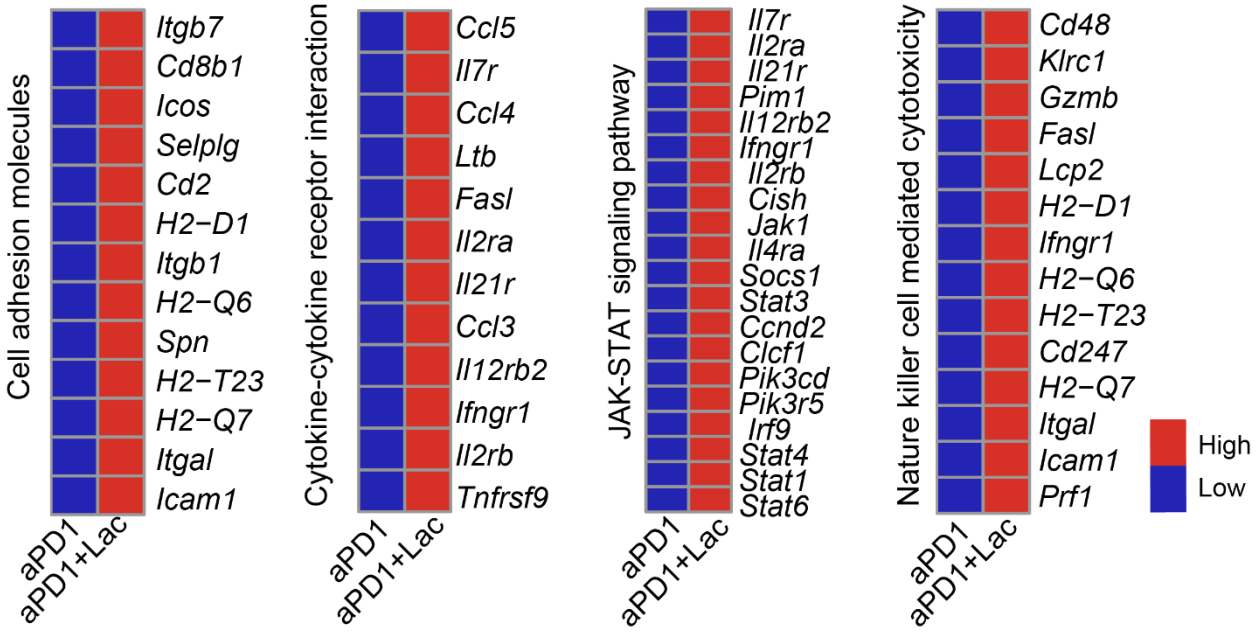
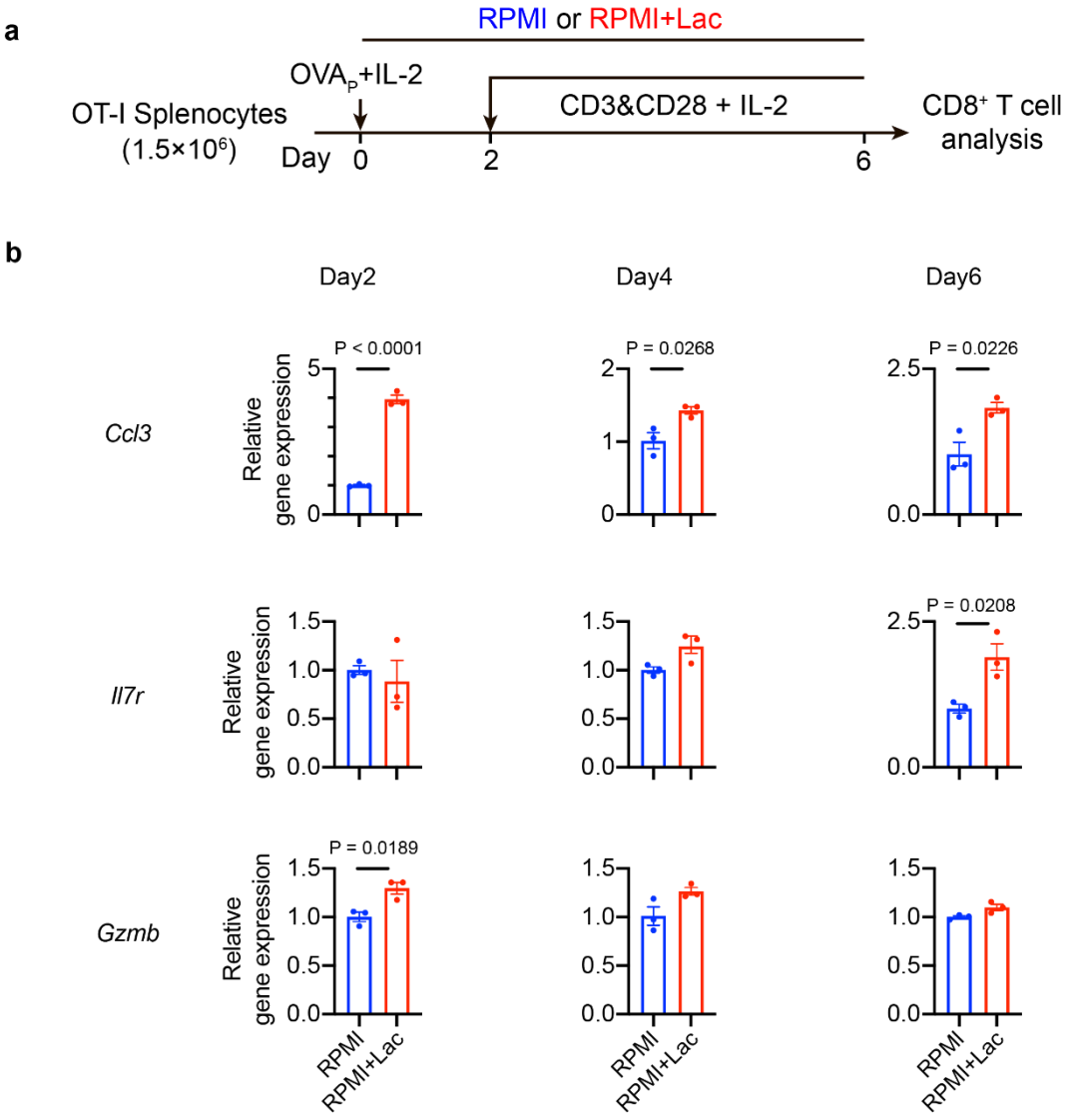
Transcription factors
Eomes
Hopx
Tbx21
Zeb2
Hif1a
Id2
Tox
Proliferation and survival markers
Mki67
Bcl2
Treg markers
Il2ra
Foxp3
Ikzf2
R O/E
aPD1+Lac
aPD1

Supplementary Fig. 2. Clustering of T cells from single cell RNA sequencing analysis.

a, The tSNE projection of 21, 826 single T cells from 2 treatment groups, showing the formation of 21 main clusters, including 11 for $CD8^+$ T cells, 7 for conventional $CD4^+$ T helper cells (Th: 1-7 of $CD4^+$ clusters) and 3 for regulatory T cells (Treg: 8-10 of $CD4^+$ clusters). Each dot corresponds to one single cell, colored according to cell cluster. **b,** Mean expression of selected T cell function-associated genes in each cell cluster. Green boxes highlight the prominent patterns defining known T cell subtypes. The ratio of observed cell number to expectation ($R_{O/E}$) of Lac effect on anti-PD-1 treatment for each cluster was calculated by one-tail Chi-square test. Source data are provided as a Source Data file.

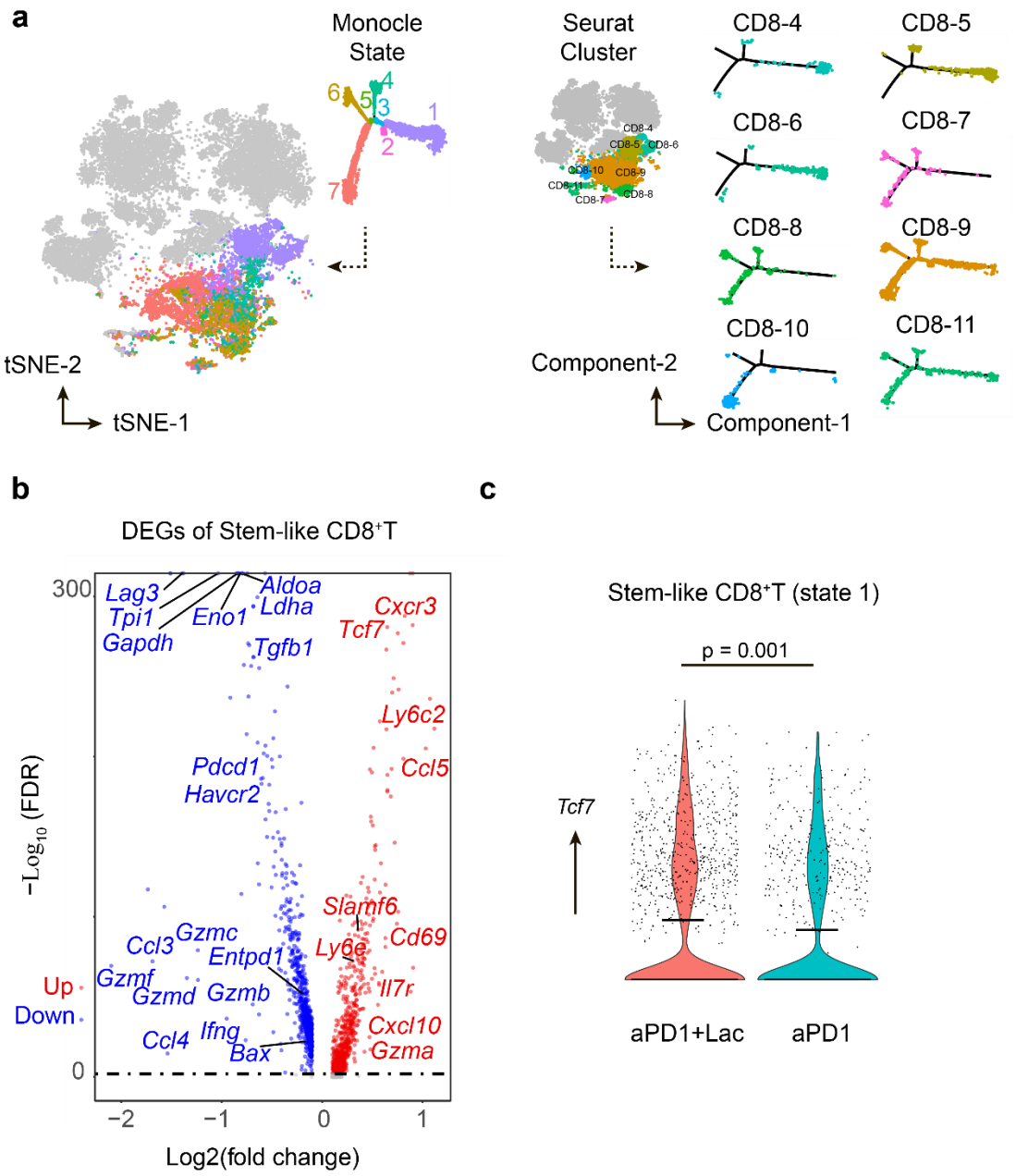


Supplementary Fig. 3. Differentially expressed genes from GSEA analysis in Figure 3. Source data are provided as a Source Data file.



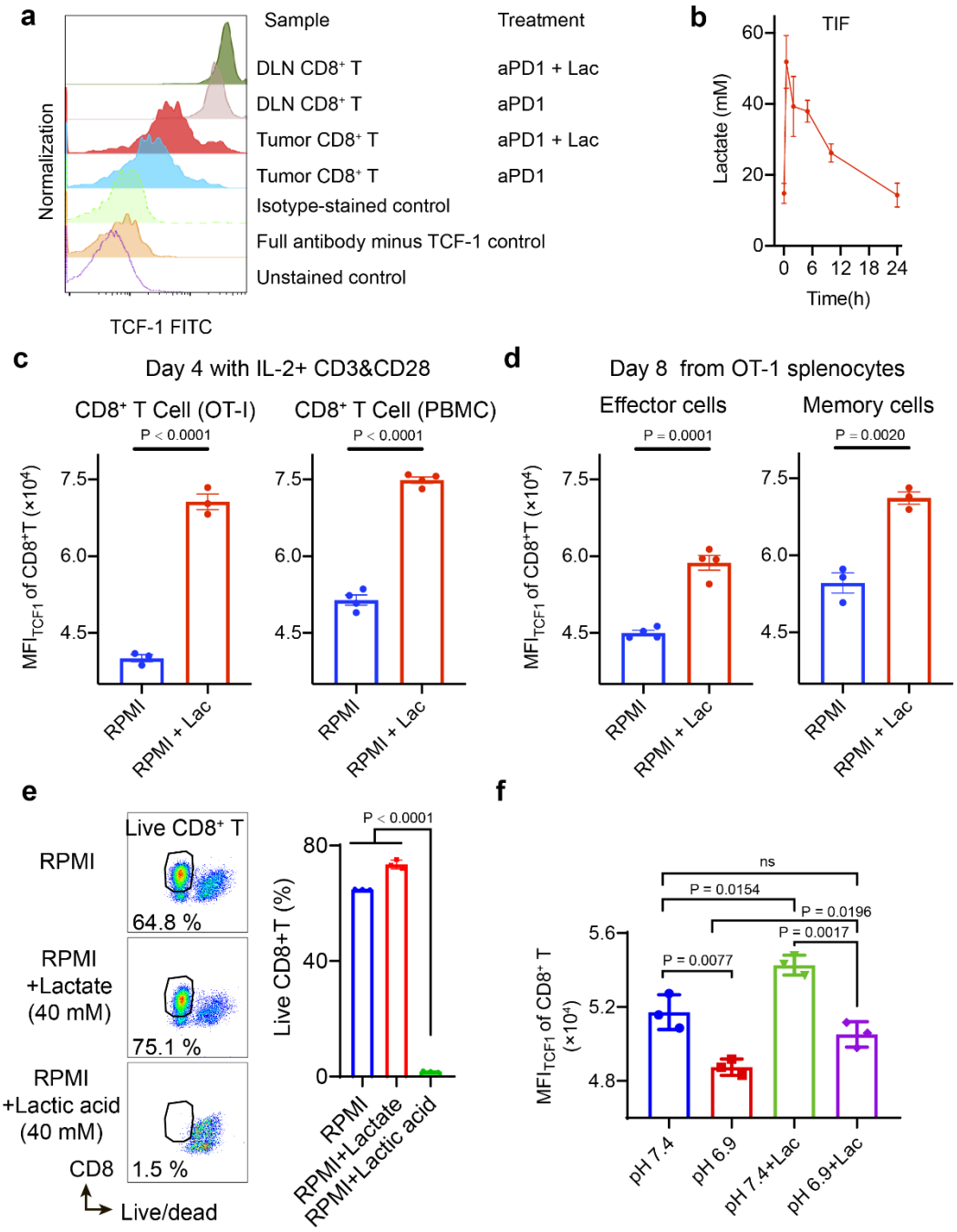
Supplementary Fig. 4. Verification of the expression of genes elevated by lactate treatment from single cell analysis.

a, Schematic illustration of the CD8$^+$ T cell culture assay. **b,** Gene expression quantified with qPCR for *Ccl3*, *Il7r*, and *Gzmb*. Data are shown as means ± SEM. n= 3 biologically independent samples. P value was determined by one-tail unpaired t-test. Source data are provided as a Source Data file.



Supplementary Fig. 5. Delineation of stem-like CD8⁺ T cell population by pseudotime analysis.

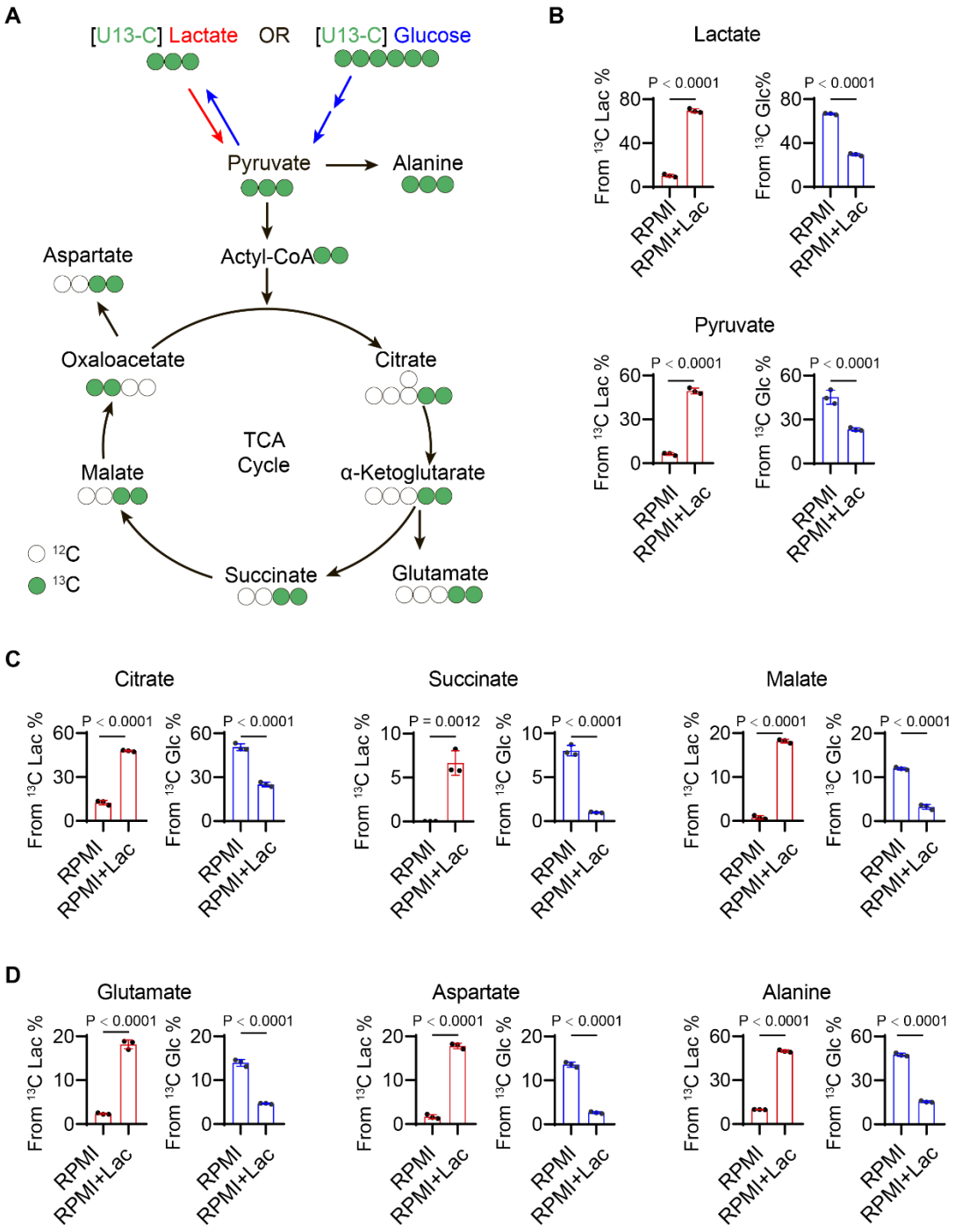
a, Correlation of Seurat clusters and monocle states. Stem-like T cells in state 1 are mostly from Seurat cluster CD8-4, CD8-5, CD8-6 and part of CD8-9. **b,** Volcano plot of differentially expressed genes by stem-like CD8⁺ T cells. **c,** *Tcf7* gene expression in stem-like CD8⁺ T cells. p value was determined by two-tail Mann Whitney U test. Source data are provided as a Source Data file.



Supplementary Fig. 6. Lactate increases the expression of TCF-1 *in vivo* and *ex vivo* while lactic acid induces T cell death.

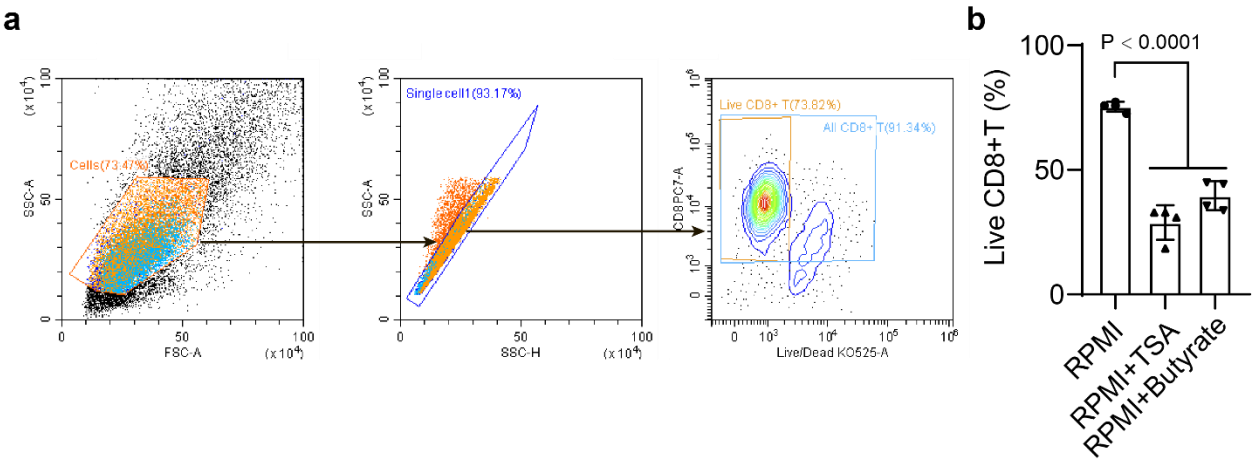
a, Flow cytometry histograms of TCF-1 expressions on $CD8^+$ T cells under different treatment conditions. Unstained control, full antibody minus anti-TCF-1 and isotype IgG were used as controls. **b,** Lactate concentration in tumor interstitial fluids (TIF) after subcutaneous lactate injection (n = 3 biologically independent samples). **c,** Quantification of TCF-1 expressions in *ex*

vivo cultured $CD8^+$ T cells from OT-I mice or human PBMCs on day 4 (n = 3 biologically independent samples for OT-I cell, n = 4 biologically independent samples for PBMC). **d,** Quantification of TCF-1 expressions in *ex vivo* cultured effector or memory $CD8^+$ T cells on day 8. For memory T cells induction, $CD8^+$ T cells from OT-1 splenocytes were activated with OVAp for two days and purified with ficoll, followed by 6-Day culture with addition of IL-15 (20 ng/mL) (n = 4 biologically independent samples for effector cell culture, n = 3 biologically independent samples for memory cell culture). **e,** Flow cytometry plot and quantification of live $CD8^+$ T cells from OT-I mice in RPMI medium with sodium lactate (pH 7.4) or lactic acid (pH 4.6) after two days (n = 3 biologically independent samples). **f,** Quantification of TCF-1 expression in $CD8^+$ T cells cultured in medium with mildly acidic pH and lactate concentrations (n = 3 biologically independent samples). Data are shown as means ± SEM. P value was determined by two-tail unpaired t-test (**c**, **d**) or one-tail one-way ANOVA (**e**, **f**). Source data are provided as a Source Data file.



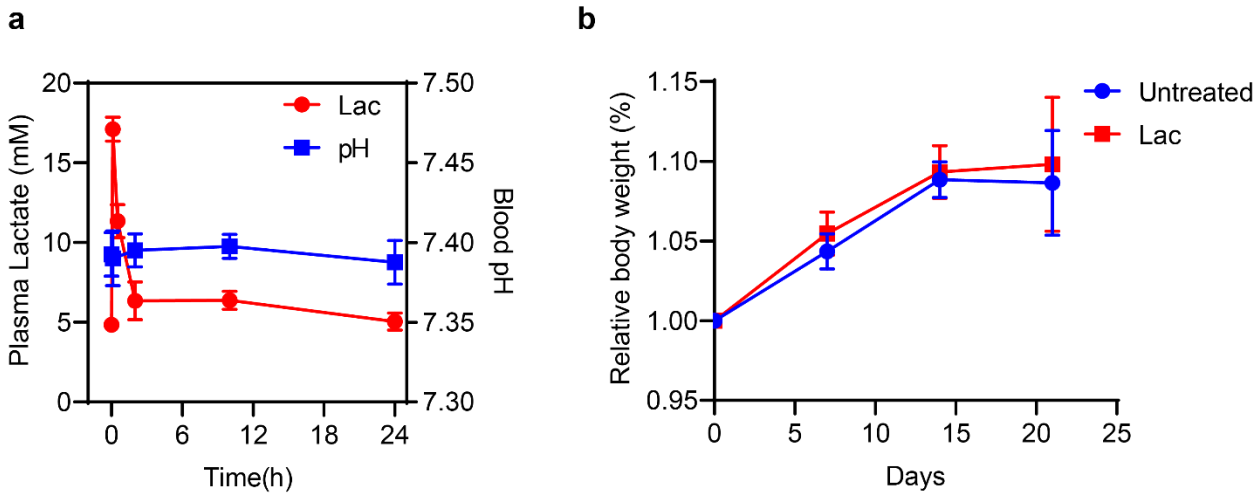
Supplementary Fig. 7. Lactate feeds TCA cycle during *ex vivo* culture of CD8$^+$ T cell.

a, Schematic illustration of isotopologues generated from ^{13}C-glucose or ^{13}C-lactate. Normalized abundance of intracellular lactate and pyruvate (**b**), TCA cycle intermediates (**c**) and derived amino acids (**d**). Data are shown as means ± SEM. n = 3 biologically independent samples. Source data are provided as a Source Data file.



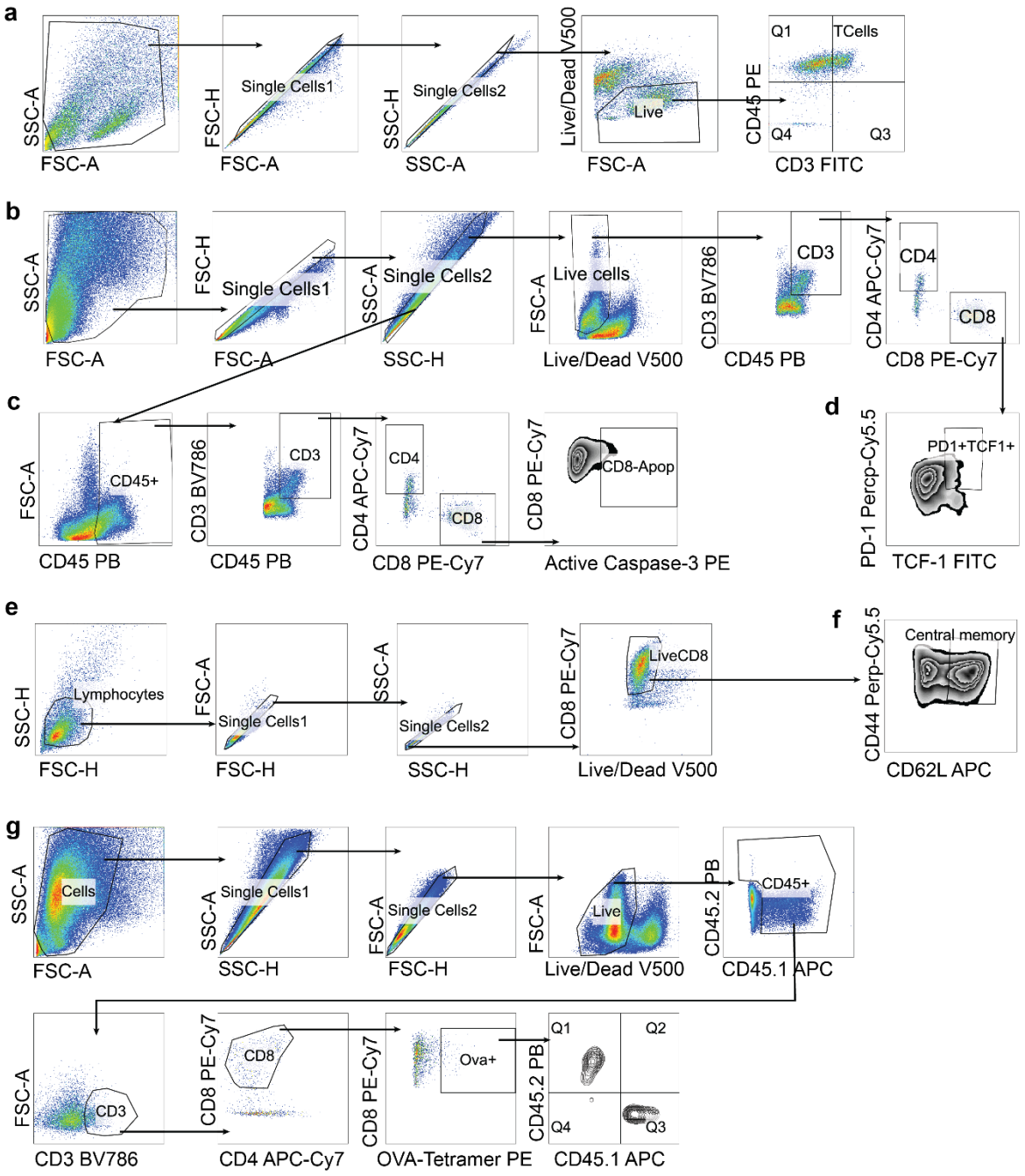
Supplementary Fig. 8. Cell viability of CD8+ T cell cultured with TSA or butyrate after 4-day culture.

a, Gating strategy for quantification of live CD8+ T cells. CD8+ T cells from OT-I mice were cultured with different drug for 4 days (TSA = 1 nM, Butyrate = 250 μM). **b**, Statistical analysis of live CD8+ T cells with different treatments. n = 4 biologically independent samples. Data are shown as means ± SD. P value was determined by one-way ANOVA and corrected by Tukey method for multiple comparisons. Source data are provided as a Source Data file.



Supplementary Fig. 9. Effect of subcutaneously injected lactate solution on blood lactate concentrations and pH levels and body weight.

a, Lactate concentration and pH in blood as a function of time after subcutaneous lactate injection (1.68 g/kg). (For lactate, n = 3 biologically independent samples, for pH, n = 4 biologically independent samples). **b**, Change of body weight after daily lactate treatment from Day 0 to 21. (n = 5 biologically independent samples). Data are shown as means ± SEM. Source data are provided as a Source Data file.



Supplementary Fig. 10. Gating strategies used for cell sorting and flow cytometry analysis.

a, Gating strategy to sort tumor infiltrating T cells for single cell analysis. Data from sorted cells were used in Fig 3b-d, Fig 4a-b, Supplementary Fig 2, 3, 5. **b**, Gating strategy for analyzing tumor infiltrating $CD8^+$ T cells number and TCF-1 expression in Fig. 2e, Fig. 3e,f, supplementary Fig. 6a. **c,** Gating strategy for analyzing apoptotic tumor infiltrating $CD8^+$ T cells in Fig. 2f. **d,** Gating

strategy for analyzing tumor infiltrating stem-like $CD8^+$ T cells number in Fig. 4d. **e,** Gating strategy for analyzing $CD8^+$ T cells in cell culture in Fig. 5 b-e, h, i, 6d, supplementary Fig. 6 c, e, f. **f,** Gating strategy for analyzing IL-15 induced memory $CD8^+$ T cells in cell culture in supplementary Fig. 6d. g, Gating strategy for analyzing tumor infiltrating ova-targeting $CD8^+$ T cells from recipients or donor in Fig. 7d-f.