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Life Sciences Reporting Summary

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For final submission: please carefully check your responses for accuracy; you will not be able to make changes later.

Experimental design

1. Sample size

Describe how sample size was determined.

Sample sizes were not predetermined; for the OT-I and CAR mouse experiments, sample sizes were chosen based on a previous study from our lab [ref. 18]. For the Nr4a3-/- and Nr4a TKO experiments in the original submission, power calculations using a one-sided Mann-Whitney-Wilcoxon Test were retroactively performed on the initial experiment, and determined that chosen sample sizes were sufficient. Summary Statement from power calculation as follows: Group sample sizes of 7 and 7 achieve 91% power to show a difference in means when there is a difference of 0.9 between the null hypothesis mean difference of 0.0 and the actual mean difference of -0.9 at the 0.050 significance level (alpha) using a one-sided Mann-Whitney-Wilcoxon Test. These results are based on 2000 Monte Carlo samples from the null distributions: Normal(M0 S) and Normal(M0 S), and the alternative distributions: Normal(M0 S) and Normal(M1 S). Because the initial Nr4a3-/- and Nr4a TKO difference was less significant than that of the WT and Nr4a TKO, we extrapolated that the previous sample sizes would be sufficient for the WT and Nr4a TKO experiments as well.

2. Data exclusions

Describe any data exclusions.

One replicate of cytokine production collected from in vivo TILs by flow cytometry was excluded due to a machine/cytometer error during data collection.

For human cell ATAC-seq analysis, three samples with less than 10 million unique, non-chrM mapped reads were excluded. Samples with low numbers of unique reads are often indicative of sample viability issues or PCR amplification artifacts. Additionally, samples from one donor had substantial signal at regulatory elements that were not apparent in other samples, and all four samples from this donor (donor 3) were excluded from further comparisons. We have found that ATAC-seq is generally very reproducible between biological replicates and substantial outliers can be indicative of sample preparation issues. These analyses were performed on previously published data from other investigators and thus exclusion criteria were not pre-determined.

3. Replication

Describe the measures taken to verify the reproducibility of the experimental findings.

All experimental findings can be and were reliably reproduced. For sequencing and flow cytometry, we performed two to six independent biological replicates of each assay and all results were reproducible. For mouse survival studies, altogether we used a minimum of 17 mice and a maximum of 39 mice (independent biological replicates) per transfer group.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Tumor-bearing mice were first tumor size-matched and then randomly allocated to groups for adoptive transfer of CAR or OT-I, or in the later experiments, CAR + empty vector (pMIN) or CAR + Cre consisting of various Nr4a-floxed genotypes.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Investigators were not blinded to group allocation during data collection and analysis; investigators were aware of the cell type transferred into tumor-bearing mice. As certain experiments already required the simultaneous participation of more than one investigator, we did not have the personnel resources to consistently perform blinding; hence blinding was not used for the course of this study.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ A statement indicating how many times each experiment was replicated
☐	☒ The statistical test(s) used and whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒ Test values indicating whether an effect is present *Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.*
☐	☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒ Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

ATAC-seq analysis: bowtie 1.0.0, samtools 0.1.8, bedtools v2.16.2, MACS2 v2.1.1.20160309, picard tools-1.94, java genomics toolkit 1.1.0, trim_galore 0.3.8, homer v4.10.1, and R v3.3.3 (with packages Biobase v2.34.0, BiocGenerics v0.20.0, Biostrings v2.42.1, data.table v1.11.4, dplyr v0.7.6, GenomeInfoDb v1.10.3, GenomicAlignments v1.10.1, GenomicRanges v1.26.4, ggplot2 v3.0.0, gtools v3.5.0, IRanges v2.8.2, limma v3.30.13, MEDIPS v1.24.0, pheatmap v1.0.8, RColorBrewer v1.1-2, Rsamtools v1.26.2, S4Vectors v0.12.2, SummarizedExperiment v1.4.0, tidyr v0.8.1, XVector v0.14.1)

RNA-seq, scRNA-seq, and GSEAs analysis: TrimGalore v0.4.5, Cutadapt v1.13, STAR v2.5.3a, R v3.3.3, GSEA v3.0; BioConductor packages: (for data analysis), rtracklayer v1.34.2, GenomicAlignments v1.10.1, DESeq2 v1.14.1; BioConductor packages: (for making figures) pheatmap, ggplot2, ggrepel, grid, RColorBrewer, MAGIC (R implementation, Rmagic v1.0.0

Flow cytometry analysis: FlowJo v.10 (Tree Star, Inc), Prism 7 (GraphPad Software)
Tumor growth curve / survival curve analysis: Prism 7 (GraphPad Software)
Assembly / layout of figures: Adobe Illustrator CS6, Affinity Designer 1.6.1

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

All unique materials will be made available by authors upon request.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

All antibodies were purchased from Biolegend, eBioscience, BD Bioscience, and Cell Signaling Technology with the exception of mouse CD3 which was purified from a monoclonal-antibody producing hybridoma (Clone 145-2C11). All Biolegend, eBioscience, BD Bioscience, and Cell Signaling Technology antibodies provide validation statements/data and relevant citations on the manufacturer's website, which can be found by searching for the catalog number of the antibody on the corresponding manufacturer's website. For flow cytometry, all antibodies were used at a final concentration of 1:200 with the exception of Ki67, which was used at a final concentration of 1:100. For ChIP, 10ug of the HA-tag antibody was used.

Catalog No. Supplier Name Antibody Clone Name Lot No.
100712 Biolegend APC anti-mouse CD8a 53-6.7 B200238
100706 Biolegend FITC anti-mouse CD8a 53-6.7 B208067, B217242
100708 Biolegend PE anti-mouse CD8a 53-6.7 B134188, B151201
100737 Biolegend BV421 anti-mouse CD8a 53-6.7 B226247, B210399
100734 Biolegend PerCP/Cy5.5 anti-mouse CD8a 53-6.7 B156856
100722 Biolegend PeCy7 anti-mouse CD8a 53-6.7 B190884
110730 Biolegend PeCy7 anti-mouse CD45.1 A20 B188237, B217246
11-0900-85 eBioscience FITC anti mouse/rat CD90.1 (Thy 1.1) HIS51 4310957
554898 BD Biosciences PE mouse anti-rat/mouse CD90.1 OX-7 2317593
202539 Biolegend BV711 anti-rat/mouse CD90.1 Thy1.1 OX-7 B223103
202516 Biolegend PerCP/ Cy5.5 anti-rat/mouse CD90.1 Thy1.1 OX-7 B202057
202519 Biolegend APC-Cy7 Anti-rat/mouse CD 90.1 OX-7 B222663
345106 Biolegend PE anti-human CD271 (NGFR) ME 20.4 B175123
345108 Biolegend APC anti-human CD271 (NGFR) ME 20.4 B204228
345112 Biolegend PerCP/Cy5.5 anti-human CD271 (NGFR) ME 20.4 B218745
135221 Biolegend BV421 anti-mouse CD279 (PD-1) 29F.1A12 B213655
135206 Biolegend PE anti-mouse CD279 (PD-1) 29F.1A12 B142906
135210 Biolegend APC anti-mouse CD279 (PD-1) 29F.1A12 n/a
125210 Biolegend APC anti-mouse CD223 (Lag3) C9B7W B176313
125223 Biolegend PE/Dazzle 594 anti-mouse CD223 (Lag3) C9B7W B224161
12-5870-81 eBioscience PE anti-mouse TIM3 RMT3-23 4301948, 4273433
119723 Biolegend BV421 anti-mouse CD366 (Tim-3) RMT3-23 B235257
119705 Biolegend APC anti-mouse CD366 (Tim-3) RMT3-23 n/a
123907 Biolegend PE anti-mouse CD200R (OX2R) OX-110 B220799
123809 Biolegend APC anti-mouse CD200 (OX2) OX-90 B203310
133507 Biolegend PE anti-mouse CD244.2 (2B4 B6 alloantigen) m2B4 (B6) 458.1 B182843
126309 Biolegend PE anti-mouse CD357 (GITR) DTA-1 B214908
506328 Biolegend BV421 anti-mouse TNF alpha MP6-XT22 B224675
503839 Biolegend PE/Dazzle594 anti-mouse IL-2 JES6-5H4 B211964
12-7021-82 eBioscience PE anti-mouse IL-2 JES6-5H4 E030634
505807 Biolegend PE anti-mouse IFN gamma XMG1.2 B178149
17-7311-82 eBioscience APC anti-mouse IFN gamma XMG1.2 E07379-1633
363004 Biolegend PE anti-human CD19 SJ25C1 B214170
9066S Cell Signaling Technology TCF1/ TCF7 Rabbit mAb (Pacific Blue Conjugate) C63D9 1
12-4875-80 eBioscience PE anti-mouse Eomes Dan11mag 4313231, 4323634
50-5825-82 eBioscience eFluor 660 anti-human/mouse Tbet 4B10 E12136-1632
65-0865-18 eBioscience Fixable Viability Dye eFluor 780 n/a E11447-1674
102112 Biolegend LEAF (TM) Purified CD28 37.51 B229179, B231127, B228119
n/a monoclonal antibody- producing hybridoma purified CD3 145-2C11 n/a
3724S Cell Signaling Technology HA-tag Rabbit mAb C29F4 8
3739S Cell Signaling Technology Myc-tag (9B11) mouse PE mAb 9B11 9
135216 Biolegend PeCy7 anti-mouse CD279 (PD-1) 29F.1A12 B227806
a12-1011-80 Invitrogen PE anti-mouse CD101 Moushi 101 4330771
17-0381-81 eBioscience APC anti-mouse CD38 90 4324890
12-5965-80 eBioscience PE anti-mouse Nur77 (Nr4a1) 12.14 E01954-1636
sc-376984AF647 Santa Cruz Biotechnology AF647 anti-mouse Nr4a2 F-5 G2517
sc-393902PE Santa Cruz Biotechnology PE anti-mouse Nr4a3 H-7 B2818
563786 BD Biosciences BUV395 rat anti-mouse CD8a 53-6.7 8072932, 7096603
652405 Biolegend APC anti-mouse Ki67 16A8 B191905
345104 Biolegend FITC anti-human NGFR ME20.4 B223717
400411 Biolegend APC Rat IgG1 k isotype control RTK2071 B238505
400511 Biolegend APC Rat IgG2a k isotype control RTK2758 n/a

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

The EL4 mouse thymoma cell line was purchased from the American Type Culture Collection (ATCC): EL4 (ATCC® TIB-39™, Mus musculus T cell lymphoma). The B16-OVA mouse melanoma cell line was a kind gift of Dr. Schoenberger (LJI). The 293T cell line was purchased from ATCC: 293T (ATCC® CRL-3216™). The Platinum-E Retroviral Packaging Cell Line, Ecotropic (PlatE) cell line was purchased from Cell BioLabs, Inc: RV-101. The MC-38 mouse colon adenocarcinoma cell line (a kind gift of A.W. Goldrath, UCSD, La Jolla, CA) was originally purchased from Kerafast, Inc (ENH204).

b. Describe the method of cell line authentication used.

The EL4 cell line stained positive for mouse Thy1.2 and PD-1; and stained negative for huCD19. The B16-OVA cell line stained negative for huCD19. The MC-38 cell line stained negative for huCD19.
The PlatE and 293T cell lines were not authenticated.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cell lines were not tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

C57BL/6J, B6.SJL-PtprcaPepcb/BoyJ, Rag 1-/- mice were obtained from Jackson Laboratories. Nr4a gene-disrupted strains were obtained from Takashi Sekiya and Akihiko Yoshimura, with permission from Pierre Chambon. Both male and female mice were used for studies. Mice were age-matched and between 8-12 weeks old when used for experiments, and tumor-bearing mice were first tumor size-matched and then randomly assigned to experimental groups. All mice were bred and/or maintained in the animal facility at the La Jolla Institute for Allergy and Immunology. All experiments were performed in compliance with the LJI Institutional Animal Care and Use Committee (IACUC) regulations.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

There were no human participants in this study.

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Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

☒ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ 3. All plots are contour plots with outliers or pseudocolor plots.

☒ 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.	Sample preparation for cell sorting of huCD19-expressing cell lines: Bulk populations of huCD19-transduced EL4, B16-OVA, or MC-38 cells were spun down and stained for cell sorting. Sample preparation for flow cytometry of in vitro CD8+ T cells: Mouse CD8+ T cells in culture were spun down and stained for phenotyping with flow cytometry or stained for cell sorting. Sample preparation for flow cytometry/cell sorting of TILs from CAR and OT-I experiments, and for flow cytometry of TILs from Nr4aTKO vs WT experiments: On Day 21, mice were euthanized and perfused with PBS prior to removal of tumor. Tumors were collected, pooled together by group, homogenized, and then dissociated using the MACS Miltenyi Mouse Tumor Dissociation kit (Miltenyi Biotec) and the gentleMACs dissociator with Octo Heaters (Miltenyi Biotec) according to manufacturer's instructions. Tumors were then filtered through a 70uM filter and spun down. Supernatant was aspirated and the tumors were resuspended in the equivalent of 4-5 grams of tumor per 5mL of 1%FBS/PBS for CD8 positive isolation using the Dynabeads FlowComp Mouse CD8 isolation kit (Invitrogen). After positive isolation, cells were either divided into equal amount for staining and phenotyping with flow cytometry, or stained for cell sorting. Sample preparation for cell sorting of TILs from WT and Nr4a TKO experiments: On Day 21, mice were euthanized and perfused with PBS prior to removal of tumor. Tumors were collected, pooled together by group, homogenized, and then dissociated using the MACS Miltenyi Mouse Tumor Dissociation kit (Miltenyi Biotec) and the gentleMACs dissociator with Octo Heaters (Miltenyi Biotec) according to manufacturer's instructions. Tumors were then filtered through a 70uM filter and spun down. Supernatant was aspirated and the tumors were resuspended in 40% Percoll/RPMI and underlaid with 80% Percoll/PBS in 15mL conical tubes to form an 80%/40% Percoll discontinuous density gradient. Samples were spun for 30min at room temperature at 1363g in a large benchtop centrifuge with a swinging bucket. TILs were collected from 80%/40% Percoll interface and further purified using CD90.2 Microbeads (Miltenyi Biotec) and magnetic separation. After positive isolation, cells were stained for cell sorting.
6. Identify the instrument used for data collection.	LSRFortessa, LSR-II, FACSAria-I, FACSAria-II, FACSAria-Fusion (BD Biosciences)
7. Describe the software used to collect and analyze the flow cytometry data.	FACSDiva8.0 (BD Biosciences), FlowJo v.10 (Tree Star, Inc), Prism 7 (GraphPad Software)

8. Describe the abundance of the relevant cell populations within post-sort fractions.

No post-sort analysis was done on sorted cell populations from TILs, which were processed immediately for ATAC-seq or RNA-seq.
EL4-huCD19, B16-OVA-huCD19, and MC38-huCD19 cell lines were expanded in vitro after cell sorting, and flow cytometry confirmed that huCD19 expression remained high in the sorted population.

9. Describe the gating strategy used.

Gating strategy for cell sorting:

huCD19 cell line sorts -> FSC-A/SSC-A -> FSC-W/FSC-H -> SSC-W/SSC-H -> huCD19+ (roughly top 16% of huCD19-expressing cells)

Nr4a1, Nr4a2, Nr4a3-expressing cell sorts -> FSC-A/SSC-A -> FSC-W/FSC-H -> SSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> NGFR+ (set expression level of NGFR-expressing CD8+ cells) for empty vector (pMIN) or Nr4a1, Nr4a2, Nr4a3

CAR sorts: FSC-A/SSC-A -> FSC-W/FSC-H -> SSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> CD45.1+ Thy1.1+ (CAR) -> PD-1hi TIM3hi CAR, PD-1hi TIM3lo CAR
OT-I sorts: FSC-A/SSC-A -> FSC-W/FSC-H -> SSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> CD45.1+ only (OT-I) -> PD-1hi TIM3hi OT-I
Corresponding Endogenous sorts: FSC-A/SSC-A -> FSC-W/FSC-H -> SSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> CD45.1- Thy1.1- (Endogenous) -> PD-1hi TIM3hi, PD-1hi TIM3lo and PD-1lo TIM3lo Endogenous

WT or Nr4a TKO sorts: FSC-A/SSC-A -> FSC-W/FSC-H -> SSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> Thy1.1+ NGFR+ (CAR + pMIN empty vector = WT or CAR + Cre = Nr4a TKO)

Gating strategy for flow cytometry:

Gating strategy for all in vitro experiments are similar; gating strategy for all in vivo experiments are similar. Specifics are detailed below for each set of experiments:

Confirming huCD19 expression on EL4-huCD19, B16-OVA-huCD19, MC38-huCD19: FSC-A/SSC-A -> FSC-W/SSC-H -> huCD19+; negative gate set on parent population EL4, B16-OVA, MC38 respectively

Assaying CAR expression in vitro:
CAR in vitro, surface marker expression: FSC-A/SSC-A -> FSC-W/SSC-H -> CD8+ Thy1.1+ (CAR) cells or CD8+ Thy1.1- (mock) -> PD-1, TIM3, LAG3
CAR in vitro, cytokine production: FSC-A/SSC-A -> FSC-W/SSC-H -> CD8+ Thy1.1+ cells (CAR) or CD8+ Thy1.1- (mock) -> TNF, IFNg; cytokine production negative gates set on mock unstimulated or CAR+ unstimulated

Assaying Nr4a1, 2, 3 expression in vitro:
Nr4a1, Nr4a2, Nr4a3 in vitro, surface marker and transcription factor expression: FSC-A/SSC-A -> SSC-A/CD8+ -> FSC-W/ live-dead dye -> SSC-W/SSC-A ->NGFR+ cells (pMIN empty vector or Nr4a1, Nr4a2, Nr4a3) -> PD-1, TIM3, LAG3, CD200, GITR, 2B4, CD101, CD38.
Nr4a1, Nr4a2, Nr4a3 in vitro, cytokine production: FSC-A/SSC-A -> SSC-A/CD8+ -> FSC-W/ live-dead dye -> SSC-W/SSC-A -> NGFR+ cells (pMIN empty vector or Nr4a1, Nr4a2, Nr4a3) -> TNF, IFNg; cytokine production negative gates set on empty vector (pMIN) unstimulated

Assaying TILs (CAR or OT-I):
TILs, surface markers or transcription factor expression: FSC-A/SSC-A -> FSC-W/ FSC-H -> SSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> CD45.1+ Thy1.1+ (CAR) or CD45.1+ Thy1.1- (OT-I) -> PD-1, TIM3, LAG3, TCF1, Eomes, T-bet.
TILs, cytokine production: FSC-A/SSC-A -> FSC-W/FSC-H -> SSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> CD45.1+ Thy1.1+ (CAR) or CD45.1+ Thy1.1- (OT-I) -> TNF, IFNg, IL-2; cytokine production negative gates set on CAR unstimulated or OT-I unstimulated.
Nr4a protein level expression: FSC-A/SSC-A -> FSC-W/FSC-H -> SSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> CD45.1+ Thy1.1+ (CAR) or CD45.1- Thy1.1- endogenous -> PD-1/TIM3 -> Nr4a1, Nr4a2, Nr4a3

Assaying TILs (WT or Nr4a TKO):

TILs, surface markers or transcription factor expression: FSC-A/SSC-A -> FSC-W/FSC-H -> SSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> Thy1.1+ NGFR+ (CAR + pMIN empty vector = WT, or CAR + Cre = Nr4a TKO) -> gate on 10^3 – 10^4 Thy1.1 + expression -> PD-1, TIM3, LAG3, TCF1, Eomes, T-bet.
TILs, cytokine production: FSC-A/SSC-A -> FSC-W/FSC-H -> SSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> Thy1.1+ NGFR+ (CAR + pMIN empty vector = WT or CAR + Cre = Nr4a TKO) -> TNF, IFNg, IL-2; cytokine production negative gates set on WT unstimulated.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☒