NR4A transcription factors limit CAR T cell function in solid tumours

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T cells expressing chimeric antigen receptors (CAR T cells) targeting human CD19 (hCD19) have shown clinical efficacy against B cell malignancies1,2. CAR T cells have been less effective against solid tumours3–5, in part because they enter a hyporesponsive (‘exhausted’ or ‘dysfunctional’) state6–9 triggered by chronic antigen stimulation and characterized by upregulation of inhibitory receptors and loss of effector function. To investigate the function of CAR T cells in solid tumours, we transferred hCD19-reactive CAR T cells into hCD19+ tumour-bearing mice. CD8+ CAR+ tumour-infiltrating lymphocytes and CD8+ endogenous tumour-infiltrating lymphocytes expressing the inhibitory receptors PD-1 and TIM3 exhibited similar profiles of gene expression and chromatin accessibility, associated with secondary activation of nuclear receptor transcription factors NR4A1 (also known as NUR77), NR4A2 (NURR1) and NR4A3 (NOR1) by the initiating transcription factor NFAT (nuclear factor of activated T cells)10–12. CD8+ T cells from humans with cancer or chronic viral infections13–15 expressed high levels of NR4A transcription factors and displayed enrichment of NR4A-binding motifs in accessible chromatin regions. CAR T cells lacking all three NR4A transcription factors (Nr4a triple knockout) promoted tumour regression and prolonged the survival of tumour-bearing mice. Nr4a triple knockout CAR tumour-infiltrating lymphocytes displayed phenotypes and gene expression profiles characteristic of CD8+ effector T cells, and chromatin regions uniquely accessible in Nr4a triple knockout CAR tumour-infiltrating lymphocytes compared to wild type were enriched for binding motifs for NF-κB and AP-1, transcription factors involved in activation of T cells. We identify NR4A transcription factors as having an important role in the cell-intrinsic program of T cell hyporesponsiveness and point to NR4A inhibition as a promising strategy for cancer immunotherapy.

Mouse B16-OVA melanoma, EL4 thymoma and MC38 colon adenocarcinoma cell lines were engineered to express hCD19 (Extended Data Fig. 1a). B16-OVA-hCD19 cells stably maintained hCD19 expression after growth in syngeneic C57BL/6J mice for 18 days and subsequent culture for 7 days ex vivo (Extended Data Fig. 1a, right). B16-OVA and B16-OVA-hCD19 cells grew at the same rate in vivo, indicating that the hCD19 antigen did not cause tumour rejection (Extended Data Fig. 1b, left). On the basis of tumour growth rate, we inoculated mice with 500,000 B16-OVA-hCD19 tumour cells (Extended Data Fig. 1b, right). Mouse CD8+ T cells retrovirally transduced with a second-generation CAR against hCD196,17 exhibited a transduction efficiency of 95.5 ± 4.0% (mean ± s.d.) (Extended Data Fig. 1c, d), produced TNF and IFN-γ upon re-stimulation with EL4-hCD19 cells, and exhibited dose-dependent lysis of B16-OVA-hCD19 cells (Extended Data Fig. 1e–g). CAR T cells did not express higher surface levels of PD-1, TIM3 or LAG3 than mock-transduced cells under resting conditions (Extended Data Fig. 1h).

C57BL/6J mice bearing B16-OVA-hCD19 tumours and adoptively transferred with CD8+CD45.1+Thy1.1+ CAR T cells (Fig. 1a, b) or CD8+CD45.1+ OT-I cells (specific for chicken ovalbumin (OVA) SIINFEKL peptide presented by H-2Kb; Extended Data Fig. 2a, b) showed similar tumour growth rates (Extended Data Fig. 2c); low numbers of CAR T cells were transferred to minimize tumour rejection (Extended Data Fig. 2d). Eight days after adoptive transfer, CAR and OT-I tumour-infiltrating lymphocytes (TILs) (Fig. 1b, Extended Data Fig. 2b, e, f) comprised around 18% and 9%, respectively, of CD8+ TILs (Fig. 1c) and exhibited similar proportions of PD-1hiTIM3lo cells compared to endogenous TILs (Fig. 1b, Extended Data Fig. 2b). All TILs produced low levels of TNF and IFN-γ upon re-stimulation with PMA and ionomycin (Fig. 1d, e), confirming their decreased function. The transcriptional profiles of ‘highly exhausted’ PD-1hiTIM3bi CAR TILs (population A, Fig. 1b) were similar to those of endogenous PD-1hiTIM3lo TILs (population C, Fig. 1b), but distinct from those of CAR and endogenous ‘antigen-specific memory precursor’ PD-1–TIM3– TILs18 (populations B and D) and naive-like endogenous PD-1–TIM3+ TILs (population E) (Fig. 1a, Extended Data Fig. 2g, Supplementary Table 1). The chromatin accessibility profiles of endogenous, OT-I and CAR PD-1hiTIM3bi and PD-1hiTIM3lo TIL subsets (A–D, F) were similar to one another, but distinct from those of PD-1loTIM3lo endogenous TILs (E), which resembled naive CD8+ T cells (Fig. 1b, Extended Data Fig. 3). PD-1hiTIM3bi TILs (B, D) resembled memory-precursor CD8+ T cells7,11,12, with accessible regions showing substantial enrichment for consensus TCF1 motifs (Fig. 2b, cluster 6). Regions that were selectively accessible in PD-1hi populations (A–D, F) were enriched for consensus NR4A (nuclear receptor, NR) as well as NFAT, NF-κB, bZIP and IRF: bZIP motifs (Fig. 2b, clusters 8, 9). NR4A protein expression was higher in PD-1hiTIM3hi than in PD-1hiTIM3lo TILs (Fig. 2c, Extended Data Fig. 4a–d).

Single-cell RNA-sequencing (RNA-seq) data from human CD8+ TILs provided further justification for studies of NR4A. In CD8+ TILs infiltrating a human melanoma14, NR4A1 and NR4A2 expression correlated positively with PD1CD1 (PD-1) and HAVCR2 (TIM3) expression, and NR4A3 showed a moderate positive correlation (Fig. 2d). PDCD1 and HAVCR2 expression correlated positively with TIGIT, CD38, CTLA4, 3JN, TOX, TOX2 and IRF4 and negatively with TCF7 (Extended Data Fig. 4e–g, Supplementary Table 2). Additionally, NR4A, NFAT, NF-κB, bZIP and IRF: bZIP motifs were enriched in regions uniquely accessible in CD8+PD-1hi TILs from human melanoma and non-small cell lung cancer15 and in HIV-antigen-specific CD8+ T cells from infected humans15 (Fig. 2e, cluster 9). The upregulation of NR4A family members and enrichment of NR4A binding motifs in differentially accessible regions of chronically stimulated human and mouse CD8+PD-1hi T cells11,12,15,19 led us to focus on NR4A family members as potential transcriptional effectors of CD8+ T cell exhaustion.

The three NR4A proteins are essential for regulatory T cell development20, indicating redundant function. We compared NR4A-sufficient (wild type) CAR TILs with NR4A triple knockout (Nr4a TKO) CAR

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Fig. 1 | CAR, OT-I and endogenous CD8⁺ TILs isolated from B16-OVA-hCD19 tumours exhibit similar phenotypes. a, Experimental design to assess CAR and endogenous TILs; 1.5 × 10⁶ CAR T cells were adoptively transferred into C57BL/6J mice 13 days after tumour inoculation. b, Left, representative flow cytometry plot identifying CD8⁺ CD45.2⁺ endogenous TILs and CD8⁺ CD45.1⁺Thy1.1⁺ CAR TILs (Thy1.1 encoded in the CAR retroviral vector). Right, flow cytometry plots showing PD-1 and TIM3 surface expression on CD8⁺ CAR and endogenous TILs. c, Bar graph showing the percentage of CAR and OT-I TILs in total CD8⁺ TILs. Bars show mean values with data points for 6, 5 and 11 independent experiments for CAR, OT-I and endogenous TILs, respectively. d, Quantification of cytokine production after re-stimulation of CAR, OT-I and endogenous (endo.) CD8⁺ T cells, compared to cultured CD8⁺ TILs (Thy1.1 encoded in the CAR retroviral vector). e, Flow cytometry plots showing PD-1 and TIM3 surface expression on CD8⁺ CAR and endogenous TILs. Bars show mean values with data points for 3 independent experiments. All P values were calculated using two-tailed unpaired t-tests with Welch’s correction. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. e, Representative flow cytometry plots of cytokine production after re-stimulation.

Neura⁻/⁻ mice with both CAR and Cre retroviruses, and naive CD8⁺ T cells from Neura⁻/⁻ Neura⁻/⁻ Neura⁻/⁻ with CAR and empty retroviruses, respectively (Extended Data Fig. 5a–c). Compared to

Fig. 2 | CAR and endogenous CD8⁺ TILs exhibit similar gene expression and chromatin accessibility profiles. a, Principal component analysis of RNA sequencing (RNA-seq) data from CAR PD-1⁻ TIM3⁻ (A) and PD-1⁻ TIM3⁺ (B) TILs and endogenous PD-1⁻ TIM3⁻ (C), PD-1⁻ TIM3⁺ (D) or PD-1⁻ TIM3⁺ (E) TILs. Data represent 3 independent experiments, each using TILs pooled from 9–14 mice. b, Top, heatmap of mouse CD8⁺ T cell assay for transposase-accessible chromatin using sequencing (ATAC-seq) data showing difference in signal (measured as log(normalized read counts)) from mean for 9 k-means clusters. Bottom, heatmap of motif enrichment analysis. Data shown for one representative member of transcription factor families enriched in at least one cluster compared to all accessible regions. Representative motifs with a fold enrichment greater than 1.5, a P value less than 1 × 10⁻¹⁰, and found in at least 5% of regions are shown. c, Quantification of Nrf4A expression (mean fluorescence intensity); P values for CAR comparisons (top) were calculated using two-tailed paired t-tests; P values for endogenous comparisons (bottom) were calculated using row-normalizing one-way ANOVA with Greenhouse–Geisser correction and Tukey’s multiple comparisons tests; for both calculations, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Data show mean ± s.d. and individual values from three independent experiments, each using TILs pooled from 9–14 mice. d, Scatterplots of RNA-seq data showing expression of PDCD1 (x axis) and HAVCR2 (y axis) in single cells of human CD8⁺ TILs, with expression of the indicated Nrf4A genes shown in the colour scale. Each dot represents a single cell. e, Top, human CD8⁺ T cell ATAC-seq data from PD-1⁻ TIM3⁺ TILs (two samples from melanoma, one sample from non-small cell lung tumour) and antigen-specific CD8⁺ T cells from individuals with HIV⁺ showing difference in signal (measured as log(normalized read counts)) from mean for 9 k-means clusters. Bottom, heatmap of motif enrichment analysis.
control tumour-bearing mice adoptively transferred with wild-type CD8+ CAR T cells, tumour-bearing mice adoptively transferred with Nr4a TKO CD8+ CAR T cells showed pronounced tumour regression and enhanced survival (Fig. 3a–c). Differences in tumour size were apparent as early as day 21 after tumour inoculation (Fig. 3b, bottom). Nr4a TKO CAR T cells promoted tumour regression and prolonged survival even in immunocompetent recipient mice (Extended Data Fig. 5d–g). Thus, NR4A transcription factors suppress tumour rejection in the CAR T cell model.

To assess NR4A redundancy, we evaluated the anti-tumour effects of CD8+ CAR T cells lacking individual NR4A proteins (Extended Data Fig. 6a). Nr4a TKO CAR T cells showed greater anti-tumour activity than CAR T cells lacking Nr4A1, Nr4A2 or Nr4A3 (Extended Data Fig. 6b–d). Moreover, retroviral expression of any NR4A transcription factor in CD8+ T cells (Extended Data Fig. 7a) resulted in increased expression of inhibitory surface receptors and decreased cytokine production upon re-stimulation (Extended Data Fig. 7b–d). In principal component analyses of RNA-seq data, the majority of the variance (78%) was between cells expressing any NR4A transcription factor compared with cells expressing the empty vector control (Extended Data Fig. 7e, Supplementary Table 3). In both RNA-seq and ATAC-seq, pairwise comparisons showed few if any differences between NR4A family members (Extended Data Fig. 7f, g). Thus, the three NR4A proteins induce similar changes in transcriptional and chromatin accessibility profiles in CD8+ T cells.

To assess phenotypic and genome-wide changes associated with anti-tumour function, we modified experimental conditions to delay tumour regression (Fig. 3d). Tumour sizes and TIL recoveries were similar between Nr4a TKO and wild type (Extended Data Fig. 8a–c). Eight days after adoptive transfer, Nr4a TKO TILs showed a mild but statistically significant decrease in PD-1 expression compared to wild-type TILs, and the total Nr4a TKO PD-1+ population was markedly skewed towards low TIM3 expression (Fig. 3e). Moreover, the percentage of cells expressing TNF or both IFNγ and TNF after re-stimulation was significantly higher in Nr4a TKO compared to wild-type TILs (Fig. 3f). TIM3+ Nr4a TKO CAR TILs were noticeably skewed towards low TCF1 expression (Extended Data Fig. 8d, top); the TIM3+TCF1hi population, which is different from the TIM3−TCF1lo memory precursor population that expands after PD-1 blockade18,21–23, may be responsible for increased effector function. There was no significant difference in the mean fluorescence intensities of TCF1, T-bet or Eomes (Extended Data Fig. 8d, bottom).

Nr4a TKO TILs showed increased expression of genes related to effector function; that is, mRNAs encoding effector proteins (IL-2, TNF and granzymes) were upregulated. Genes expressed in naive or memory T cells compared to effector populations (for example, SELL and CD69) were downregulated in Nr4a TKO compared to wild-type TILs in cluster 2). Cluster 4 contains genes

**Fig. 3** NR4A-deficient CAR TILs promote tumour regression and prolong survival. a, Experimental design; 3 × 10^5 wild-type or Nr4a TKO CAR T cells were adoptively transferred into Rag1−/− mice 7 days after tumour inoculation. PBS was injected as a control. b, Top, tumour growth in individual mice. Bottom, tumour sizes of individual mice at day 21 (mean ± s.d.). P values were calculated using one-way ANOVA with Tukey’s multiple comparisons test. c, Survival curves; ****P < 0.0001 calculated using log-rank (Mantel–Cox) test. Surviving mouse numbers at days 7, 21 and 90 were n = 21, 14 and 0 (PBS); n = 35, 25 and 1 (wild type); n = 39, 36 and 27 (Nr4a TKO). d, Experimental design; 1.5 × 10^6 wild-type or Nr4a TKO CAR T cells were adoptively transferred into Rag1−/− mice 13 days after tumour inoculation and then analysed 8 days later. e, Surface expression of PD-1 and TIM3 on CAR ‘NGFR+’ cells with a set level of CAR expression (10^3–10^6). Representative flow cytometry plots (top), histograms (middle and bottom, left) and mean ± s.d. and individual values (right) of 6 independent experiments, each using TILs pooled from 3–8 mice. P values were calculated using two-tailed paired t-tests with Welch’s correction. f, Top, representative flow cytometry plots for TNF and IFNγ production. Bottom, quantification showing the mean ± s.d. and individual values of 5 independent experiments, each using TILs pooled from 3–8 mice. P values were calculated using two-tailed paired t-tests between stimulated wild-type and Nr4a TKO CAR TILs. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
A smaller subset contained NEAT-binding sites without an adjacent AP-1 site, suggesting that NR4A maintains the accessibility of ‘exhaustion-related’ regions that bind NEAT without AP-1 (Fig. 4c). Regions more accessible in Nr4a TKO compared to wild-type TILs were enriched for consensus bZIP (71%) and Rel/NFκB (25%) binding motifs, confirming the established role of bZIP (for example, Fos, Jun, ATF and CREB) and Rel/NFκB family members in T cell activation and effector function, and consistent with negative crosstalk between NR4A and NFκB family members in T cell activation and effector function.

We previously used an engineered NFAT protein, CA-RIT-NFAT1, to mimic a dephosphorylated nuclear NFAT that cannot form cooperative transcriptional complexes with AP-1 (Fos–Jun) and NFκB, TNF and IL-21, cytokines involved in effector functions, are more highly expressed in Nr4a TKO compared to wild-type TILs (Fig. 4a). Two bZIP-motif-containing regions of the Il21 promoter gain accessibility and the Traf4 locus shows increased accessibility across the promoter and the entire gene in Nr4a TKO compared to wild-type TILs (Extended Data Fig. 9b).

We investigated the binding of haemagglutinin (HA)-tagged NR4A proteins to selected differentially accessible regions in CD8+ T cells by chromatin immunoprecipitation and quantitative PCR (ChIP–qPCR). Ccr7, which is highly expressed in naive and memory T cells and decreased in effector T cells, is expressed at lower levels in effector-like Nr4a TKO compared to wild-type TILs (Fig. 4a). The Ccr7 distal 5′ region contains two ATAC-seq peaks that are less prominent in Nr4a TKO than in wild-type TILs and contain adjacent NEAT- and NR4A-binding motifs (Extended Data Fig. 9a, middle panel, peach lines). These regions bind NR4A (Extended Data Fig. 9a, right, bar plots). In contrast, two ATAC-seq peaks in the Ccr7 proximal promoter and first intron are more prominent in Nr4a TKO compared to wild-type TILs and contain bZIP and NFκB motifs (Extended Data Fig. 9a, middle panel, blue lines; and additional examples Ccr6 and Ifng). TNF and IL-21, cytokines involved in effector functions, are more highly expressed in Nr4a TKO compared to wild-type TILs (Fig. 4a). Two bZIP-motif-containing regions of the Il21 promoter gain accessibility and the Traf4 locus shows increased accessibility across the promoter and the entire gene in Nr4a TKO compared to wild-type TILs (Extended Data Fig. 9b).

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Each NR4A protein binds to and is partly responsible for increased accessibility of an enhancer located at around 23 kb 5′ of the Pdcd1 transcription start site (Fig. 4d), noted in all mouse models of exhaustion or dysfunction investigated so far11–13,15,19. The ATAC-seq peak marking this enhancer is diminished in Nr4a2 T cells and increased in T cells ectopically expressing NR4A1, NR4A2 or NR4A3 compared to cells transduced with empty vector alone (Fig. 4d). Deletion of this enhancer results in a decrease in the mean fluorescence intensity of PD-1 staining in the EL4 thymoma cell line19. PD-1 blockade19 caused a significant (two-fold) decrease in levels of Nrdt2 mRNA, with a smaller decrease in Nrdt1 and Nrdt3 mRNAs (Extended Data Fig. 10a). These data indicate that, together with NFAT, the three NR4A transcription factors are prominent and redundant effectors of the CD8+ T cell hyporesponsive program downstream of NFAT (Fig. 4e). Moreover, because NR4A deficiency results in downregulation of the inhibitory receptors PD-1 and TIM3, the effect of NR4A deficiency is functionally similar to that of PD-1 blockade19, but NR4A deficiency affects a wider range of regulatory elements than PD-1 blockade alone (Fig. 4e).

Although immune cell therapies offer considerable promise for the treatment of cancer, treatment with individual blocking antibodies against targets such as PD-1 and CTLA4 rarely achieve complete cures. We have shown that the NFAT–NR4A axis controls the expression of multiple inhibitory receptors, and that treatment of tumour-bearing mice with CAR T cells lacking all three NR4A transcription factors resulted in tumour regression and prolonged survival. Inhibiting the function of NR4A family members in tumour-infiltrating T cells could be a promising strategy in cancer immunotherapy as it would be expected to mimic combination therapies with blocking antibodies against multiple inhibitory receptors31.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-019-0985-x.

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Author Contributions J.C. designed and performed experiments, analysed data, wrote the paper, prepared the figures and wrote the manuscript; I.F.-M. performed computational analyses of the RNA-seq and scRNA-seq data; H.S. assisted with in vivo mouse experiments and in vitro experiments; C.W.L. performed ChIP and ChIP–qPCR; L.J.H. assisted with in vivo mouse experiments; T.S. and A.Y. gave advice and provided the Nr4a-gene-disrupted mice (with permission from P. Chambon); J.P.-S. conceived the mouse CAR T cell model, designed experiments and performed computational analyses of the ATAC-seq data; A.R. supervised the project; J.C., J.P.-S. and A.R. interpreted data and wrote the manuscript, with all authors contributing to writing and providing feedback.

Competing interests The La Jolla Institute of Immunology has a pending patent, PCT/US2018/062354, covering the use and production of engineered immune cells to disrupt the NFAT–AP1 pathway transcription factors, including the NR4A family members, with J.C., H.S., J.P.-S. and A.R. listed as inventors. A.R. receives funding from Takeda for subsequent research related to this subject matter. None of the other authors has any competing interests.

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METHODS

Data reporting. No statistical methods were used to predetermine sample size. The investigators were not blinded to allocation during experiments and outcome assessment.

Construction of retroviral vector (MSCV-myc-CAR-2A-Thyl.1) containing chimeric antigen receptor (CAR). The chimeric antigen receptor was pieced together using published portions of the clone FMC63 human CD19 single chain variable fragment, and the published portions of the murine CD8 and CD3-sequences.2 The sequence for the tag on the N terminus was obtained from published work.3 This chimeric antigen construct was then cloned into an MSCV-puro (Clontech) murine retroviral vector in the place of the puro-gene.2

Sequence of CAR construct (aa): (490 aa), 5′-ATGGCTTTGCCAGTGACA

Construction of mouse tumour cell lines expressing hCD19. B16-OVA-hCD19 cells were cultured in Dulbecco’s medium (DMEM) with 10% (vol/vol) FBS, 1% L-glutamine, 1% penicillin/streptomycin and passed three times before inoculation. At the time of injection, cells were trypsinized and resuspended in Hanks balanced salt solution without phenol red at 10 million cells per millilitre. C57BL/6J male or female mice (8–12 weeks old) were injected intradermally with 5 × 10⁶ B16-OVA-hCD19 cells (50 µl per injection).

Preparation of MC38-hCD19 colon adenocarcinoma cells for tumour inoculation. MC38-hCD19 cells were cultured in Dulbecco’s medium (DMEM) with 10% (vol/vol) FBS, 0.1mM non-essential amino acids, 1 mM sodium pyruvate, 10mM Hepes, 1% L-glutamine, 1% penicillin/streptomycin and passed two times before inoculation. At the time of injection, infection and resuspended in Hanks balanced salt solution without phenol red at 10 million cells per millilitre. C57BL/6J male or female mice (8–12 weeks old) were injected intradermally with 5 × 10⁶ MC38-hCD19 cells (50 µl per injection).

B16-OVA-hCD19 tumour model. For analysis of CAR CD8⁺ TILs and endogenous CD8⁺ TILs: on day 0, 8–12-week-old C57BL/6J mice were injected intradermally with 5 × 10⁶ B16-OVA-hCD19 cells. After tumours became palpable, tumour measurements were recorded with a manual caliper every other day and tumour area was calculated in centimeters squared (length × width). On day 13, 1.5 million CAR-transduced CD45.1⁺ CD8⁺ T cells were adoptively transferred into tumour size-matched tumour-bearing mice. On day 21, tumours were collected from mice. For analysis of CAR CD8⁺ TILs lacking NR4A family members: because the Nr4a gene-disrupted mice were originally derived from Jackson laboratories, and their genetic background might not have been fully compatible with that of inbred C57BL/6J mice despite stringent backcrossing, we used Rag1-deficient mice as recipients in most experiments to avoid variable rejection. On day 0, 8–12-week-old Rag1⁻/⁻ mice were injected intra-dermally with 5 × 10⁶ B16-OVA-hCD19 cells and tumours were measured every other day after they became palpable. On day 13, 1.5 million CAR- and empty vector pMIN-transduced CD8⁺ Thy1.1⁺ GFP⁺ NR4a⁺/⁻ Nr4a2⁺/⁻ Nr4a3⁺/⁻ (wild type) or CAR- and Cre-transduced CD8⁺ Thy1.1⁺ GFP⁺ Nr4a1⁺/⁻ Nr4a2⁺/⁻ Nr4a3⁺/⁻ (Nr4a TKO) T cells were adoptively transferred into tumour size-matched tumour-bearing mice. On day 21, tumours were collected from the mice. For monitoring of tumour growth for survival studies after adoptive transfer of CAR T cells lacking NR4A family members: again, because the Nr4a-gene-disrupted mice were originally derived from 129/SvJ embryonic stem cells,3 and their genetic background might not have been fully compatible with that of inbred C57BL/6J mice despite stringent backcrossing, we used Rag-deficient mice as recipients in most experiments to avoid variable rejection. On day 0, 8–12-week-old Rag1⁻/⁻ mice were injected intra-dermally with 5 × 10⁶ B16-OVA-hCD19 cells and tumours were measured every other day after they became palpable. On day 13, 1.5 million CAR- and empty vector pMIN-transduced CD8⁺ Thy1.1⁺ GFP⁺ NR4a⁺/⁻ Nr4a2⁺/⁻ Nr4a3⁺/⁻ (wild type) or CAR- and Cre-transduced CD8⁺ Thy1.1⁺ GFP⁺ Nr4a1⁺/⁻ Nr4a2⁺/⁻ Nr4a3⁺/⁻ (Nr4a TKO) T cells were adoptively transferred into tumour size-matched tumour-bearing mice. On day 21, tumours were collected from the mice.

Eukaryotic cell lines. 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 expressing the ovalbumin transgene was purchased from the American Type Culture Collection (ATCC). Scherer et al.22 previously described the 293T cell line was purchased from ATCC: 293T (ATCC CRL-3021). The Platinum-E Retroviral Packaging Cell Line, Ectoport (Plate) cell line was purchased from Cell Biolabs, Inc.: RV-10. The MC38 mouse adenocarcinoma cell line (a gift from A.W. Goldrath, UCSD, La Jolla, CA) was originally purchased from Keratap, Inc. (ENH204). The EL4 cell line stained positive for mouse Thy1.2 and PD-1 and stained negative for hCD19. The B16-OVA cell line stained negative for hCD19. The MC38 cell line stained negative for hCD19. The PlatE and 293T cell lines were not authenticated. Cell lines were not tested for mycoplasma contamination.
MC38-hCD19 tumour model. The monitoring of tumour growth for survival studies after adoptive transfer of CAR T cells lacking NR4A4 family members into C57BL/6j mice bearing MC38-hCD19 tumours was performed as described for the B16-OVA-hCD19 model using immunocompetent recipients.

Preparation of cells for adoptive transfer. CD8+ T cells were isolated and activated with 1 μg ml−1 anti-CD3 and 1 μg ml−1 anti-CD28 for 1 day, then removed from activation and transduced with retrovirus expressing CD8+ T cells. After the transduction, cells were replaced with media containing 100 U of IL-2 per ml. One day after the first transduction, a second transduction was performed and immediately after the transduction, cells were replaced with media containing 100 U of IL-2 per ml. On the day of adoptive transfer (either day 3 or day 5 post activation), cells were analysed by flow cytometry and cell counts were obtained using a haemocytometer. The number of CAR-transduced cells was obtained using the cell counts from the haemocytometer and the population percentages obtained from flow cytometry. Cells were then collected, washed with PBS and resuspended at a concentration equivalent of 1.5 million, 3 million or 6 million CAR-transduced cells per 200 μl of PBS. Mice were then adoptively transferred with 200 μl of retro-orbital intravenous injections each.

Isolation of TILs for subsequent analyses. Sample preparation for flow cytometry and cell sorting of TILs from CAR and OT-1 experiments and for flow cytometry of TILs from Nraa TKO versus wild-type experiments: on day 21, mice were killed and perfused with PBS before removal of tumour. Tumours were collected, pooled together by group, homogenized, and then dissociated using the MACS Miltenyi Mouse Tumour Dissociation kit (Miltenyi Biotech) and the gentleMACS dissociator with Octo Heaters (Miltenyi Biotec) according to manufacturer’s instructions. Tumours were then filtered through a 70 μm filter and spun down. Supernatant was aspirated and the tumours were resuspended in the equivalent of 4–5 g of tumour per 5 ml 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 amounts for staining and phenotyping with flow cytometry or stained for cell sorting. Sample preparation for cell sorting of TILs from Nraa wild-type and Nraa TKO experiments: on day 21, mice were killed and perfused with PBS before removal of tumours. Tumours were collected, pooled together by group, homogenized, and then dissociated using the MACS Miltenyi Mouse Tumour Dissociation kit (Miltenyi Biotech) and the gentleMACS dissociator with Octo Heaters (Miltenyi Biotech) according to manufacturer’s instructions. Tumours were then filtered through a 70 μm filter and spun down. Supernatant was aspirated and the tumours were resuspended in 40% Percoll/RPMI and underlaid with Octo Heaters (Miltenyi Biotec) and using the pCL10A1 and pCL-Eco packaging vectors (the former for the hCD19 model, the latter for all other viruses produced). Immediately after the transduction, cells were replaced with media containing 100 U of IL-2 per ml. One day after the first transduction, a second transduction was performed and immediately after the transduction, cells were replaced with media containing 100 U of IL-2 per ml. On the day of adoptive transfer (either day 3 or day 5 post activation), cells were analysed by flow cytometry and cell counts were obtained using a haemocytometer. The number of CAR-transduced cells was obtained using the cell counts from the haemocytometer and the population percentages obtained from flow cytometry. Cells were then collected, washed with PBS and resuspended at a concentration equivalent of 1.5 million, 3 million or 6 million CAR-transduced cells per 200 μl of PBS. Mice were then adoptively transferred with 200 μl of retro-orbital intravenous injections each.

Statistical analyses. Statistical analyses on flow cytometric data and tumour growth data were performed using the appropriate statistical comparison, including paired or unpaired two-tailed t-tests with Welch’s correction as needed, one-way ANOVA with multiple comparisons test (Tukey’s or Dunnett’s), row-matching (RM) one-way ANOVA with Greenhouse-Geisser correction, or ordinary two-way ANOVA (Prism 7, GraphPad Software). Statistical analyses for survival curves were performed using the log-rank (Mantel–Cox) test (Prism 7, GraphPad Software). P ≤ 0.05 was considered statistically significant.

In vitro killing assay. Approximately 10,000 B16-OVA-hCD19 cells (target cells) were plated in 100 μl of T cell media (or media only for background) in each well in E-plate 96 (ACEA Biosciences Inc.). Plate was placed in xCELLigence Real-Time Cell Analysis (RTCA) instrument (ACEA Biosciences Inc.) after 30 min and incubated overnight. The following day, the plate was removed from xCELLigence RTCA machine and CD8+ CAR T cells (effector cells) were added in an additional 100 μl of T cell media for 30 min (for lysis positive control, 0.2% TritonX was used, for lysis negative control, only media was used). The plate was then placed back into the incubator, and data acquisition began. 5 h after, the cell index (CI) was obtained from each well. Percentage of specific lysis was calculated for each well as follows: percentage of specific lysis = 100 × (CD8+ CAR T cells with effector cells − CD8+ CAR T cells alone)/CD8+ CAR T cells alone.

Chromatin immunoprecipitation and quantitative PCR (ChIP–qPCR). ChIP was performed as previously described21. In brief, CD8+ T cells were isolated from C57BL/6j mice as above, activated with plate-bound anti-CD3/CD28, transduced with either empty vector control or retrovirus expressing NR4A1, NR4A2, or NR4A3 with HA-tag on the N terminus. Cells were cultured for a total of 5 days post-transduction. For fixation, formaldehyde (16%, ThermoFisher) was added directly to the cells to a final concentration of 1% and incubated at room temperature for 10 min with constant agitation. Glycine (final 125mM) was added to quench the fixation and the cells were washed twice with ice-cold PBS. Cell pellets were snap-frozen with liquid nitrogen and stored at −80 °C until use. For nuclei isolation, cell pellets were thawed on ice and lysed with buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP40, 0.25% Triton-X100) supplemented with 1% Halt protease inhibitor (ThermoFisher) for 10 min at 4 °C with constant rotation. Pellets were washed once with washing buffer (10 mM Tris-HCl pH 7.5, 280 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.2% sodium deoxycholate, 0.2% Triton-X100, 1% Halt protease inhibitor) and twice with shearing buffer (10 mM Tris-HCI pH 8.0, 1 mM EDTA, 0.1% SDS, 1% Halt protease inhibitor). Nuclei were resuspended in 1 ml shearing buffer, transferred to 1 ml milliTUBE (Covaris, Woburn, MA), and sonicated with Covaris E220 for 18 min (Duty Cycle 5%, intensity 140 Watts, cycles per burst 200). After sonication, insoluble debris was removed by centrifugation at 20,000g for 10 min at 4°C. The concentration of chromatin was quantified using Qubit DNA BR assay (ThermoFisher). For immunoprecipitation, 25 μg of chromatin was removed and mixed with equal volume of 2 × Conversion buffer (10 mM Tris-HCl pH 7.5, 280 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.2% sodium deoxycholate, 0.2% Triton-X100, 1% Halt protease inhibitor) in a 2-ml low-binding tube (Eppendorf). Either 5% or 6% of input chromatin was saved as control. Chromatin was pre-cleared using 30 μl washed protein A magnetic dynabeads (ThermoFisher) for 1h at 4°C with constant rotation. Pre-cleared chromatin was transferred to new tube, added with 10 μg rabbit monoclonal anti-HA (C29F4, Cell Signaling Technology) and 30 μl washed protein A magnetic dynabeads, and incubated at 4°C overnight with constant rotation. Read-bound chromatin was washed twice with RIPA buffer (50 mM Tris-HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS), once with high salt washing buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS), and once with Lithium washing buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% NP-40), and once with TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). All washes were incubated for 5 min at 4°C with constant rotation. Chromatin was eluted from beads by incubating with elution buffer (100 mM NaHCO3, 1% SDS) at room temperature for 30 min in the presence of 0.5 mg ml−1 of RNaseA (Qiagen). To de-crosslink protein and DNA, proteinase K (final 0.5 mg ml−1) and NaCl (final 200 mM) were added to the recovered supernatant and incubated at 65°C overnight with constant shaking (1,000 r.p.m.) in a Thermomixer (Eppendorf). DNA was purified using Zymo CHIP DNA clean and concentration kit (Zymo Research) according to the manual from the manufacturer. Eluted DNA was analysed by qPCR using Power SYBR Green PCR Master
Mix (Roche) and StepOne Real Time PCR system (ThermoFisher). The signals from ChIP sample was normalized to those from the input and calculated as ‘percentage of input’. A value of ‘undetected’ was recorded as zero.

ChIP-qPCR primers (all coordinates are for mm10), (1) chrX:7584283–7584409

127 bp: Fp3-CN2-qf (forward) 5'-CCCACACAGAGTCGAGAA3'- (reverse) Fp3-CN2-qr 5'-TGGTGGCTACTGAGTGTA3' - (c) chr1: 99163437–99163636 192 bp: CRR7_E1_R1 (forward) 5'–GGCTCCTTGGTCAAT AATGAGT7– (c) chr1:99168432–99168614 183 bp: CRR7_E2_F1 (forward) 5'-GGACAGACAGGTTGAGTTT3'- CRR7_E2_R1 (reverse) 5'–GGCCTGTGGTCTAA ATGAGT7– (5) chr7:8196147–8196301 155 bp: CRR6_F1 (forward) 5'-GGCAGGTAGTTTTGTTTGTCTGACCA3'- CRR6_T1_R1 (reverse) 5'–CCGTATGTTGATGGTTGTTGCT7– (6) chr10:118460432–118460610 179 bp: IFng_E1_R1 (forward) 5'-GCGCC TAGAAGTTCAGTGCT-3'– IFng_E1_R1 (reverse) 5'-TGTAGGATGCA GCAGTTTGGTGTG-3'.

Cell sorting. Cell sorting was performed by the LJI Flow Cytometry Core, using the FACSaria-I, FACSaria-II, or FACSaria-Fusion (BD Biosciences). For ATAC-seq, 50,000 cells were sorted from the isolated CD8+ TILs, with the exception of the OT-I samples, for which 15,000–30,000 cells were sorted. In most cases, a second ATAC-seq technical replicate using 50,000 additional cells was prepared in parallel. For RNA-seq, two technical replicates of 10,000 cells each were sorted from the isolated CD8+ TILs. For the CAR and OT-I experiments, the populations sorted were as follows: CD8+ CD45.1+ Thy1.1+ PD-1- TIM3- CAR (population A), CD8+ CD45.1+ Thy1.1+ PD-1+TIM3+ CAR (population B). The RNA-seq samples were made up of two replicates of population A and two replicates of population B.

Technical replicates were removed. RNA-seq analysis was performed at the gene level, employing the transcript annotations of the mouse mm10 genome. Reads aligning to annotated features were counted using the summarizeOverlaps function (mode = ‘Union’) of the Bioconductor package GenomicAlignments41 v1.10.1. The DESeq2 package42 v1.14.1 was used to normalize the raw counts and identify differentially expressed genes (FDR cutoff of P < 0.1 and fold change (log2 scale) ≥ 2 or ≤ –2, as specified). Genes with less than 10 reads total were further filtered in all comparisons as an initial step. Transformed values (rlog) were calculated within DESeq2 for data visualization.

Single-cell RNA-seq analysis. Data were obtained from a previously published study on the cellular ecosystem of human melanoma tumours43. In brief, malignant and non-malignant cells (including immune, stromal, and endothelial cells) were profiled by single-cell RNA-seq. Normalized expression values (\(E_i = \log_2(\text{TPM}_i/10 + 1)\), where \(\text{TPM}_i\) refers to transcripts per million (TPM) for gene \(i\) in cell \(j\)) were obtained from Gene Expression Omnibus (GSE72056). For the analysis, we only kept genes with non-zero expression values in at least 10 cells. Given the technical noise and gene dropout associated with single-cell RNA-seq data, we used the MAGIC algorithm44 for imputation in the matrix of normalized expression values, with diffusion parameter \(t = 2\). An R implementation of the MAGIC method was downloaded from (https://www.krishnaswamylab.org/magic-project).

Tumour-infiltrating T cells were selected based on the inferred cell type annotation in a previous study45. CD8+ T cells were selected based on the expression of CD8A (cells with imputed values ≥ 4) and CD4 (cells with imputed values ≤ 1.5). Imputed values were used for gene expression visualizations.

Gene set enrichment analysis. Gene set enrichment analysis (GSEA) was performed employing the GSEA Preranked function, ranking genes by fold change according to the pertinent comparison, with number of permutations of 10,000 and allowing for gene set size up to 2,000 genes. Gene sets were defined from differentially expressed genes obtained from pairwise comparisons between effector, memory, and exhausted CD8+ T cells from a previously published study43. In this context, differential gene expression was identified employing DESeq2 with FDR cutoff of P < 0.01 and fold change (log scale) cutoff of 1.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability All data generated and supporting the findings of this study are available within the paper. RNA-seq and ATAC-seq data are available in the Gene Expression Omnibus (GEO) database under the SuperSeries reference number GSE123739. Source Data for Figs. 2, 4 and Extended Data Figs. 2, 4, 7, 8, 9 are provided in Supplementary Tables 1–5. Additional Source Data are provided in the online version of the paper. Additional information and materials will be made available upon request.

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Extended Data Fig. 1 | Functional assessment of a hCD19-reactive chimeric antigen receptor (CAR). a, Left, three panels, EL4, MC38 and B16-OVA cell lines expressing hCD19. Grey, parental; black, hCD19-expressing cells. Right, B16-OVA-hCD19 cells recovered after growth in a C57BL/6J mouse followed by culture for 7 days. Grey, isotype control; black, anti-hCD19. Data from one biological replicate in each case. b, Left, growth curves (mean ± s.e.m., 15 mice per group) of 250,000 B16-OVA parental or B16-OVA-hCD19 tumour cells in vivo after inoculation into C57BL/6J mice. There is no significant difference at any time point (ordinary two-way ANOVA, \( P > 0.9999 \) at day 19). Right, growth curves (mean ± s.e.m.) of 250,000 (\( n = 5 \) mice) or 500,000 (\( n = 6 \) mice) B16-OVA-hCD19 tumour cells in vivo after inoculation into C57BL/6J mice. There is no significant difference between the two groups (ordinary two-way ANOVA; \( P = 0.0146 \)). c, Diagram of the CAR construct. LS, leader sequence; SS, signal sequence; myc, myc epitope-tag; scFv, single chain variable fragment against human CD19; followed by the mouse (m) CD28 and CD3\( \zeta \) signalling domains. The 2A self-cleaving peptide and the mouse Thy1.1 reporter. d, CAR surface expression monitored by myc epitope-tag and Thy1.1 expression. Mock-transduced CD8\( ^{+} \) T cells were used as controls. e, Cytokine (TNF, IFN\( \gamma \)) production by CAR CD8\( ^{+} \) T cells after re-stimulation with EL4-hCD19 cells or with PMA and ionomycin. f, Quantification of the data shown in e; \( P \) values (TNF: ****\( P < 0.0001 \), IFN\( \gamma \): ***\( P = 0.0009 \)) were calculated using a two-tailed unpaired t-test. g, In vitro killing assay (mean ± s.e.m.) of CD8\( ^{+} \) CAR and mock-transduced T cells; data from two biologically independent experiments, each with three technical replicates. h, Inhibitory surface receptor expression on CAR- and mock-transduced CD8\( ^{+} \) T cells cultured in vitro for 5 days; data representative of three biological replicates. Grey shading, isotype control; black line, mock or CAR. Data in d, e and h are representative of 3 independent experiments. *\( P \leq 0.05 \), **\( P \leq 0.01 \), ***\( P \leq 0.001 \), ****\( P \leq 0.0001 \).
Extended Data Fig. 2 | Adoptively transferred CD8⁺ CAR T cells infiltrating B16-OVA-hCD19 tumours exhibit phenotypes and gene expression profiles similar to those of OT-I and endogenous CD8⁺ TILs. a, b, Experimental design to assess CD8⁺CD45.1⁺ OT-I and CD8⁺CD45.2⁺ endogenous TILs; 1.5 × 10⁶ OT-I T cells were adoptively transferred into C57BL/6J mice 13 days after tumour inoculation. c, Tumour growth curves (mean ± s.e.m.) of mice adoptively transferred with CAR or OT-I CD8⁺ T cells; graph is a compilation of 3 independent experiments. At days 7 and 21, mouse numbers were: CAR, n = 24, 17; OT-I, n = 21, 20. d, Tumour growth curves (mean ± s.e.m.) of mice adoptively transferred with CAR or PBS; graph is a compilation of 3 independent experiments. At days 7 and 21, mouse numbers were: CAR n = 35, 35; PBS n = 8, 6. c, d, For tumour sizes on day 21 after tumour inoculation, P = 0.3527 for CAR compared to OT-I (c) and P = 0.6240 for PBS compared to CAR (d); P values were calculated using a two-tailed unpaired t-test with Welch’s correction, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. e, f, Flow cytometry gating scheme for CAR (e) and OT-I (f) CD8⁺ TILs. g, Mean average plots of genes differentially expressed in the indicated comparisons. Wald test was performed to calculate P values, as implemented in DESeq2; P values were adjusted using the Benjamini–Hochberg method. Genes differentially expressed (adjusted P < 0.1 and fold change (log2 scale) ≥ 1 or ≤ −1) are highlighted. Selected genes are labelled. Top row, comparisons of the CAR TIL populations amongst themselves and to endogenous PD-1loTIM3lo TILs; middle row, comparisons within the endogenous TIL populations; bottom row, comparisons of CAR and endogenous PD-1hiTIM3hi TILs (left), and CAR and endogenous PD-1loTIM3lo TILs (right).
Extended Data Fig. 3 | Adoptively transferred CD8⁺ CAR T cells infiltrating B16-OVA-hCD19 tumours exhibit chromatin accessibility profiles similar to those of endogenous CD8⁺ TILs. a, Pair-wise euclidean distance comparisons of log₂ transformed ATAC-seq density (Tn5 insertions per kilobase) between all replicates at all peaks accessible in at least one replicate. b, Scatterplot of pairwise comparison of ATAC-seq density (Tn5 insertions per kb) between samples indicated. c, Genome browser views of sample loci, Pdcd1 (left), Itgav (right); scale range is from 0–600 for all tracks and data are the mean of all replicates. CD8⁺ TIL populations are as indicated and defined in Fig. 1b, Extended Data Fig. 2b: (A) PD-1⁺TIM3⁺ CAR, (B) PD-1⁺TIM3⁻ CAR, (C) PD-1⁺TIM3⁺ endogenous, (D) PD-1⁺TIM3⁻ endogenous, (E) PD-1⁻TIM3⁻ endogenous, (F) PD-1⁺TIM3⁻ OT-I.
Extended Data Fig. 4 | Mouse and human CD8+ TILs exhibit increased expression of NR4A1, NR4A2, NR4A3. 

a, b, Flow cytometry gating scheme for CAR (a) and endogenous (b) CD8+ TILs. c, Representative flow cytometry histograms of NR4A proteins in PD-1hiTIM3hi TILs, PD-1hiTIM3lo TILs, and PD-1loTIM3lo TILs and their corresponding fluorescence minus one controls (in off-white). Data are representative of 3 independent experiments in which the sample from each independent experiment is comprised of TILs pooled together from 9–14 mice. 

d, Representative flow cytometry histograms for NR4A protein expression, comparing CAR and endogenous TIL populations (A–E) defined in Fig. 1b. e–g, Plotting in single cells the expression of PDCD1 and HAVCR2 (x and y axis, respectively) and (displayed by the colour scale) the expression of the following: e, Genes differentially upregulated in PD-1hiTIM3hi TILs relative to PD-1loTIM3lo TILs. f, Genes coding for selected transcription factors showing differential expression in the comparison of PD-1hiTIM3hi TILs relative to PD-1loTIM3lo TILs. g, Genes differentially downregulated in PD-1hiTIM3hi TILs relative to PD-1loTIM3lo TILs. Each dot represents a single cell. Human CD8+ TILs data are from ref. 14.
Extended Data Fig. 5  Prolonged survival of immunocompetent tumour-bearing mice adoptively transferred with CD8+ Nr4a TKO CAR T cells compared to mice transfected with CD8+ wild-type CAR T cells. a, CD8a only staining control (previously tested to be the same as fluorescence minus one controls for CAR expression and NGFR expression) of CAR T cells before adoptive transfer. b, CAR and NGFR expression of CD8+ wild-type CAR T cells before adoptive transfer. c, CAR and NGFR expression of CD8+ Nr4a TKO CAR T cells before adoptive transfer. Data in a–c are representative of 4 independent experiments for adoptive transfer into Rag1−/− recipient mice; preparation of adoptive transfer into immunocompetent mice was the same except for the use of GFP-expressing Cre and empty vector. d, 6 × 10^6 CAR T cells were adoptively transferred into C57BL/6J mice 7 days after tumour inoculation. e, Growth of B16-OVA-hCD19 (left; 13–15 mice per condition) and MC38-hCD19 (right; 10 mice per condition) tumours in individual mice. f, B16-OVA-hCD19 (left) and MC38-hCD19 (right) tumour sizes (mean ± s.d.) at day 21 and 19 post inoculation respectively. P values were calculated using an ordinary one-way ANOVA with Tukey’s multiple comparisons test: B16-OVA-hCD19, no significant difference; MC38-hCD19, PBS versus Nr4a TKO **P = 0.0001; PBS versus wild type, P = 0.3252; wild type versus Nr4a TKO, *P = 0.0120. g, Survival curves for mice bearing B16-OVA-hCD19 tumours (left) and MC38-hCD19 tumours (right). P values calculated using log-rank (Mantel–Cox) test. For B16-OVA-hCD19, surviving mouse numbers at day 7, day 21, day 90 were: PBS, n = 13, 11, 0; wild type, n = 15, 11, 0; Nr4a TKO, n = 14, 13, 2; *P = 0.0026. For MC38-hCD19, surviving mouse numbers at day 7 and day 19 were: PBS, n = 10, 9; wild type, n = 10, 7; Nr4a TKO, n = 10, 10; all mice died by day 23; *P = 0.0138. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.
Extended Data Fig. 6 | Tumour-bearing mice adoptively transferred with CD8+ CART cells lacking all three NR4A family members exhibit prolonged survival compared to mice transferred with wild-type CD8+ CART cells or CD8+ CART cells lacking only one of the three NR4A family members. a. Experimental design. 3 x 10^6 wild-type, Nr4a1 TKO, Nr4a1 KO, Nr4a2 KO or Nr4a3 KO CART cells were adoptively transferred into Rag1−/− mice 7 days after tumour inoculation. b. Growth of B16-OVA-hCD19 tumours in individual mice, comprised of 17 or more mice per condition (these data include the wild type and Nr4a TKO data from Fig. 3). c. Graph shows mean ± s.d. and the individual values of B16-OVA-hCD19 tumour sizes at day 21 after inoculation. P values were calculated using an ordinary one-way ANOVA with Tukey’s multiple comparisons test; PBS versus wild type, *P = 0.0395; wild type versus Nr4a1 KO, P = 0.0511 (not significant); wild type versus Nr4a2 KO, **P = 0.002, wild type versus Nr4a3 KO, *P = 0.0161; and wild type versus Nr4a TKO, ****P < 0.0001. d. Survival curves. ****P < 0.0001, calculated using log-rank (Mantel–Cox) test. Surviving mouse numbers at day 7, day 21 and day 90 were n = 31, 14, 0 for PBS; n = 35, 25, 1 for wild type; n = 17, 12, 0 for Nr4a1 KO; n = 17, 15, 1 for Nr4a2 KO; n = 32, 22, 11 for Nr4a3 KO; and n = 39, 36, 27 for Nr4a TKO. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Phenotypic and genomic features of mouse CD8+ T cells expressing NR4A1, NR4A2 or NR4A3. Mouse CD8+ T cells were isolated, activated, transduced with empty retrovirus or retroviruses encoding HA-tagged Nr4a1, Nr4a2 or Nr4a3 with human NGFR reporter, and assayed on day 5 post activation. a, Flow cytometry gating of CD8+ NGFR+ empty vector control, NR4A1-, NR4A2- and NR4A3-expressing cells at a constant expression level of NGFR reporter. b, Quantification of surface receptor expression (data from 3 independent replicates), showing geometric MFI normalized across experiments to the average of all samples within each experiment. c, Representative flow cytometry plots of cytokine production upon re-stimulation with PMA and ionomycin. d, Quantification of the data in c, showing geometric MFI normalized across experiments to the average of all samples within each experiment. All P values were calculated using an ordinary one-way ANOVA with Dunnett’s multiple comparisons test; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. e, PCA plot of RNA-seq data from in vitro resting mouse CD8+ T cells ectopically expressing NR4A1, NR4A2, NR4A3 and empty vector control. f, Mean average plots of genes differentially expressed in the comparisons of ectopic expression of NR4A1, NR4A2 or NR4A3 against empty vector (top row), and pairwise comparisons between the ectopic expression of various NR4A family members (bottom row). Wald test was performed to calculate P values, as implemented in DESeq2. P values were adjusted using the Benjamini–Hochberg method. Genes differentially expressed (adjusted P < 0.1 and fold change (log2 scale) ≥ 1 or ≤ −1) are highlighted using different colours as indicated in the PCA plot as in e. Selected genes are labelled. g, Scatterplot of pairwise comparison of ATAC-seq density (Tn5 insertions per kb) between the indicated samples. Data in a–d are from three independent experiments; data in e–g from two independent experiments, each with two technical replicates.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | CD8⁺ Nr4a TKO CAR TILs show increased effector function compared to CD8⁺ wild-type CAR TILs. a, Tumour growth curves (mean ± s.e.m.) after adoptive transfer of 1.5 × 10⁶ CAR T cells into Rag1−/− mice on day 13 after tumour inoculation. Mouse numbers at day 7 and day 21 were: wild type, n = 47, 35; Nr4a TKO, n = 41, 32. P values were calculated using an ordinary two-way ANOVA with Tukey’s multiple comparisons test; for wild type versus Nr4a TKO, P = 0.5463. b, Flow cytometry gating scheme for surface markers, cytokines, and transcription factors expressed by wild-type (top) and Nr4a TKO (bottom) TILs. All samples are gated on cells with a set level of CAR expression (10⁴–10⁵) within the CAR⁺ NGFR⁺ population. c, Bar plots (mean ± s.d.) showing (left) number of wild-type and Nr4a TKO CAR TILs per g of tumour (5 independent experiments; P value was calculated using a two-tailed ratio paired t-test) and (right) mean fluorescence intensity of Ki67 of wild-type and Nr4a TKO CAR TILs (2 independent experiments). d, Top, representative flow cytometry plots for TIM3 and TCF1 expression in wild-type and Nr4a TKO CAR TILs (2 independent experiments). Bottom, bar plots (mean ± s.d.) of transcription factor expression by wild-type and Nr4a TKO CAR TILs (6 independent experiments). P values were calculated using two-tailed paired t-tests. For all P value calculations, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. e, PCA plot of RNA-seq data from Nr4a TKO or wild-type CAR TILs. f, Normalized enrichment scores (NES) of gene sets defined from pairwise comparisons of effector, memory and exhausted CD8⁺ T cells from LCMV-infected mice¹¹. Enrichment score was calculated using a Kolmogorov–Smirnov test, as implemented in gene set enrichment analysis (GSEA). g, GSEA of RNA-seq data from Nr4a TKO and wild-type CAR TILs displayed as enrichment plots, ranking genes by fold change in expression between those conditions. The false discovery rate (FDR) for both (f, g) is controlled at a level of 5% by the Benjamini–Hochberg correction. For e–g, data are from two independent experiments each consisting of 1–2 technical replicates.
Extended Data Fig. 9 | NR4A family members bind to predicted NR4A-binding motifs that are more accessible in wild-type CAR TILs compared to the Nr4a TKO CAR TILs, and regions more accessible in wild-type compared to Nr4a TKO CAR TILs are more accessible in CA-RIT-NFAT1- and NR4A1/2/3-transduced cells. 

a, Top right, histogram view showing expression of NR4A in cells ectopically expressing HA-tagged versions of NR4A1, NR4A2, NR4A3; data are representative of 2 independent experiments. Middle, genome browser views of the Ccr7, Ccr6, Ifng loci for wild-type CAR TILs compared to Nr4a TKO CAR TILs, including binding motifs for NFAT, NR4A, bZIP and NFκB. Scale range is 0–600 for all tracks and data are mean of two independent experiments. Right, bar plots showing enrichment of NR4A at regions probed; data representative of 2 independent experiments consisting of three technical replicates each.

b, Genome browser views of the Il21 (top), Tnf (bottom) loci incorporating wild-type CAR TILs compared to Nr4a TKO CAR TILs, including binding motifs for NFAT, NR4A, bZIP and NFκB. Scale range is 0–600 for Il21 and 0–1000 for Tnf; data are mean of two independent experiments.

c, Top four panels, ATAC-seq data from Nr4a TKO and wild-type CAR TILs compared with data from cells ectopically expressing CA-RIT-NFAT1, NR4A1, NR4A2 or NR4A3. Bottom panel, ATAC-seq data from Nr4a TKO and wild-type CAR TILs compared with data from cultured cells re-stimulated with PMA and ionomycin.
Extended Data Fig. 10 | Nr4a family members show a moderate decrease in mRNA expression in antigen-specific cells from LCMV-infected mice treated with anti-PDL1 or IgG control. a. Mean average plots of genes differentially expressed in cells treated with anti-PDL1 compared to cells treated with IgG control, highlighting two different categories of differentially expressed genes: those with adjusted \( P < 0.1 \) and fold change (log2 scale) ≥ 0.5 or ≤ −0.5 (lighter colours); and those with adjusted \( P < 0.1 \) and fold change (log2 scale) ≥ 1 or ≤ −1 (darker colours). Selected genes are labelled. Displayed are the number of genes in each category. The sequencing data in this analysis were obtained from ref. 19. Wald test was performed to calculate \( P \) values, as implemented in DESeq2; \( P \) values were adjusted using the Benjamini–Hochberg method.
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Please do not complete any field with “not applicable” or n/a. Refer to the help text for what text to use if an item is not relevant to your study. 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.*

- 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, BiocStrings v2.42.1, data.table v1.11.4, dplyr v0.7.6, GenomenInfoDb v1.10.3, GenomicAlignments v1.10.1, GenomeInfoDB v1.10.3, GenomicRanges v1.26.4, ggplot2 v3.0.0, gtools v3.5.0, IRanges v2.24.0, 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 v1.35.2, GenomeAlignments 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 B134388, 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 231745
202539 Biolegend BV711 anti-rat/mouse CD90.1 Thy.1 OK-7 B223103
202516 Biolegend PerCP/ Cy5.5 anti-rat/mouse CD90.1 B245102, B245103
202519 Biolegend APC-Cy7 Anti-rat/mouse CD90.1 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) B29.1 A12 B231365
135206 Biolegend PE anti-mouse CD279 (PD-1) A29.1 B1242906
135210 Biolegend APC anti-mouse CD279 (PD-1) A29.1 A12 n/a
125210 Biolegend APC anti-mouse CD233 (lag3) C97PW B176313
125223 Biolegend PE/Dazzle594 anti-mouse CD233 (lag3) C97PW B224161
12-5870-81 eBioscience PE anti-mouse TIM3 RMT3-23 A301948, 4273433
119705 Biolegend Anti-mouse CD366 (tim-3) RMT3-23 A301948
123907 Biolegend PE anti-mouse CD200R (Ox2) Ox-110 B220799
123809 Biolegend Anti-mouse CD200 (Ox2) Ox-90 B203310
133507 Biolegend PE anti-mouse CD44.2 (2B4 B6 alloantigen) m2B4 (B6) 458.1 B182843
126309 Biolegend PE anti-mouse CD357 (Gitr) DTA-1 B241900
506328 Biolegend BV421 anti-mouse TNF alpha mp6-XT22 B224675
503839 Biolegend PE/Dazzle594 anti-mouse IL-2 JE56-SH4 B211964
12-7021-82 eBioscience PE anti-mouse IL-2 JE56-SH4 E030634
505807 Biolegend PE anti-mouse Ifn gamma Xmg1.2 B178149
17-7311-82 eBioscience APC anti-mouse Ifn gamma Xmg1.2 E07397-1633
363004 Biolegend PE anti-human CD19 SJ25C1 B214170
90665 Cell Signaling Technology TCF1/ TCF7 Rabbit mAb (Pacific Blue Conjugate) C63D9 1
12-4875-80 eBioscience PE anti-mouse Fomes Dan11mag 431321, 4323634
50-5825-82 eBioscience efluor 660 anti-human/mouse Tbet 480 B12136-1632
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
37245 Cell Signaling Technology HA-tag Rabbit mAb C294F B
3739S Cell Signaling Technology Myc-tag (B811) mouse PE mAb B811 9
135216 Biolegend PeCy7 anti-mouse CD279 (PD-1) B29.1 A12 B227806
12-1011-82 Invitrogen PE anti-mouse CD101 mAb CD101 Moushi 101 4330771
17-0381-81 eBioscience APC anti-mouse CD38 90 4324890
12-5965-80 eBioscience PE anti-mouse Nur77 (Nru41) 12.14 E01954-1636
sc-376984AF647 Santa Cruz Biotechnology AF647 anti-mouse Nr4a2 P-5 G2517
sc-393902PE Santa Cruz Biotechnology PE anti-mouse Nr4a3 H-7 B2818
563786 BD Biosciences BV355 rat anti-mouse CD8a 53-6.7 8072932, 7096603
652405 Biolegend APC anti-mouse K67 16A8 B191905
345104 Biolegend FITC anti-human Ngfr ME20.4 B232717
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.
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, FACSArta-I, FACSArta-II, FACSArta-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/SSC-H -> huCD19+ (roughly top 16% of huCD19-expressing cells)

- Nr4a1, Nr4a2, Nr4a3-expressing cell sorts: FSC-A/SSC-A -> FSC-W/SSC-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/SSC-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/SSC-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/SSC-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

Gating strategy for flow cytometry:

- 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 -> FSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> CD45.1+ Thy1.1+ (CAR) or CD45.1+ Thy1.1- (OR-I) -> PD-1, TIM3, LAG3, CD200, GITR, 2B4, CD101, CD38.

- Nr4a1, Nr4a2, Nr4a3 in vitro, cytokine production: FSC-A/SSC-A -> FSC-W/SSC-H -> CD8+ Live/dead dye neg. -> CD45.1+ Thy1.1+ (CAR) or CD45.1+ Thy1.1- (OR-I) -> 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/SSC-H -> (Live) CD8+ Live/dead dye neg. -> CD45.1+ Thy1.1+ (CAR) or CD45.1+ Thy1.1- (OR-I) -> PD-1, TIM3, LAG3, TCF1, Erns, T-bet.

- TILs, cytokine production: FSC-A/SSC-A -> FSC-W/SSC-H -> (Live) CD8+ Live/dead dye neg. -> CD45.1+ Thy1.1+ (CAR) or CD45.1+ Thy1.1- (OR-I) -> TNF, IFNg, IL-2; cytokine production negative gates set on CAR unstimulated or OR-I unstimulated.

- Nr4a protein level expression: FSC-A/SSC-A -> FSC-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. ☒