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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	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 all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	No commercial, open source, or custom code was used for this study.
Data analysis	For RNA sequencing analysis, 1) Fastq files were first evaluated with FASTQC v0.11.8, 2) Cutadapt v1.18 was used to remove Illumina adapters, 3) paired end reads for each condition were aligned with STAR v2.5.4b usin g the genome reference consortium human build 38 patch release 13 (GRCh38.p13) downloaded from NCBI (https://www.ncbi.nlm.nih.gov/), 4) HTSeq (Python 3.6.5) was used to generate expression counts for each gene, 5) DESeq2 (R v3.6.1, https://www.R-project.org/) was used to normalize the data and calculate differential expression based on statistical cutoffs, 6) volcano plots were generated with the R package EnhancedVolcano (https://bioconductor.org/packages/devel/bioc/vignettes/EnhancedVolcano/inst/doc/EnhancedVolcano.html), 7) Heat maps were generated with the R package pheatmap (https://cran.r-project.org/web/packages/pheatmap/index.html), and 8) pathway analysis was conducted with Qiagen IPA (QIAGEN, Redwood City, CA, USA). For analysis of ATAC sequencing data, 1) Quality check was performed first with FASTQC v0.11.8, 2) Nextera adapter sequences were trimmed with Cutadapt v1.18, 3) paired-end reads for each sample were aligned to the UCSC reference genome for human genome 38 (hg38), patch release 13 (https://hgdownload.soe.ucsc.edu/downloads.html#human) with Bowtie2 v2.5.0, 4) Bam files were sorted with samtools v1.17 and mitochondrial reads were removed with the BAMQC program v0.6.5, 5) Peak calling was performed with the MACS2 package v2.2.7, 6) differential peak accessibility analysis was conducted with DESeq2 in the DiffBind package (Bioconductor v3.16), 7) Differentially accessible peaks were annotated with the R package "ChIPSeeker" (Bioconductor v3.16), 8) motif analysis was conducted using MEME suites, and 9) BigWig files were created with the "bamCoverage" tool and averaged BigWig files were generated with the tool "Wiggletools". For CRISPR screen analysis, 1) Paired-end files were merged with the tool "bbmerge", 2) MAGeCK-VISPR was used to perform quality and differential expression analysis, 3) Volcano plots were generated with ggplot, and 4) pathway analysis was conducted with Enrichr (https://maayanlab.cloud/Enrichr/) and QIAGEN IPA (QIAGEN, Redwood City, CA, USA). All of these softwares and tools are publicly available. Manual analysis of flow cytometric data was completed with Kaluza Analysis 2.1 software (Beckman Coulter) or FlowJo X10.0.7r2 software

(Becton Dickenson).

In vivo bioluminescence data was analyzed using Living Image version 4.8.2software (PerkinElmer).

All other statistical analyses were performed with GraphPad Prism 10 (GraphPad Software). The specific statistical tests performed are specified in figure legends.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The raw and processed files from the in vitro genome-wide CRISPR screen are available in the Gene Expression Omnibus (GEO) repository under accession code GSE273299. The raw and processed files from RNA sequencing of baseline and chronically stimulated healthy donor CART19-28ζ cells are available in GEO under accession code GSE273294. The raw and processed files from ATAC sequencing of baseline and chronically stimulated healthy donor CART19-28ζ cells are available in GEO under accession code GSE273297. Due to patient privacy, we have provided processed files from RNA and ATAC sequencing of pre-infusion patient CART cells. For RNA sequencing, we have provided a supplementary file with non-normalized counts, and for ATAC sequencing we have provided supplementary peak files. Source data from in vitro and in vivo experiments are provided as a supplementary file.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	This information has not been collected for this study.
Reporting on race, ethnicity, or other socially relevant groupings	This information has not been collected for this study.
Population characteristics	This information has not been collected for this study.
Recruitment	This does not apply to our study.
Ethics oversight	This does not apply to our study.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample sizes were determined by the rule of three in statistics where it is stated that if a certain event or outcome did not occur in a sample with n subjects, the interval from 0 to 3/n is the 85% confidence interval in the population. With this, all of our experiments required at least 3 replicates per group to be able to determine statistical significance carried out in our analyses. As such all (n) number of replicates were > or = 3. For animal studies, sample size was determined based on the estimated effect of the experimental group compared to the control group. Based on such assumptions, we need to include 5-10 mice per group to achieve 80% power and a type I error of 0.05. This is based on our prior experience for evaluation of CART cell therapy in vivo (PMID: 38561490, PMID: 37879074, PMID: 37378662, PMID: 35440691)
Data exclusions	No data was excluded from these analyses.
Replication	All data included in this manuscript were successfully replicated two or more times.
Randomization	Randomization was performed for in vivo studies. Mice were randomized into groups based on tumor burden as assessed with bioluminescent imaging.
Blinding	Researchers were not blinded in animal studies as groups were assigned and analyzed through randomization by tumor burden at

engraftment, excluding the possibility of blinding.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

1. APC-Cy7 anti-human CD3, clone SK7, BioLegend, catalog #344818, dilution 1:100
2. APC anti-human CD3, clone UCHT1, eBioscience, catalog #17-0038-42, dilution 1:100
3. BV605 anti-human CD3, clone SK7, BioLegend, catalog #344836, dilution 1:100
4. BV785 anti-human CD4, clone OKT4, BioLegend, catalog #317442, dilution 1:100
5. FITC anti-human CD4, clone OKT4, eBioscience, catalog #11-0048-42, dilution 1:100
6. PerCP anti-human CD8, clone SK1, BioLegend, catalog #344708, dilution 1:100
7. PerCP anti-human CD20, clone 2H7, BioLegend, catalog #302324, dilution 1:100
8. APC anti-human CD20, clone 2H7, BioLegend, catalog #302310, dilution 1:100
9. APC-eFluor780 anti-mouse CD45, clone 30-F11, eBioscience, catalog #47-0451-82, dilution 1:100
10. BV421 anti-human CD45, clone HI30, BioLegend, catalog #304032, dilution 1:100
11. PE-CF594 anti-human IL-2, clone 5344.111, BD Biosciences, catalog #562384, dilution 2.5:100
12. APC anti-human IL-4, clone MP4-25D2, BD Biosciences, catalog #554486, dilution 1:500
13. AF700 anti-human TNF-alpha, clone MAb11, BioLegend, catalog #502928, dilution 2.5:100
14. APC-efluor780 anti-human IFN-gamma, clone 4S.B3, eBioScience, catalog #47-7319-42, dilution 2.5:100
15. BV421 anti-human GM-CSF, clone BVD2-21C11, BD Biosciences, catalog #562930, dilution 2.5:100
16. BV421 anti-human PD-1, clone EH12.2H7, BioLegend, catalog #329920, dilution 1:100
17. FITC anti-human PD-1, clone MIH4, eBioscience, catalog #11-9969-42, dilution 1:100
18. PE anti-human TIM-3, clone F38-2E2, BioLegend, catalog #345006, dilution 1:100
19. PE-Cy7 anti-human CTLA-4, clone BNI3, BioLegend, catalog #369614, dilution 1:100
20. FITC anti-human LAG-3, clone 3DS223H, eBioscience, catalog #11-2239-42, dilution 1:100
21. PerCP anti-human CCR4, clone L291H4, BioLegend, catalog #359406, dilution 1:100
22. APC anti-human CCR6, clone 11A9, BD Biosciences, catalog #560619, dilution 1:100
23. APC-Cy7 anti-human CXCR3, clone G02H7, BioLegend, catalog #353722, dilution 1:100
24. LIVE/DEAD Aqua, Invitrogen, catalog #L34966, dilution 3:500
25. Biotin ProteinL, GenScript, catalog #M00097, dilution 1ug per 200uL
26. PE Streptavidin, BioLegend, catalog #405203, dilution 6.25:100
27. APC anti-human IL4R, clone G077F6, BioLegend, catalog #355005, dilution 1:100

Validation

1. APC-Cy7 anti-human CD3, tested by flow cytometry on human peripheral blood lymphocytes (manufacturer)
2. APC anti-human CD3, tested by flow cytometry on normal human peripheral blood cells (manufacturer)
3. BV605 anti-human CD3, tested by flow cytometry on human peripheral blood lymphocytes (manufacturer)
4. BV785 anti-human CD4, tested by flow cytometry on human peripheral lymphocytes (manufacturer)
5. FITC anti-human CD4, tested by flow cytometry on human peripheral blood mononuclear cells(manufacturer)
6. PerCP anti-human CD8, tested by flow cytometry on human peripheral blood lymphocytes(manufacturer)
7. PerCP anti-human CD20,tested by flow cytometry on human peripheral blood lymphocytes (manufacturer)
8. APC anti-human CD20, tested by flow cytometry on human peripheral blood lymphocytes (manufacturer)
9. APC-eFluor780 anti-mouse CD45, tested by flow cytometry on C57BL/6 bone marrow cells (manufacturer)
10. BV421 anti-human CD45, tested by flow cytometry on human peripheral blood lymphocytes (manufacturer)
11. PE-CF594 anti-human IL-2, tested by flow cytometry on human peripheral blood mononuclear cells (manufacturer)
12. APC anti-human IL-4, tested by flow cytometry on human peripheral blood mononuclear cells (manufacturer)
13. AF700 anti-human TNF-alpha, tested by flow cytometry on human peripheral blood lymphocytes (manufacturer)
14. APC-efluor780 anti-human IFN-gamma, tested by flow cytometry on normal human peripheral blood cells (manufacturer)
15. BV421 anti-human GM-CSF, tested by flow cytometry on human peripheral blood mononuclear cells (manufacturer)
16. BV421 anti-human PD-1, tested by flow cytometry on human peripheral blood lymphocytes (manufacturer)

17. FITC anti-human PD-1, tested by flow cytometry on normal human peripheral blood cells (manufacturer)
18. PE anti-human TIM-3, tested by flow cytometry on Th1-polarized human peripheral blood mononuclear cells (manufacturer)
19. PE-Cy7 anti-human CTLA-4, tested by flow cytometry on human peripheral blood mononuclear cells (manufacturer)
20. FITC anti-human LAG-3, tested by flow cytometry on normal human peripheral blood cells (manufacturer)
21. PerCP anti-human CCR4, tested by flow cytometry on human peripheral blood lymphocytes (manufacturer)
22. APC anti-human CCR6, tested by flow cytometry on human lysed whole blood (manufacturer)
23. APC-Cy7 anti-human CXCR3, tested by flow cytometry on human peripheral blood lymphocytes (manufacturer)
24. LIVE/DEAD Aqua, tested by flow cytometry on Jurkat (manufacturer)
25. Biotin ProteinL, tested by flow cytometry on human T-cells (Supplementary Figure S26)
26. PE Streptavidin, tested by flow cytometry on C57BL/6 splenocytes (manufacturer)
27. APC anti-human IL4R, tested by flow cytometry on human peripheral blood lymphocytes (manufacturer)

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	The mantle cell lymphoma cell line, JeKo-1, the acute lymphoblastic leukemia cell line, NALM6, and the epithelial-like cell line, 293T, were purchased from ATCC, Manassas, VA, USA (cat. #CRL-3006, cat. #CRL-3273, and cat. #CRL-3216). The multiple myeloma cell line, OPM-2, was purchased from DSMZ, Braunschweig, Germany (cat. #ACC 50). Primary human T cells were isolated from de-identified healthy donor blood samples that were obtained from apheresis donor cones collected during apheresis platelet collection at Mayo Clinic. When requesting blood samples, sex was not specified as a criteria for selection.
Authentication	Cell lines were authenticated by the manufacturer and routinely checked for phenotype by flow cytometry.
Mycoplasma contamination	Cell lines were tested monthly and confirmed negative for mycoplasma contamination.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used in this study.

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	All mouse experiments were conducted under the Mayo Clinic Institutional Animal Care and Use Committee (IACUC)-approved animal protocols. The mouse strains used in this study include male or female 6- to 8-week-old NOD-SCID-IL2rγ-/- (NSG) mice from Jackson Laboratories. The mice were housed in a pathogen-free, biosafety level 2+ animal facility in social housing (2-5 mice per cage). As part of their housing, the mice were exposed to a 12-hour dark/12-hour light cycle with an ambient temperature at 21 +/- 1 degrees Celsius and a humidity of 50% +/- 10%.
Wild animals	No wild animals were used in this study.
Reporting on sex	Mouse experiments were performed on both male and female sexes of mice with each individual experiment being performed on only one sex of mice at a time.
Field-collected samples	No field-collected samples were used in this study.
Ethics oversight	Animal studies were performed according to the Institutional Animal Care and Use Committee (IACUC) at the Mayo Clinic animal facilities (IACUC # A00001767-16-R22).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	Plants were not used.
Novel plant genotypes	Plants were not used.
Authentication	Plants were not used.

Flow Cytometry

Plots

Confirm that:

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

☒ 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).

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

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

Methodology

Sample preparation	For panels related to CART cell proliferation, Th1/Th2 phenotype, and the expression of inhibitory receptors: All well contents were spun and washed in flow buffer (Phosphate-buffered saline (PBS), 2% (v/v) fetal bovine serum (FBS), and 1% (v/v) sodium azide) before staining with desired antibodies for 15 minutes in the dark at room temperature. Cells were next washed in flow buffer to then be analyzed by flow. For CAR detection: Protein L staining with a Biotin Protein L primary antibody (cat. #M00097, GenScript, Piscataway, NJ, USA) and a secondary antibody for streptavidin (cat. #405203, BioLegend, San Diego, CA, USA) were used to detect CAR on lentivirally generated CART cells and an anti-Whitlow linker antibody was used to detect CAR expression on retrovirally generated CART cells used for RNA and ATAC sequencing experiments. Staining was performed according to the manufacturer protocol. For intracellular staining of cytokines: Briefly, CART cells were co-cultured with JeKo-1, NALM6, or OPM-2 target cells at a 1:5 effector-to-target cell ratio for four hours with the addition of CD28 (clone L293, cat #348040, BD Biosciences, San Diego, CA, USA), CD49d (clone L25, cat #340976, BD Biosciences, San Diego, CA, USA), monesin (cat #420701, BioLegend, San Diego, CA, USA), and CD107a (clone H4A3) FITC (cat #555800, BD Biosciences, San Diego, CA, USA). After four hours of co-culture, intracellular staining of T cells was performed by first staining with Live/Dead Aqua (cat #L34966, Invitrogen, Carlsbad, CA, USA) before using the FIX & PERMTM Cell Permeabilization Kit (cat #GAS001S100 and cat #GAS002S100, Life Technologies, Oslo, Norway). Then, intracellular staining was performed with the desired antibodies.
Instrument	Flow cytometry was performed on three-laser CytoFLEX (Beckman Coulter, Chaska, MN, USA) where cells were gated by singlet discrimination and live cells were determined by live/dead aqua staining (Cat#L34966, Thermo Fisher Scientific, Waltham, MA, USA).
Software	Analyses were performed using Kaluza 2.1 software and FlowJo Version 10
Cell population abundance	FACs sorting was not used.
Gating strategy	Following final wash steps, cells were analyzed for desired surface markers. Absolute cell count numbers were obtained using volumetric measurement. Cells were gated on SSC vs. FSC plots for cell separation by size and complexity, FSC-H vs. FSC-A plots making a diagonal gate to exclude doublets, and live/dead Aqua staining (Cat#L34966, Thermo Fisher Scientific, Waltham, MA, USA) to exclude dead cells, followed by cell subset characterization based on predesigned antibody panels which were optimized and used to stain samples. The gates establishing positivity for these cell subset markers were determined through negative gating by fluorescence minus one (FMO) control wells for all evaluations except for CAR percentage evaluations. CAR positivity was determined through staining of untransduced T cells. CFSE negative gating was determined based on stained CART cells kept in media without stimulation.

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