

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 (<i>n</i>) 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 <i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>
☒	☐ 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. <i>F</i> , <i>t</i> , <i>r</i>) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted <i>Give P values as exact values whenever suitable.</i>
☒	☐ 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 <i>d</i> , Pearson's <i>r</i>), 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	FACSDiva BD CELESTA™ version 8.0.1.1; FORTESSA™ and SYMPHONY™ cell analyzer, IncuCyte® S3 system (Sartorius), QUIAgility (QUIAGEN), QuantStudio™ 3 (Applied Biosystems), GloMax® Discover instrument model GM3000, software version 4.0.0 (Promega)
Data analysis	SnapGene version 8.1, FlowJo™ version 10.9.0 (BD Life Sciences), GraphPad Prism version 8 and version 10 f, Microsoft Excel, Biorender (biorender.com); R environment.

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](#)

Source data are provided with this paper

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	Sex and gender not considered for the study design
Reporting on race, ethnicity, or other socially relevant groupings	Donor's blood samples are deidentified with no reporting on race, ethnicity or other socially relevant groupings
Population characteristics	Healthy donors
Recruitment	The study did not involve subject enrollment in clinical studies
Ethics oversight	Ethical approval from Azienda Ospedaliera Universitaria Federico II Protocol number# 8925 and Universitätsklinikum Regensburg protocol n° 20-2040-101

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](https://www.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	No formal sample size calculation was performed for this study. Instead, the sample size was determined based on practical considerations, including resource availability and the scope of the study. The chosen sample size aligns with similar studies in the field and is deemed sufficient to provide meaningful insights while ensuring a diverse and representative set of participants. Additionally, the sample size was considered adequate to capture relevant trends and patterns without overextending beyond logistical constraints. While a formal power analysis was not conducted, the selected sample size allows for a robust preliminary exploration of the research question. For experiments performed in primary cells or in vivo, biological replicates were defined as independent donors (n=3/4) or individual mice (n=6 for conditions). For transfection and electroporation experiments, biological replicates were defined as independent experimental repeats performed on different days (n=3-5). Technical replicates represent repeated measurements of the same sample. No statistical methods were used to predetermine sample size.
Data exclusions	for transfection/electroporation, no data were excluded from this study. All collected data were included in the analysis to ensure a comprehensive and unbiased representation of the results. Samples excluded from the analysis when cells did not survive during the experimental procedures is applied to a subset of donors and where applicable to individual mice. Exclusions were based on cell viability and were determined prior to data analysis.
Replication	The reproducibility of the experimental findings was ensured by following standardized procedures and methodologies. All experiments were conducted under consistent conditions, and key analyses were repeated to confirm reliability. The findings were consistent across repeated trials, supporting the robustness of the results. Biological replicates were defined as independent experiment for transfection and electroporation, or of independent donors and individual mice. Technical replicates consisted of repeated measurements of the same sample.
Randomization	Randomization was not applicable to this study due to the nature of the research design. However, efforts were made to minimize potential biases by ensuring consistency in data collection and analysis.
Blinding	Blinding was not used in this study because it was not applicable to the research design. However, to minimize potential biases, objective criteria were used for data collection and analysis.

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	LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit (L34959, Life Technologies Inc.) 1:1000; anti-CD3-BV510 clone HIT3a (RUO) (564713, BD Horizon, Lot 3135539, 2ul), anti-CD8-BB700 clone RPA-T8 (RUO)(566452, BD Horizon, Lot 2250819, 2ul), anti-CD39-BV421 (2241070, Sony, 1ul), anti-TIGIT-BV786 (243680, Sony, 1ul), anti-TIM3-BV711 (565566, BD biosciences, 2ul), anti-PD1-BUV650 (2249750, Sony, 1ul), anti-LAG3-Pe-CF594 (565718, BD Horizon, 2ul), anti-NR4A2-FITC Rabbit polyclonal (orb464231, Biorbyt, 2ul), anti-TOX-PE (130120716, Miltenyi biotec, 2ul); LIVE/DEAD™Near IR 1:1000, anti-CD8-BUV 805 (742030, BD biosciences, 0.5ul), anti-TCR/ Vb13.1-VB R720 (130131138, Miltenyi biotec, 2ul), anti-CD39- BV650 (563681, BD biosciences, 1ul), anti-TIGIT- PerCP/eFluor/710 (46950042, ThermoFisher, 2ul), anti-TIM3-BUV421 (565562, BD biosciences, 1ul), anti-PD1-BUV737 (565299/612791 , BD Pharmingen, 5ul), anti-LAG3-BUV615 (752362, BD biosciences, 5ul), anti-NR4A2-AF647 sc-376984, Santa Cruz, 1ul), anti-NGFR-BV421 (345131, BioLegend, 2ul)
Validation	Antibody titers were validated through a series of optimization experiments, including serial dilution assays and signal-to-noise ratio assessments. Validation was further supported by manufacturer-provided specifications, previously published studies using the same antibodies, and internal controls to confirm specificity and reproducibility in the intended application. All primary antibodies used in this study were validated for human flow cytometry by the manufacturers. Controls including fluorescence minus one (FMO) were used where appropriate.

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	The cell lines used in this study were obtained from ATCC: Jurkat cells ATCC-TIB-152, Clone E6-1; HEK 293 FT cells CRL-1573. SK-MEL-23 NY-ESO-1* cells, which were kindly provided by Dr. Steven A. Rosenberg (National Institutes of Health, USA). Primary cells taken by healthy donors were provided by Hospitals. The sex of the donors is not provided.
Authentication	None of the cell lines used in this study were authenticated
Mycoplasma contamination	All cell lines were routinely tested for mycoplasma contamination using PCR-based assays, and no contamination was detected.
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines, as listed in the ICLAC register, 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	Laboratory experiments were performed using NOG IL-15 mice (NOD.Cg-Prkdcscid Il2rgtm1Sug Tg(CMV-IL2/IL15)1-1Jic/JicTac; Taconic Biosciences). Mice were 8 weeks of age at the time of tumor implantation. Animals were maintained under specific pathogen-free (SPF) conditions with ad libitum access to food and water. Mice were housed under standard laboratory conditions in a temperature- and humidity-controlled animal facility: mice were maintained on a 12 h light/12 h dark cycle, with ambient temperature maintained at 22 ± 2°C and relative humidity at 45–65%. Mice were monitored twice weekly in accordance with institutional animal guidelines.
Wild animals	This study did not involve wild animals.
Reporting on sex	Only female mice were used to reduce variability in tumor growth kinetics.
Field-collected samples	This study did not involve field-collected samples.
Ethics oversight	All animal procedures were approved by Government of lower Franconia under protocol number RUF-55.2.2-2532-2-1610 and were conducted in accordance with national and institutional animal welfare regulations.

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

Plants

Seed stocks	NA
Novel plant genotypes	NA
Authentication	NA

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

HEK 293FT cells were detached with 50 µl of Trypsin and resuspended in 150 µl of FACS Buffer (1xPBS, 1% FBS and 2mM EDTA), while Jurkat cells were analyzed directly.

For experiments using human T cells, ethical approval was provided from Azienda Ospedaliera Universitaria Federico II under the Protocol number# 8925.

Human CD8+ T cells were isolated from buffy coats of blood donors by magnetic sorting, activated with Dynabeads™ Human T-Activator CD3/CD28 at 1:1 beads:cells ratio, and cultured in R10 medium (RPMI-1640 supplemented with 10% FBS (Life Technologies), 1% penicillin/streptomycin (Gibco), 2mM GlutaMax (Gibco), 10mM HEPES, Penicillin-Streptomycin (100 U/ml) and 1% Non-Essential Amino Acids (HyClone) in the presence of IL-2 (30 U/ml). Lentivirus transduction was carried out in 6-well plates in stimulated T cells after 24-48h from stimulus. Cells were transduced with lentivirus for SPs at a MOI of 5 and for TCR at MOI of 5 kept at a concentration of one million cells/ml.

These CD8+ T cells were collected at specific time points for staining.

For in vitro characterization, T cells were incubated with LIVE/DEAD™ Fixable Yellow Dead Cell Stain (L34959, Life Technologies Inc.) for 20 min at RT FOR DEAD CELL EXCLUSION. CD8+ T cells were then stained for extracellular markers with the following antibodies: anti-CD3-BV510 (564713, BD Horizon), anti-CD8-BB700 (566452, BD Horizon), anti-CD39-BV421 (2241070, Sony), anti-TIGIT-BV786 (243680, Sony), anti-TIM3-BV711 (565566, BD biosciences), anti-PD1-BUV650 (2249750, Sony), anti-LAG3-Pe-CF594 (565718, BD Horizon). Cells were fixed and permeabilized using the Transcription factor buffer set (562574, BD Pharmingen), following the manufacturer's instruction, and then stained for 40 min at 4°C with anti-NR4A2-FITC (orb464231, Biorbyt), anti-TOX-PE (130120716, Miltenyi biotec). Cells were then washed once with Perm/Wash, once with 1x PBS and resuspended in 150 µl of 1xPBS.

For co-cultured of SP73/TCR engineered primary T cells (Figure 6) and in vivo experiments (Figure 7) we used LIVE/DEAD™ Near IR, for dead cell exclusion. CD8+ T cells were then stained for extracellular markers with the following antibodies: anti-CD8-BUV 805 (742030, BD biosciences), anti-TCR/ Vb13.1-VB R720 (130131138, Miltenyi biotec), anti-CD39- BV650 (563681, BD biosciences), anti-TIGIT- PerCP/eFluor/710 (46950042, ThermoFisher), anti-TIM3-BUV421 (565562, BD biosciences), anti-PD1-BUV737 (565299/612791, BD Pharmingen), anti-LAG3-BUV615 (752362, BD biosciences). Cells were fixed and permeabilized using the Transcription factor buffer set (562574, BD Pharmingen), following the manufacturer's instruction, and then stained for 40 min at 4°C with anti-NR4A2-AF647 sc-376984, Santa Cruz). Cells were then washed once with Perm/Wash, once with 1x PBS and resuspended in 150 µl of 1xPBS.

A fraction of the cells was acquired on flow cytometer to assess mCherry expression according to the engineered construct and the specific experimental setup. In Dynabeads-based activation experiments, CD8+ T cells were stained with anti-NGFR-BV421 (345131, BioLegend) and mCherry expression was analyzed within the LNGFR+ population to selectively identify transduced cells. For tumor co-culture and in vivo experiments, cells were first stained with LIVE/DEAD™ Near IR viability dye and subsequently labeled with anti-CD8-BUV805 (742030, BD Biosciences) and anti-TCR Vb13.1-VBR720 (130-131-138, Miltenyi Biotec). EGFP fluorescence was additionally acquired to enable gating on live CD8+ TCR+ EGFP+ cells, within which mCherry fluorescence was quantified.

Instrument	BD FACSCelesta™, model number 660345. FACSFortessa™, FACSSymphony™,
Software	FACSDiva version 8.0.1.1; FlowJo 10 version 10.9.0
Cell population abundance	Cells were acquired with a BD FACSCelesta™ cell analyzer (BD Biosciences) 48h after transfection/electroporation. For each

Cell population abundance

sample, >10000 singlet events were collected and a compensation matrix created using unstained cells (wild type) or single-color controls (EBFP only, EGFP only and mCherry only). The transfection efficiency was determined by the fluorescence acquisition of a transfection marker.

CD8⁺ T cells transduced with a lentiviral construct carrying a synthetic promoter driving mCherry expression were stimulated using activation beads or used in tumor co-culture and in vivo experiments, and collected at the indicated time points for flow cytometry analysis. For in vitro activation experiments, a minimum of 20,000 singlet events per sample were collected. For tumor co-culture and in vivo experiments, at least 10,000 singlet events per sample were acquired. A compensation matrix was generated using unstained cells and single-color/compensation beads controls, according to the experimental setup. Transduction efficiency and synthetic promoter (SP) activity were determined by mCherry fluorescence acquisition. Cell population abundance was quantified as the frequency of CD8⁺ T cells within the live population. In Dynabeads-based activation experiments, mCherry expression was analyzed within the LNGFR⁺ population to selectively identify transduced cells. In tumor co-culture and in vivo experiments, mCherry fluorescence was quantified within the engineered CD8⁺ TCR⁺ EGFP⁺ population. For in vivo experiments, each mouse was considered an independent biological replicate. Data analysis was performed using FlowJo (BD Life Sciences).

Gating strategy

For transfection/electroporation experiments: Population of live cells and single cells were selected according to FSC/SSC parameters, followed by a gate strategy selecting the fluorescent cells sequentially (see Supplementary Figure S5). Fluorescent protein expression was analyzed first selecting the live cell population followed by single cells. mCherry was then observed in the EGFP population (transfection marker). Data containing percentage and Geometric Mean were extrapolated from this analysis and used for calculation of SP basal and inducible activity. Data analysis was performed with FlowJo™ v10.9.0 Software (BD Life Sciences).

For CD8 T cells experiments: Population of live single cells was selected based on FSC/SSC parameters, followed by doublet exclusion. Dead cells were excluded using LIVE/DEAD™ viability dyes according to the experimental condition. For in vitro characterization experiments, gating was performed sequentially as follows: live cells → singlets → CD3⁺CD8⁺ T cells → engineered/transduced population (LNGFR⁺ or EGFP⁺_TCR Vβ13.1⁺, depending on the construct) → mCherry⁺ cells. For tumor co-culture and in vivo experiments, gating was performed as follows: live cells → singlets → CD8⁺ cells → EGFP⁺_TCR Vβ13.1⁺ engineered cells → mCherry⁺ SP cells. Expression of functional markers (including CD39, PD-1, TIGIT, ...) was analyzed within the gated EGFP⁺_TCR Vβ13.1⁺ engineered cells population. Intracellular transcription factors were quantified following fixation and permeabilization within the same gated population. Percentage and Mean Fluorescence Intensity (MFI) values were extrapolated from this analysis and used for calculation of SP activity (see corresponding Supplementary Figures).

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