nature portfolio

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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	Modeling protein structures: Rosetta (release-340);
Data analysis	Flow cytometry: Flowjo 10.8.1; BLI analysis: ForteBio analysis 9.1.0; GraphPad Prism 10; Structure visualization: PyMOL v2.5.2

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

All protein sequences are included in supplementary table 1. The crystal structure has been deposited in the Protein Data Bank with accession code 9DL1

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	We used cell lines originated from human HBL-1 - Female HLY-1 - Unspecified SUDHL4 - Male SUDHL5 - Female
Reporting on race, ethnicity, or other socially relevant groupings	HBL-1 - Unspecified HLY-1 - Unspecified SUDHL4: Caucasian SUDHL5: Caucasian
Population characteristics	N/A
Recruitment	We don't recruit people for this study. The donor primary cells were provided by Stanford Blood Center and the human Immunology Core at the University of Pennsylvania.
Ethics oversight	Blood related research has Biosafety Project approval by the Environmental Health & Safety Office at Stanford and approval from University of Pennsylvania review board. A written informed consent to participates in this study was provided by the participants.

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	2-3 biological replicates were performed, which was sufficient to show data reproducibility. No sample size calculations were performed.
Data exclusions	no data were excluded
Replication	pMHC titration on yeast is replicated three times for each TRACeR clone (Fig. 1e). CAR T experiment (Fig. 4c, Supplementary Fig. S24) were performed with primary T cells from 2 independent donors, which 4-5 technical replicates per donor per E:T ratio. Mice immunogenicity study (Fig. S12, S13) was performed with 8 biological replicates (8 mice per group) 4 biological replicates for naive mice (4 mice per group). BLI analysis was performed once for each reported interaction. Deep sequencing of yeast libraries were performed once per library. All other experiments if not specifically stated are replicated with 2-3 biological replicates.
Randomization	Randomization is needed for interventional trials. This study was an observational study without a therapeutic intervention and not an interventional study
Blinding	Blinding is needed to control for a placebo effect in interventional studies or for interpretation bias for subjective outcomes. Neither is applicable to this study

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

FITC labeled anti-cmyc Ab (Miltenyi biotech 130-116-485); 1:100 diliution ;
PE labeled anti-human Fc (biolegend 410707); 1:100 dilution
Anti-V5 (clone SV5-Pk1, produced in-house using Addgene #218107), 1 ug/m
TCR Vβ13.1 Antibody, anti-human, APC, REAfinity™ (Miltenyi Biotech 130-118-966) 1:100 dilution
FITC Mouse Anti-Human CD8 (BD 551347) 1:100 dilution
anti-CD16/CD32 (Clone 2.4G2, BioXCell BE0307) 10 ug/ml
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher L34957)1:500 dilution
Zombie UV Fixable Viability Kit (Biolegend 423107) 1:1000 dilution
CD44 BUV395 (clone IM7, BD Bioscience 568507) 1 ug/ml 1:200 dilution
Ly-108 BUV737 (Clone 13G3, BD Bioscience 741893) , 2 ug/ml 1:100 dilution
CD127 BV421 (Clone A7R34, Biolegend 135027) 2 ug/ml 1:100 dilution
CD27 BV510 (Clone LG.3A10, Biolegend 124229) 2 ug/ml 1:100 dilution
CX3CR1 BV605 (Clone SA011F11, Biolegend 149027), 2 ug/ml 1:100 dilution
CD4 BV785 (Clone RM4-5, Biolegend 100552), 2 ug/ml, 1:100 dilution
KLRG1 FITC (Clone 2F1, Biolegend 138410), 2 ug/ml 1:250 dilution
CD8 PE (Clone 53-6.7,, Biolegend 100708), 1 ug/ml 1:200 dilution
CD8 PerCP-Cy5.5 (Clone 53-6.7, Biolegend 100734), 1 ug/ml 1:200 dilution
CD69 PE/Cy7 (Clone H1.2F3, Biolegend 104512) , 2 ug/ml 1:100 dilution
CD3 AF700 (Clone 17A2, Biolegend 100216), 5 ug/ml 1:100 dilution
CD62L APC-eFluor 780 (Clone MEL-14, Invitrogen 47-0621-82), 1.25 ug/ml 1:160 dilution
V beta 8.1, 8.2 TCR FITC (Clone MR5-2, BD Biosciences 51-01344L) , 10 ug/ml 1:20 dilution
V beta 8.1, 8.2 TCR PE (Clone MR5-2, BD Biosciences 553186), 10 ug/ml 1:20 dilution
V beta 8.3 TCR FITC (Clone 1B3.3, BD Biosciences 51-09044L) , 10 ug/ml 1:20 dilution
V beta 8.3 TCR PE (Clone 1B3.3, BD Biosciences 553664), 10 ug/ml 1:20 dilution
CD107a BUV395 (Clone 1D4B, BD Biosciences 565533), 2 ug/ml 1:100 dilution
IFN-γ BV605 (Clone XMG1.2, Biolegend 505840) , 0.5 ug/ml 1:400 dilution
Ki-67 FITC (Clone 11F6, Biolegend 151212), 2 ug/ml 1:250 dilution
IL-2 PE (Clone JES6-5H4, Biolegend 503808), 0.5 ug/ml 1:400 dilution
TNFa APC (Clone MP6-XT22, Biolegend 506308) 0.5 ug/ml 1:400 dilution
CellTrace Yellow (Invitrogen Cat: C34567) 1:2000 dilution
CellTrace Violet (Invitrogen, Cat: C34557) 1:2000 dilution
Live/Dead NIR (Thermo Fisher, Cat: L10119) 1:500 dilution
anti-human CD8a – FITC (Clone SK1, Cat: 980908) 1:200 dilution

Validation

ll antibodies used throughout this study were validated by the vendors. Details in validation can be found
FITC labeled anti-cmyc Ab: https://www.miltenyibiotec.com/US-en/products/c-myc-antibody-anti-human-mouse-rat-sh1-26e7-1-3.html#conjugate=fitc:size=100-tests-in-200-ul
V beta 8.3 TCR PE (Clone 1B3.3, 10 g/ml, BD Biosciences) – 553664 https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/pe-hamster-anti-mouse-v-8-3-t-cell-receptor.553664
PE labeled anti-human Fc: https://www.biolegend.com/fr-lu/products/pe-anti-human-igg-fc-11933
Anti-V5: https://www.addgene.org/218107/
TCR Vβ13.1 Antibody, anti-human, APC, REAfinity™: https://www.miltenyibiotec.com/US-en/products/tcr-vb13-1-antibody-anti-human-reafinity-rea560.html#conjugate=vio-bright-r720:size=100-tests-in-200-ul
FITC Mouse Anti-Human CD8 : https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/fitc-mouse-anti-human-cd8.551347
anti-CD16/CD32 : https://bioxcell.com/invivomab-anti-mouse-cd16-cd32-be0307
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit:https://www.thermofisher.com/order/catalog/product/L34957
Zombie UV Fixable Viability Kit: https://www.biolegend.com/fr-fr/products/zombie-uv-fixable-viability-kit-9336
CD44 BUV395: https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/buv395-rat-anti-mouse-cd44.568507
Ly-108 BUV737: https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/buv737-mouse-anti-mouse-ly-108.741893
CD127 BV421: https://www.biolegend.com/nl-nl/products/brilliant-violet-421-anti-mouse-cd127-il-7ralpha-antibody-7193
CD27 BV510: https://www.biolegend.com/nl-nl/products/brilliant-violet-510-anti-mouse-rat-human-cd27-antibody-11907
CX3CR1 BV605: https://www.biolegend.com/nl-nl/products/brilliant-violet-605-anti-mouse-cx3cr1-antibody-12110
CD4 BV785: https://www.biolegend.com/nl-nl/products/brilliant-violet-785-anti-mouse-cd4-antibody-7954
KLRG1 FITC: https://www.biolegend.com/nl-nl/products/fitc-anti-mouse-human-klrg1-mafa-antibody-6865
CD8 PE: https://www.biolegend.com/nl-nl/products/pe-anti-mouse-cd8a-antibody-155

CD8 PerCP-Cy5.5:https://www.biolegend.com/nl-nl/products/percp-cyanine5-5-anti-mouse-cd8a-antibody-4255
CD69 PE/Cy7 : https://www.biolegend.com/nl-nl/products/pe-cyanine7-anti-mouse-cd69-antibody-3168
CD3 AF700: https://www.biolegend.com/nl-nl/products/alexa-fluor-700-anti-mouse-cd3-antibody-3375
CD62L APC-eFluor 780: https://www.thermofisher.com/antibody/product/CD62L-L-Selectin-Antibody-clone-MEL-14-Monoclonal/47-0621-82
V beta 8.1, 8.2 TCR FITC : https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/panels-multicolor-cocktails-ruo/anti-mouse-tcr-v-screening-panel.557004
V beta 8.1, 8.2 TCR PE (https://www.bdbiosciences.com/content/dam/bdb/products/global/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/553xxx/5531xx/553186_base/pdf/553186.pdf '
V beta 8.3 TCR FITC: https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/panels-multicolor-cocktails-ruo/anti-mouse-tcr-v-screening-panel.557004
V beta 8.3 TCR PE: https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/pe-hamster-anti-mouse-v-8-3-t-cell-receptor.553664
CD107a BUV395: https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/buv395-rat-anti-mouse-cd107a.565533
IFN-γ BV605: https://www.biolegend.com/ja-jp/products/brilliant-violet-605-anti-mouse-ifn-gamma-antibody-8114
Ki-67 FITC: https://www.biolegend.com/en-us/products/fitc-anti-mouse-human-ki-67-antibody-18405
IL-2 PE: https://www.biolegend.com/en-us/products/pe-anti-mouse-il-2-antibody-954
TNFa APC: https://www.biolegend.com/en-us/products/apc-anti-mouse-tnf-alpha-antibody-975
CellTrace Yellow: https://www.thermofisher.com/order/catalog/product/C34567
CellTrace Violet: https://www.thermofisher.com/order/catalog/product/C34557
Live/Dead NIR: https://www.thermofisher.com/order/catalog/product/L10119
anti-human CD8a – FITC: https://punchout.biolegend.com/en-gb/products/fitc-anti-human-cd8-19355

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	SUDHL-5: Jonathan Yewdell, NIAID SUDHL-4: Louis Staudt, NCI HBL-1: Louis Staudt, NCI HLY-1: Louis Staudt, NCI Drosophila melanogaster S2: Purchased from Thermo Fisher https://www.thermofisher.com/order/catalog/product/R69007?ef_id=EAIaIQobChMI25CM4NfPiQMVqGlBAh0r7wq2EAAYAiAAEgIXVPD_BwE:G:s&s_kwcid=AL!3652!3!533624062684!!!g!!!383441308!122660977177&cid=bid_clb_pex_r01_co_cp0000_pjt0000_bid00000_0se_gaw_dy_pur_con&s_kwcid=AL!3652!3!533624062684!!!g!!&gad_source=1&gbraid=0AAAADxi_GRDvBQT_-RRJsOWRVkB5mH0o&gclid=EAIaIQobChMI25CM4NfPiQMVqGlBAh0r7wq2EAAYAiAAEgIXVPD_BwE
Authentication	SUDHL5 was obtained directly from the ATCC. SUDHL4 was confirmed as 100% match to SUDHL4 via ATCC STR profiling. HBL1 and HLY1 were confirmed by a PCR-based copy number variant fingerprinting of 16 loci from genomic DNA (see Phelan et al. Nature 2018), but were not STR profiled.
Mycoplasma contamination	The cell lines have been routinely confirmed mycoplasma negative with the ATCC Mycoplasma PCR Detection kit (cat# 30-1012K).
Commonly misidentified lines (See ICLAC register)	None of the cell lines used belong to the commonly misidentified lines.

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	The animal studies received approval from the Institutional Animal Care & Use Committee of the Office of Animal Welfare at the University of Pennsylvania (protocol 803042). BALB/c mice were purchased from Jackson Labs (Strain #:000651). All animals used were female mice, between age 5-7 weeks.
Wild animals	No wild animals are used in this study
Reporting on sex	mouse, BALB/c, female, 5-7 weeks of age
Field-collected samples	No field-collected samples are used in this study
Ethics oversight	Mice were used under protocols approved by The animal studies received approval from the Institutional Animal Care & Use Committee of the Office of Animal Welfare at the University of Pennsylvania (protocol 803042).

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

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	Transformed yeast cells were grown in SDCAA media. For induction of expression, yeast cells were centrifuged at 2000g for 5 min and resuspended in SGCAA medium supplemented with 0.2% glucose at the cell density of 1e7 cells per ml and induced at 30 °C for 16-24 h. Cells were washed with PBSA (phosphate buffer saline (PBS) with 0.5% BSA) and labeled with pMHC tetramer/monomers, together with anti-c-myc fluorescein isothiocyanate (FITC, Miltenyi Biotech). After incubation for ~1 hr under room temperature, cells are washed twice and resuspended in PBSA, then run on Sony SH800 cell sorter.
Instrument	Sony SH800 sorter, BioRad Ze5
Software	Raw data were collected from the instrument supplied software. Data were analyzed by FlowJo X (10.9)
Cell population abundance	The cell population after sorting is gradually enriched. The abundance is monitored by the presence of double positive signals. Using yeast surface display as an engineering step does not require population analysis. We sequence everything that comes through by NGS.
Gating strategy	We used single labeled (on expression level) cells to establish the background threshold, and collect any cell that has higher signal than the background threshold. We do not use the collected double positive population for analysis, but just used for enrichment steps to isolate high affinity binders.

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