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Reporting Summary

Nature Research 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 Research policies, see Authors & Referees 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	Flow cytometry data acquisition was performed using BD FACS Diva software. Seahorse XFe96 Bioanalyzer (Agilent) was collected with Seahorse Wave software 2.6.1. Microscopy data was collected with Olympus cellSens software. Real time PCR data was collected with Applied Biosystems StepOne software. Sequencing was done using NextSeq 500 System.
Data analysis	Flow cytometry data analysis was performed using FlowJo Version 10 (Treestar) and graphed using Graphpad Prism version 8. Seahorse XFe96 Bioanalyzer (Agilent) was analyzed with Seahorse Wave software and graphed with Graphpad Prism version 8. Immunoblot and microscopy data were analyzed with ImageJ and graphed with Graphpad Prism version 8. Real time PCR data was analyzed with Applied Biosystems StepOne software and Microsoft Excel and graphed with Graphpad Prism version 8. Sequencing reads were trimmed for adapters using Cutadapt prior to being aligned to Mus musculus reference genome (mm10) using the RNA-seq aligner HISAT2. Subread's featureCounts function was used for gene level quantification and results were normalized to Transcripts Per Kilobase Million (TPM). Using the raw quantification, differential genes were found with the R package DESeq2, Thermo Compound Discoverer 3.0 software suite.

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/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research 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 list of figures that have associated raw data
- A description of any restrictions on data availability

RNA-seq data have been deposited to the Gene Expression Omnibus with the accession code GSE155192. Source data for Fig 3d,e, and Extended data 3a,b are

available in the GEO repository GSE122713 and source data for Extended data 9e,f are available in the GEO repository GSE109125. The data that support the findings of this study are available from the corresponding authors upon request.

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	No pre-determined statistics were used to dictate sample size; sample size was guided by previous experiments: Scharping et al 2016 Immunity, Menk et al 2018 J Exp Med, Scharping et al 2017 Cancer Immunol Res., Najjar et al 2019 Cancer Immunol Res. These sample sizes are sufficient because they allowed for the determination of statistical significance between groups and minimized the number of animals or replicates needed for each experiment.
Data exclusions	Data obtained from in vivo samples were excluded from analysis if mouse tumors were small at time of sacrifice (<6mm) or CD8+ TIL had no PD1+Tim3+ terminally exhausted cells (Figure 1g, 4h, 5b)
Replication	Multiple replicates were included in each experiment (at least 2 times), including both biological replicates in one experiment and replicating the experiment on different days. Replication attempts were successful.
Randomization	Mice or cells were randomly allocated to experimental groups.
Blinding	Blinding was not possible as most of the data acquisition and analysis was done by a single person. Blinding would have required at least two individuals for each experiment which was not feasible during the course of our 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
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

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

Antibodies

Antibodies used	Anti-CD8 (clone 53.6.7, 1:1000), anti-Tim3 (clone RMT3-23, 1:250), anti-PD1 (clone 29F.1A12, 1:500), anti-Lag3 (clone C9B7W, 1:200), anti-TIGIT (clone 1G9, 1:200), anti-CD39 (clone Duha59, 1:500), anti-IL2 (clone JES6-5H4, 1:250), anti-TCF1 (clone W16175A, 1:250), anti-CD44 (clone IM7, 1:500), anti-Thy1.1 (clone OX-7, 1:1000), anti-IFNγ (clone XMG1.2, 1:500) and anti-TNFα (clone MP6-XT22, 1:500) antibodies were obtained from BioLegend. Anti-Hif1α (clone 241812, 1:250) was obtained from R&D Systems. Anti-NFAT1 antibody (clone 25A10.D6.D2, 1:50), CV- ATP5A, CIII- UQCRC2, CII- SDHB from Total Rodent OXPHOS Rodent WB antibody cocktail (cat ab110413, 1:250) was obtained from Abcam. Anti-Tox (clone TXRX10, 1:250) and anti-Blimp1 (clone 5E7, 1:100) was obtained from Fisher. pAKT (S473) (clone D9E, 1:4000), pS6 (S235/236) (clone D57.2.2E, 1:1000), BNIP3 #3769 (1:1000), LCK #2752 (1:1000), pTyr-100 #9411 (WB 1:2000, flow 1:3200) was obtained from Cell Signaling. NDUFS4 (clone 1-E-4, 1:200) and beta-actin (clone C4, 1:2000) was obtained from Santa Cruz Biotechnology. In vivo hypoxia staining was detected with an antibody to pimonidazole (1:500), obtained from Hypoxyprobe. In vivo antibodies anti-PD1 (clone J43), anti-CTLA4 (clone 9H10), and isotype controls were obtained from Bio X Cell.
Validation	Antibodies were validated by manufacturers: Biolegend: ll newly developed clones at BioLegend undergo validation testing for multiple applications. This serves as a cross-check for specificity and provides clarity for research uses. Validate with proper controls: Endogenous positive and negative expressing cells, knockdown/knockout cell lines, Isotype controls, biological testing, multiple donor samples for human cells,

side-by-side comparsions of currently available clones. Each lot of these antibodies is quality control tested by immunofluorescent staining with flow cytometric analysis.
R&D Systems: Hif1a antibody tested via CoCl2 treated MCF7 human breast cancer cell line, fixed with paraformaldehyde, andpermeabilized with saponin
Abcam: We include relevant controls, routinely running unstained, positive, negative, isotype, viability, Fc-blocking, fluorescence minus one (FMO), and single-staining controls. For an FMO control, we stain all our samples with fluorescent conjugates except the one that is being tested. This shows the contribution of the other fluorescent conjugates in the signal of the unlabeled channel. This control is important for determining non-specific binding of an antibody. Isotype controls are a good negative control that allows us to determine background signal from the signal given by specific antibody binding. These controls use primary antibodies matching the isotype of the primary antibody you are validating but which do not have specificity for the target. We also use many KO cell lines in our flow cytometry validation whenever these are available. If an antibody gives a positive flow cytometry signal and passes all of our control experiements we will make this antibody available for purchase and state that it is suitable for use in flow cytometry.
Fisher: Undergo rigorous 2-part testing approach:
Part 1—Target specificity verification: This helps ensure the antibody will bind to the correct target. Our antibodies are being tested using at least 1 of the following methods to ensure proper functionality in researcher's experiments.
Knockout—expression testing using CRISPR-Cas9 cell models
Knockdown—expression testing using RNAi to knockdown gene of interest
Independent antibody verification (IAV)—measurement of target expression is performed using two differentially raised antibodies recognizing the same protein target
Cell treatment—detecting downstream events following cell treatment
Relative expression—using naturally occurring variable expression to confirm specificity
Neutralization—functional blocking of protein activity by antibody binding
Peptide array—using arrays to test reactivity against known protein modifications
SNAP-ChIP™—using SNAP ChIP to test reactivity against known protein modifications
Immunoprecipitation-Mass Spectrometry (IP-MS)—testing using immunoprecipitation followed by mass spectrometry to identify antibody targets
Part 2—Functional application validation: These tests help ensure the antibody works in a particular application(s) of interest, which may include (but are not limited to):
Western blotting
Flow cytometry
ChIP
Immunofluorescence imaging
Immunohistochemistry
Cell Signaling: CST Hallmarks of Antibody Validation Description:
Binary Model: Antibody signal is measured in model systems with known presence/absence of target signal. Includes wild-type vs. genetic knockout, targeted induction or silencing.
Ranged Expression: Antibody signal strength is measured in cell lines or tissues representing a known continuum of target expression levels. Includes siRNA and heterozygous knockout assays.
Orthogonal Data: Antibody signal is correlated to target expression in model systems measured using antibodyindependent assays. Includes mass spectrometry and in situ hybridization.
Multiple Antibodies: Antibody signal is compared to the signal observed using antibodies targeting nonoverlapping epitopes of the target. Includes IP, ChIP, and ChIP-seq.
Heterologous Expression: Antibody signal is evaluated in cell lines following heterologous expression of native (or mutated) target protein.
Complementary Assays: Antibody specificity may be validated using complementary assays. Includes competitive ELISA, peptide dot blots, peptide blocking, or protein arrays.
Hypoxyprobe: Antibody Reagents - Protein adducts of reductively-activated pimonidazole proved to be effective immunogens for the production of polyclonal and monoclonal antibodies. The antibodies have now been used in a variety of immunochemical analyses including
a) immunoperoxidase analysis of formalin fixed, paraffin embedded sections;
b) immunofluorescence analysis of frozen fixed sections;
c) cytometry with directly labeled or secondary fluorescent antibodies;
d) flow cytometry with directly labeled or secondary fluorescent antibodies;
e) Western blotting;
f) enzyme linked immunosorbent (ELISA)assays.
Antibodies to pimonidazole adducts are very robust. For example, aqueous solutions of the IgG1 monoclonal antibody against pimonidazole adducts (clone 4.3.11.3) are stable indefinitely when stored at -20oC and are stable for at least 4 months at 4oC when supplemented with 10 mg/mL of bovine serum albumin and 10 millimolar sodium azide. Pimonidazole adducts in vivo are robust lasting for days in hypoxic tissue. This feature provides flexibility in the timing of tissue harvesting which is an advantage in clinical studies.
Bio X Cell: Bio X Cell Products Features
Exceptional Purity Our optimized proprietary antibody manufacturing method ensures an ultra-pure antibody solution without added proteins or chemicals. Each lot is QC tested for purity using SDS-PAGE.
Ultra-low Endotoxin Levels The level of endotoxin is QC tested for each lot. Our InVivoMAb products are at or below 2EU/mg and InVivoPlus products are at or below 1EU/mg. If endotoxin levels below 1EU/mg are required, please simply contact our technical support for details.
Pathogen Free Each lot of InVivoPlus product is screened for an exhaustive panel of murine pathogens. The results are detailed on product-specific datasheets, to help you adhere to IACUC and Animal Facility requirements.
Advanced Binding Validation We utilize a library of recombinant proteins and our bioassay expertise to validate that each lot of applicable InVivoPlus antibody binds strongly and specifically to its target antigen.
Low Protein Aggregation Our proprietary antibody manufacturing method ensures an antibody solution with very low levels of protein aggregation. Additionally, each lot of InVivoPlus product is QC tested for aggregate level and guaranteed to be below 5% of the total protein.

Matching Isotype Control Antibodies Bio X Cell carries a wide selection of non-binding isotype control antibodies. This takes the guesswork out of finding the correct control for your antibody.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	B16-F10 and 293T were obtained from ATCC. B16-OVA (MO5) was obtained from P. Basse and L. Falo, originally generated by L. Falo. Plat-E was obtained from L. Kane. Ndufs4-deficient B16-F10 was generated by the Delgoffe lab.
Authentication	B16-F10, 293T, plat-E, and B16-OVA were not authenticated. Ndufs4-deficient B16-F10 was generated using transient transfection of Cas9-GFP and Ndufs4 targeted gRNA, single cell sorting of GFP+ cells, and authenticated via extracellular flux analysis and selection of a clone that had lost GFP expression and lacked Ndufs4 protein.
Mycoplasma contamination	Cell lines tested negative for Mycoplasma in 2015.
Commonly misidentified lines (See ICLAC register)	None of the cell lines used are listed in the ICLAC database.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	All mice were on C57/BL6 background and used at 6-8 weeks of age. Both male and female mice were used. C57/BL6, SJ/L (Thy1.1), Cd4Cre, Tg(TcraTcrb)1100Mjb/J (OT-I), B6.Cg-Thy1a/Cy Tg(TcraTcrb)8Rest/J (Pmel) and Hif1af/f mice were obtained from The Jackson Laboratory. Prdm1f/f mice were a gift from Amanda Poholek. E8I-CreERT2 GFP x Rosa26-LSL-TdTomato mice and spleens from Tg(Nr4a1-EGFP/cre)820Khog/J (Nur77-GFP reporter) mice were a gift from Dario Vignali. All mice were housed in specific pathogen free conditions at an ambient temperature 20-26°C and humidity of 30-70% with a 12:12 hour light:dark cycle prior to use.
Wild animals	Study did not involve wild animals.
Field-collected samples	Study did not include samples collected from the field.
Ethics oversight	Animal work was done in accordance with the Institutional Animal Care and Use Committee of the University of Pittsburgh.

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	Lymph node T cells were isolated from 6- to 8-week-old B16-bearing mice and mechanically disrupted. To obtain single-cell suspensions of tumor infiltrating lymphocytes, we injected whole tumors repeatedly using 20G needles with 2 mg/mL collagenase type IV, 2 U/mL hyluronidase (Dispase), and 10 U/mL DNase I (Sigma) in buffered RPMI with 10% FBS and incubated them for 25 min at 37°C. Tumors were mechanically disrupted between frosted glass slides and filtered to remove particulates, then vortexed for 30 seconds. For live flow, metabolic dyes were loaded into cells by culturing T cells in serum-free media at 37 degrees for 20 minutes, followed by antibody staining in FACS buffer (PBS+2%FBS) on ice. For intercellular transcription factor staining, surface staining was done with FACS buffer on ice, followed by 4% PFA fixation, then by nuclear staining (Foxp3 fix/permeabilization; Fisher) and intercellular antibodies were stained overnight using permeabilization buffer (Fisher). For cytokine staining after stim, surface staining was done with FACS buffer on ice, followed by 4% PFA fixation, then cytoplasmic staining (Cytofix/Cytoperm; Fisher) and intercellular antibodies were stained overnight using BD PermWash.
Instrument	Becton Dickinson LSR Fortessa
Software	FlowJo version 10
Cell population abundance	Sorted samples were sorted directly into lysis buffer and sort purity information was not obtained.

Gating strategy	Cells were gated on lymphocytes in FSC vs SSC, singlets on FSC-A vs FSC-W, then live CD8+ T cells by CD8 positive, Viability Dye negative.

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