nature portfolio

Corresponding author(s): Eduard Batlle, Holger Heyn and Alejandro Prados

Last updated by author(s): 23/05/2025

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	Histological images acquisition: NanoZoomer-2.0 HT C9600 scanner, Leica Inverted SP5 confocal microscope, PhenoImager HT. Ex-vivo histological images: NIKON LIPSI high content and high speed screening platform equipped with an Eclipse Ti2 inverted microscope, a Yokogawa W1 confocal spinning disk unit, a Prior stage and two Prime BSI Photometrics sCMOS cameras Flow Cytometry and cell sorting: BD FACS DIVA v8.0.1 Real Time PCRs: StepOne software v2.3. , QuantStudio™ Real-Time PCR Software Intravital bioluminescence imaging (BLI): Living image x4.5.2
Data analysis	We used R (v3.5.1), RStudio (v1.1.383) MASS R package, R package limma, drc R package, function hcluster of the amap R package. For image analysis, FIJI/ImageJ (v1.53d), Trackmate and have custom codes that are available upon request. In addition, we used QuPath (v0.5.1), GraphPad Prism (v10.2.3), FlowJo (v10.7), BD FACS DIVA, Sambamba (v0.5.9), Living image x4.5.2, StepOne software v2.3.

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

Expression arrays and RNA-seq data are available at Gene Expression Omnibus (GEO): GSE272376

Count matrices for single cell RNAseq experiments were deposited at Gene Expression Omnibus (GEO): GSE273148

Source Data are available in the on-line version of the paper. Computer code is available upon request. Further information and requests for resources and reagents should be directed to the corresponding author.

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	*Use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Indicate if findings apply to only one sex or gender; describe whether sex and gender were considered in study design; whether sex and/or gender was determined based on self-reporting or assigned and methods used.* *Provide in the source data disaggregated sex and gender data, where this information has been collected, and if consent has been obtained for sharing of individual-level data; provide overall numbers in this Reporting Summary. Please state if this information has not been collected.* *Report sex- and gender-based analyses where performed, justify reasons for lack of sex- and gender-based analysis.*
Reporting on race, ethnicity, or other socially relevant groupings	*Please specify the socially constructed or socially relevant categorization variable(s) used in your manuscript and explain why they were used. Please note that such variables should not be used as proxies for other socially constructed/relevant variables (for example, race or ethnicity should not be used as a proxy for socioeconomic status).* *Provide clear definitions of the relevant terms used, how they were provided (by the participants/respondents, the researchers, or third parties), and the method(s) used to classify people into the different categories (e.g. self-report, census or administrative data, social media data, etc.)* *Please provide details about how you controlled for confounding variables in your analyses.*
Population characteristics	*Describe the covariate-relevant population characteristics of the human research participants (e.g. age, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."*
Recruitment	*Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.*
Ethics oversight	*Identify the organization(s) that approved the study protocol.*

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	No statistical test was used to determined sample size upfront. Instead sample size was determined empirically according to previous knowledge of the variation in experimental setup. Treatment experiments were designed to have n >= 5 per group (1 or more cages). We used at least 4 mice per group for the in vivo settings, which is sufficient to detect meaningful differences based on historical record with similar experiments.
Data exclusions	No data from in vivo experiments were excluded, except for mice that were excluded if liver metastasis did not present proper engraftment four days after intra-splenic injections tracked with in vivo bioluminescence. Then, animals were randomized and treated as indicated in each experiment.

Replication	All data included in this manuscript have been reproduced in at least two separate experiments.
Randomization	For in vivo experiments, animals were randomized, so that each group received mice with similar bioluminescence levels.
Blinding	Treatments were assigned after injections. The person administrating the treatments was blinded to the treatment arms.

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

Only commercial antibodies have been used.
For IF/IHC, the following list was used:
- Primary antibodies: CD3 (Abcam, ab16669), αSMA-A488 (eBioscience, 53-9760-82), CD8 (Abcam , ab209775), Ki67 (BD Biosciences, 550609).
- Secondary antibodies: donkey anti-goat conjugated to Alexa 488/568/647 (Life Technologies A11055, A11057, A21447), donkey anti-rabbit conjugated to Alexa 488/568/647 (Life Technologies A21206, A10042, A31573) and donkey anti-mouse conjugated to Alexa 488/568/647 (Life Technologies A-21202, A10037, A31571)

Flow cytometry antibodies:
anti-mouse CD16/32 (clone 2.4G2) Tonbo Cat. No. 70-0161-U500
anti-mouse CD45-BV605 (clone 30-F11) BioLegend Cat. No. 103155
anti-mouse CD104b (PDGFRβ) - APC (clone APB5) Invitrogen Cat. No. 17-1402-82
anti-mouse CD326 (EpCAM) - BV605 (clone G8.8) Biolegend Cat. No. 118227
anti-mouse CD326 (EpCAM) – APC-Cy7 (clone G8.8) Biolegend Cat. No. 118227
anti-mouse CD31 (PECAM-1) - AF700 (clone 390) BioLegend Cat. No. 102443
anti-mouse CD3e – BV605 (clone 145-2C11) BioLegend Cat. No. 100351
anti-mouse CD4 – BV785 (clone GK1.5) BioLegend Cat. No. 100453
anti-mouse CD8 – BV711 (clone 53-6.7) BioLegend Cat. No. 100748
anti-mouse CD62L – PE-Cy7 (clone MEL- 14) BD Pharmingen Cat. No. 560516
anti-mouse CD44 – AF700 (clone G44-26) BioLegend Cat. No. 103026
anti-mouse CD279 – PE-Cy7 (PD-1) (clone 29F.1A12) BioLegend Cat. No. 135216
anti-mouse CD279 – BV785 (PD-1) (clone 29F.1A12) BioLegend Cat. No. 135215
anti-mouse CD183 (CXCR3) – APC (clone CXCR3-173) ThermoFisher Scientific Cat. No. 17-1831-80
anti-mouse CD355 (CRTAM) – PE-Cy7 (clone 11-5/CRTAM) BioLegend Cat. No. 142013
anti-mouse Ly-6A/E (Sca-1) AF700 (clone D7) BioLegend Cat. No. 108142
Anti-mouse CD64 – BV421 (clone X54-5/7.1) BioLegend Cat. No. 139309
anti-mouse I-A/I-E (MHC-II) (clone M5/114.15.2) BioLegend Cat. No. 107641
anti-mouse CD24 – BV711 (clone M1/69) Biolegend Cat. No. 563450
anti-mouse CD11c – BV785 (clone N418) BioLegend Cat. No. 117335
anti-mouse Ly6C – PerCp-Cy5.5 (clone HK1.4) eBioscience Cat. No. 45-5932-80
anti-mouse Ly6G – AF700 (clone 1A8) BioLegend Cat. No. 127622
anti-mouse CD11b – APC-Cy7 (clone M1/70) BioLegend Cat. No. 101225
anti-mouse Ki-67 (clone SolA15), AF488 eBioscience Cat. No. 53-5698-82
anti-mouse Ki-67 (clone SolA15) - BV421 eBioscience Cat. No. 404-5698-82
LIVE/DEAD™ Fixable Aqua ThermoFisher Scientific Cat. No. L34957
LIVE/DEAD™ Fixable Yellow ThermoFisher Scientific Cat. No. L34959

Validation

IF/IHQ and flow cytometry Antibodies were validated extensively by the provider (including westernblot analysis), used in multiple studies previously and confirmed by the authors for specific localization.

Extra validation was performed by comparing expression in internal controls (known negative populations).

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	Mouse tumor organoids were derived from genetic models in our lab (Tauriello et al., Nature 2018).
Authentication	Tumor organoids were genotyped frequently to avoid miss-annotations.
Mycoplasma contamination	Mycoplasma contamination was tested for weekly and was always negative.
Commonly misidentified lines (See ICLAC register)	N/A

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	Injection and transplantation of MTOs was perfomed in C57BL/6J hosts aged 7-8 weeks, sex matched with the organoid to be injected. Tgfbr2LoxP (B6.129-Tgfbr2tm1Karl/J; stock 012603), LysMCre (B6.129P2-Lyz2tm1(cre)Ifo/J; stock 004781), dLckCre (B6.Cg-Tg(Lck-icre)3779Nik/; stock 012837), Spp1-/- (B6.129S6(Cg)-Spp1tm1Blh/J; stock 004936), Ccr2CreERT2 (C57BL/6-Ccr2tm1(cre/ERT2,mKate2)Arte/Orl; stock EM:12698), and Rosa26mTmG (B6.129(Cg)-Gt(ROSA)26Sortm4 (ACTB-tdTomato-eGFP)Luo/J; stock 007676) mouse strains were obtained from The Jackson Laboratory. Mice were inbred on a C57BL/6J background and successive crosses were performed to combine alleles. The latter allele allowed us to detect recombination using the shift from tdTomato to eGFP expression. All mice were closely monitored by authors, facility technicians (during treatments and experiments) and by an external veterinary scientist responsible for animal welfare. Mice were maintained in a specific-pathogen-free (SPF) facility with a 12h light–dark cycle and given ad libitum access to standard diet and water.
Wild animals	N/A
Reporting on sex	Regarding Gender Dimension, there are no sex differences in the incidence or mortality from CRC worldwide (source: Globocan). Also, mouse tumor organoids (MTOs) were derived both from female and male quadruple mutant mice and we did not observe differences influenced by gender (Tauriello et al., 2018). In this work, we matched the sex of the syngeneic recipients with that of the origin of the tumor.
Field-collected samples	N/A
Ethics oversight	All experiments with mouse models were approved by the Animal Care and Use Committee of Barcelona Science Park (CEEA-PCB) and the Catalan government under protocols CEEA-PCB-14-000053, CEEA-PCB-14-000275, CEEA-PCB-14-000276, CEEA-PCB-14-000285, and CEEA-PCB-15-000075, all included in project 9162.

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

Plants

Seed stocks	*Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.*
Novel plant genotypes	*Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.*
Authentication	*Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.*

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	Blood was collected either by cardiac puncture at the time of sacrifice or through the mandibular vein, and transferred into a tube containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant (Monlab, JP-1501126). Red blood cells were then lysed using RBC lysis buffer (Invitrogen, 00-4300), incubating for 10 min at room temperature and then wash with PBS, according to the manufacturer's recommendations. Draining liver LNs were harvested and submitted to mechanical dissociation by passing through a 20µm cell strainer. Macrodissected liver metastases were chopped with scalpels. Subsequent enzymatic digestion was performed with LiberaseTM (Roche, 5401119001), 10mg/ml DNase I (Roche, 10104159001) in DMEM (Invitrogen, 41966-029) for 90 min at 37°C, in a shaking water bath. Digested tissue fragments were quenched, filtered through 70µm cell strainer and subsequently, washed with FACS Buffer (PBS with 1%BSA (Roche, 10735086001), 2mM EDTA (Sigma, E5134-500G), 0.05% NaN3 (Sigma, S2002-25G)).
Instrument	Flow cytometry analysis and cell separation were performed in a FACSAriaFusion flow cytometer (Beckton Dickinson).
Software	Data were analyzed using FlowJo software v10.7.
Cell population abundance	Sorted TME was obtained as DAPI-, Epcam- Sorted CAFs were obtained as DAPI-, Epcam-, CD31-, Pdgfrb+, CD45- Sorted TILs were obtained as DAPI-, Epcam-, CD31-, Pdgfrb-, CD45+, CD3+ Sorted TAMs were obtained as Aqua-, CD45+, Ly6G-, CD11b+, Cd11c+, MCH-II+, CD64+, CD24- Sorted blood T cells were obtained as DAPI-, CD45+, CD3+ and either CD4+/CD8- or CD4-/CD8+
Gating strategy	CD8+ TME-infiltrating T cells (see Extended Data Figure 1): CD8+ TILs were first gated based on FSC/SSC parameters and as CD3+. Then, cytotoxic TILs were gated as CD4-/CD8+. Naïve CD8+ TILs were defined as PD-1-negative and TPR as PD-1-positive. CXCR3 expression was higher in Naïve and TPR compared to PD-1hi cells. Then, PD-1hi cells were further checked for SCA1 and CRTAM expression. CRTAM expression was used to gate TTR cells. Finally, CRTAM- cells were classified as SCA1-positive TEFF and SCA1-negative TEX cells. CD8+ blood populations (see Figure 4): CD8+ T cells were first gated based on FSC/SCC parameters and as CD3+. Then, cytotoxic TILs were gated as CD4-/CD8+. Naïve T cells were gated as CD44- and CD62L mid to high. TEM were gated as CD62L-CD44+ and TCM as CD62L+CD44+. Transfered T cells (see Figure 4): Transfered T cells were first gated based on FSC/SCC parameters and as CD3+. Then, cells were defined as Tgfbr2WT/TOM based on a Tomato+GFP- gating, and Tgfbr2dLck-KO/GFP based on a Tomato-GFP+ gating. Immune Populations (see Extended Data Figure 5): Immune cells were gated based on FSC parameter and as CD45+. Neutrophils were gated as CD11b+/Ly6G+. From the Ly6G- population, CD11b-/CD11c- were labeled as lymphocytes and further separated as MCH-II+/CD24+ B cells and MCH-II-/CD24- T cells. The rest of the Ly6G- population, is checked MHC-II and CD24. From MHC-II-/CD24- cells, monocytes are further defined as CD64+/Ly6C+. Finally, MHC-II-/CD24- cells are further divided into CD64+ macrophages and CD24+ DCs.

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