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Corresponding author(s): Dario AA Vignali

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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	For flow cytometry data collection, we utilized BD LSRIIFORTESSA with DIVA software, and exported the data in fcs3.0 version. RNAseq data collection was performed according to the instruction of illumina on NextSeq500 system. For single cell RNAseq, cDNA library preparation was performed using and following the instruction of the 10X Genomics platform and the sequencing data was collected on NextSeq500 system.
Data analysis	For flow cytometry data analysis, we utilized FlowJo (version 10 (most recently updated to 10.5.3 for Mac)) and numerical data exported to Excel sheet were further analyzed by using GraphPad Prism (version 7 and 8 for Mac) to generate graphical representation and for ANOVA statistical analysis. Furthermore, comma separated value (csv) files exported from Excel sheet upon multi-parameter flow cytometry analysis was import to SPICE program (NIH-NIAID) to generate SPICE plots to visually represent the expression level and co-expression combination of multiple inhibitory receptors analyzed. For Treg in vitro suppression assay, FlowJo legacy version 9 for Mac was used in order to utilize the proliferation analysis module. RNASeq data were aligned to the mm10 genome using the STAR aligner,and quantified against the Refseq gene models using featureCounts. The raw data counts were processed for differential expression using the “voom” function in the limma R package, with the robust model option. Gene set enrichment testing was performed using the “RankSumWithCorrelation” function in the limma R package, which automatically corrects enrichment statistic inflation due to correlation among genes. We used the GSEA-style enrichment score for visualization of pathway enrichment results. For single cell RNAseq data analysis, we used Cell Ranger (v2.0) (10X Genomics) to analyze sequencing data generated from Chromium Single Cell 3’ RNA-seq libraries40. We first ran “cellranger mkfastq” on the Illumina BCL output folder to generate fastq files. We next generated the UMI count matrix with “cellranger count” for each library. The data were normalized with R package cellrangerRkit and visualized via t-distributed stochastic neighbor embedding (t-SNE). The diffusion pseudo-time analysis was run with R package Destiny41,42. Briefly, we built a transition matrix for all cells based on their adjacency using a locally-scaled Gaussian kernel and determined the diffusion components according to the eigenvectors of the matrix. Then, a new matrix M was generated by removing the first eigenvalue of the original transition matrix and the diffusion pseudo-time was calculated as a distance metric between the rows of

M. Lastly, we plotted the trajectory lines, which were fitted by the locally weighted scatterplot smoothing (LOESS) regression using the previously calculated values. For the differential expression analyses between the IL10 and Ebi3 gene-expressing cells in LN and Tumor Tregs, we used the "sseq" method from the standard R package CellrangerRkit.

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

Bulk RNAseq and single cell RNAseq data will be deposited in the Gene Expression Omnibus (GEO), and the accession codes will be provided. Other data are available from the corresponding authors upon appropriate and reasonable 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	The numbers of animals in experiments are determined by formal statistical procedures based on preliminary or internal pilot data. For example, the mean growth rate of the Foxp3Cre-YFP mice (considered WT in this manuscript) were estimated by applying a mixed-effects linear model to the log-transformed tumor volumes; this is similar to (but not exactly the same as) fitting a line to each animal's log (tumor volume) versus day data, and treating the per-animal slopes as the analytic variable. From the mixed model, we determined that the mean growth rate of the Foxp3Cre-YFP animals was 0.100, and that the standard error of that estimate was 0.007 (on 250 degrees of freedom). We also determined that the estimate of the standard error was consistent across the other experimental groups. From this, we can determine the number of animals required to test the null hypothesis of no difference between growth rates of different experimental groups when the true difference in growth rates is any given percentage (α =0.05, 80% power).
Data exclusions	No data were excluded from the analyses.
Replication	Replications were successful once the experimental conditions were first optimized by pilot experiments.
Randomization	For all the experiments used in the study, we prepared minimum of 4-6 breeder pairs from which we took litters randomly that were age matched within 1 week between the WT and other mutant groups. For flow cytometry and tumor growth curve experiments, we analyzed each of individual mice separately so that any variation that may exist within a group can be truthfully reported in an unbiased manner. For both bulk and single cell RNAseq experiments, we pooled tumors and lymph nodes within groups for each experimental replicate, and the cells of interest were purify by using BD Aria sorter.
Blinding	For tumor growth curves, the measurement was conducted by a blinded, second personnel for the duration of each experiment.

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

CD90.1 BV650 (OX-7, Biolegend 202533, lot# B212183, dilution factor = 1:1500), CD90.2 APC/Cy7 (30-H12, Biolegend 105328, lot# 201135, dilution factor = 1:1500), TCRb BV605 (H57-597, Biolegend 109241, lot# B256848, dilution factor = 1:500), CD4 BUV395 (GK1.5, BD Biosciences 563790, lot# 8213920, dilution factor = 1:500), CD8a BUV737 (53-6.7, BD Biosciences 564297, lot# 8032815, dilution factor = 1:500), PD-1 (RMP1-30, Biolegend 109110, lot# B235159, dilution factor = 1:400), TIM3 APC (RMT3-23, eBioscience 17-5870-82, lot# 4342179, dilution factor = 1:300), LAG3 eFluor450 (eBioC9B7W, eBioscience 48-2231-82, lot# 4295329, dilution factor = 1:300), TIGIT PerCP-eFluor710 (GIGD7, eBioscience 46-9501-82, lot# 4274784, dilution factor = 1:300), 2B4.2/CD244.2 APC-Vio770 (REA388, Miltenyi 130-105-991, lot# 5180219484, dilution factor = 1:100), Foxp3 FITC (FJK-16s, eBioscience 11-5773-82, lot# 4340671, dilution factor = 1:200), BLIMP1 Alexa Fluor647 (5E7, Biolegend 150004, lot# B222216, dilution factor = 1:50), IL7R/CD127 PE-Cyanine7 (A7R34, eBioscience 25-1271-82, lot# E07599-1635, dilution factor = 1:250), TCF1/TCF7 Pacific Blue (C63D9, Cell Signaling 9066S, ref# 11/2018, dilution factor = 1:100), CD27 PE-Cyanine7 (LG.3A10, Biolegend 124216, lot# B253693, dilution factor = 1:300), CD80 BUV737 (16-10A1 , BD Biosciences 564670, lot# 8074730, dilution factor = 1:300), CD86 APC (GL-1, Biolegend 105012, lot# B185439, dilution factor = 1:300), PD-L1 (CD274)PE-Cyanine7 (MIH5, eBioscience 25-5982-82, lot# 4341651, dilution factor = 1:400), MHC-II (I-Ab) APC-efluor780 (M5/114.15.2, eBioscience 47-5321-82, lot# 4310394, dilution factor = 1:400), CD226 (DNAM-1) APC (10E5, eBioscience 17-2261-80, lot# 1923189, dilution factor = 1:300), CD4 Biotin (RM4-5, Biolegend 100508, lot# 13192084, dilution factor = 1:500), CD11b Biotin (M1/70, Biolegend 101204, lot# 8241116, dilution factor = 1:300), CD11c Biotin (N418, Biolegend 117304, lot# B253707, dilution factor = 1:300), CD19 Biotin (6D5, Biolegend 115504, lot# B258381, dilution factor = 1:500), CD25 Biotin (PC61, Biolegend 102004, B229183, dilution factor = 1:500), CD45R/B220 Biotin (RA3-6B2, Biolegend 103204, lot# B254523, dilution factor = 1:500), gdTCR Biotin (eBioGL3, eBioscience 13-5711-85, lot# 4335132, dilution factor = 1:500), CD49b Biotin (DX5, eBioscience 13-5971-85, lot# 4295252, dilution factor = 1:500), TER-119 Biotin (TER-119, Biolegend 116204, 13256623, dilution factor = 1:500), I-Ab Biotin (KH74, Biolegend 115303, lot# B257680, dilution factor = 1:300), CD16/32 Biotin (93, eBioscience 13-0161-82, lot# E02430-1631, dilution factor = 1:500), CD105 Biotin (MJ7/18, Biolegend 120404, lot# 4344738, dilution factor = 1:500), STAT1 (D1K9Y, Cell Signaling 14994S, ref# 04/2018), STAT3 (D3Z2G, Cell Signaling 9139S, ref# 05/2018), STAT4 (C46B10 Cell Signaling 2653S, ref# 05/2018), CD44 FICT (IM7, Biolegend 103006, lot# B200236, dilution factor = 1:800), KLRG1 PerCP-eFluor710 (2F1, eBioscience 46-5893-82, lot# 4273256, dilution factor = 1:300), CD206 APC (C068C2, Biolegend 141707, lot# B253486, dilution factor = 1:500), CD62L APC (MEL-14, Biolegend 104412, lot# B223862), BrdU APC (Bu20A, eBioscience 17-5071-42, lot# 4319920, dilution factor = 1:100), Ki67 PerCP-Cy5.5 (B56, BD Biosciences 561284, lot# 7345700, dilution factor = 1:70)

Validation

All the antibodies used in our study are well-validated antibodies that are commercially readily available. In addition to commercial vendors' staining validation data contrasting to the isotype IgG stained counterparts, we have also carefully monitored non-specific binding of those antibodies by examining cells that do not express the antigen.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

B16F10 cell line (referred to as B16 throughout the manuscript) and EL4 T cell lymphoma (referred to as EL4 in the manuscript) were purchased from ATCC (Manassas, Virginia). B16-OVA cell line and BrafPten (clone24) cell line were kindly provided by Greg Delgoffe (University of Pittsburgh). BrafPten (clone 24) was originally generated by A.V. Menk and G.M. Delgoffe by cloning a cell line from a tumor harvested from BRafCA.PtenL/L.TyrCreERT2 mouse induced by 4-OHT administration.

Authentication

Those cell lines are not subsequently authenticated by ourselves.

Mycoplasma contamination

The cell lines used in this study have been tested for and are free of mycoplasma contamination.

Commonly misidentified lines
(See ICLAC register)

No commonly misidentified cell lines were used in this study.

Animals and other organisms

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

Laboratory animals

We utilized the following mouse models for the study:
Unless otherwise specified, all experimental procedures were performed on 5-8 week old laboratory mice housed in Helicobacter and MNV free SPF facilities at University of Pittsburgh in accordance with guideline of National Institute of Health (NIH) under the approved protocol and guideline from the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Female mice were used for tumor growth and RNA sequencing experiments, while both male and female mice were used for tumor-infiltrating lymphocyte flow cytometry analysis.
Il10GFP.Ebi3Tom.Foxp3Cre-YFP mice were generated by crossing Il10GFP (Vert-X; Jackson Laboratory) to Ebi3Tom.Foxp3Cre-YFP (developed in our laboratory). Ebi3L/L-Tom mice were developed in our laboratory and have been previously described. Il10L/L (provided by Werner Muller (Germany)) and Ebi3L/L-Tom were crossed to Foxp3Cre-YFP mice to generate Treg cell-specific cytokine deletion strains. Prdm1YFP and Prdm1L/L strains were provided by Amanda Poholek at University of Pittsburgh. E8ICre mice (provided by Dan R. Littman, New York University; previously described) and OT-I transgenic mice (obtained from Jackson Laboratory) were crossed to Prdm1L/L and Prdm1YFP strains, respectively, to generate E8ICre.Prdm1L/L and Prdm1YFP.OT-I mice. Rag1–/– and IL-10R–/– (Il10rb–/–) mice were purchased from Jackson Laboratory. IL-35R–/– (CD4Cre.Il6stL/L.IL12rb2–/–) mice were generated as described previously.

Wild animals	No wild animals were used in this study.
Field-collected samples	No field-collected sampled were used in this study.
Ethics oversight	All experimental procedures performed on mice used in this study are in accordance with guideline of National Institute of Health (NIH) under the approved protocol and guideline from the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh.

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

Human research participants

Policy information about studies involving human research participants

Population characteristics	All samples were from non-small cell lung cancer patients (age range of 45-80) with either adeno or squamous cell carcinoma (early stage—I and II) and no prior immunotherapy. Solid primary tumors were collected via surgical resection and were placed into sterile collection media (RPMI+ 10%FBS+ 1%Penstrep) and kept on ice until further dissection (1-2 hours post-surgery). Peripheral blood (PBL) was drawn pre-operatively into EDTA tubes and was kept on a rocker at room temperature until processing (1-2 hours post collection). Tumor-infiltrating lymphocytes and peripheral blood mononuclear cells were collected via Ficoll gradient and cryopreserved in FBS containing 10% DMSO.
Recruitment	Subjects were first approached by their treating surgeon, radiologist, pulmonologist or medical oncologist who determined their willingness to participate in research studies. If a patient was willing to be a subject, a complete explanation of the research was given to them by a research coordinator and informed consent was obtained and documented. The goal of the research study and its methods was discussed and all pages of the informed consent document was reviewed with the subject and any questions answered. Once there was a thorough understanding by the subject of the contents of the consent form and a reiteration of the nature of the research study, the patient was asked to sign and date the form. A signed and dated copy, including the signature of the person obtaining the consent was given to the patient at the time consent was obtained, and the subject was again informed that participation would not influence medical care and that withdrawal was possible at any time. There were no self-selection bias or other biases that would have impacted results as the research group received all patient samples in a de-identified manner.
Ethics oversight	All specimens were acquired under University of Pittsburgh approved Institutional Review Board (IRB) protocol, and informed consent was obtained from all patients.

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	For mouse specimen processing: To obtain single cell suspension samples for flow cytometry analysis, the samples were processed as following: Spleens or lymph nodes were processed through 40µm cell strainers to obtain single suspension. Tumor samples were first mechanically minced into small pieces with scissors, followed by enzymatic digestion with Collagenase IV and Dispase in complete RPMI1640 medium for 30 minutes. Digested tumors were then processed through 70µm cell strainer. Red blood cells in spleens and tumors were lysed with ammonium chloride solution. All the samples were first stained with live/dead discrimination dye in PBS prior to antigen staining in FACS buffer containing 5% FBS and 5% normal mouse serum. For human specimen processing: All specimens were acquired under a University of Pittsburgh approved and exempt Institutional Review Board (IRB) protocol with a non human subjects designation as all specimens were collected in a blinded fashion, and informed consent was obtained from all patients and healthy donors. We collected peripheral blood from healthy donor to obtain PBL samples, and human tumor samples were collected from patients with non-small cell lung carcinoma (NSCLC). For enzymatic digestion, Liberase (Sigma Aldrich) was used at 50 µg/mL for 15 minutes at 37°C in RPMI medium (Lonza) to release tumor infiltrating lymphocytes (TIL). For healthy donor peripheral blood samples, Ficoll Paque Plus (GE Healthcare) was used to separate peripheral blood leukocytes (PBL) and lysis buffer (BD biosciences) was used to remove red blood cells when necessary. Lung TIL and HD PBL were incubated in complete RPMI medium (10% FBS (Atlanta biologicals), non-essential amino acids (Sigma Aldrich), L-glutamine (Lonza) and penicillin/streptomycin (Corning) and sodium pyruvate (Sigma Aldrich)) with TCR stimulation: plate-bound anti-CD3 (0.5 µg/mL, Invitrogen, clone: OKT3) and soluble anti-CD28 (1 µg/mL Invitrogen, clone: CD28.2) and 200U/ml rhIL-2 overnight followed by four hours of stimulation with PMA/ionomycin and GolgiStop. The cells were then harvested and stained with surface antibodies (CD3, CD4, CD8, CD25) and viability dye. A fixation/permeabilization kit (eBiosciences) was used

	according to manufacturer's protocol, followed by intracellular staining.
Instrument	BD LSRFORTESSA for flow cytometry analysis and BD Aria sorter for cell purification.
Software	FlowJo was used to gate the target populations, and Microsoft Excel, Graphpad Prism, and SPICE were used for further analyses.
Cell population abundance	For RNAseq experiments where cells were purified by FACS-sorting, we ensured the purity of the sorted samples by sequential double-sorting. The purity was determined by re-analyzing the double-sorted samples on the same instrument (BD FACSAria). All the fractionated subpopulations used for RNAseq experiments were uniformly 500 cells per sample, which was directly sorted into 96-well plate containing lysis buffer for cDNA synthesis purpose. For adoptive transfer experiments, we purified the target cells by streptavidin-magnetic bead negative selection. The purity of the isolated cells was determined on BD LSRFORTESSA prior to transfer.
Gating strategy	For all the experiments involving flow cytometry (either for analyzing or FACS-sorting), the following gating strategy was applied to eliminate non-specifically stained cells. First, lymphocyte gate was applied on the SSC-A/FSC-A window. The gating size was determined by comparing the lymphocyte cluster from lymph node samples to the matched tumor samples in order to adjust for the blasted tumor infiltrating lymphocyte cell size. Subsequently, sequential singlet/doublet discrimination was applied via SSC-W/SSC-H and FSC-W/FSC-H. From the selected singlets, dead cells were discriminated out by gating on live/dead dye negative population. For our study is focused on tumor infiltrating T cells, we have subsequently gated on TCRb+CD4+CD8- or TCRb+CD8+CD4- for further analysis. In order to set the gating for positive populations, we used unstained samples and also stained lymph node samples for inhibitory receptor staining as lymph node counterparts do not express them compared to tumor infiltrating lymphocytes.

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