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# Reporting Summary

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## 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<br>*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<br>*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 were collected on an LSR II cytometer using FACS Diva v8.0 (BD) or an Aurora cytometer (Cytek) using SpectroFlo v2.2.0.3 (Cytek).<br>ELISA data were recorded with Synergy HTX plate reader (BioTek).<br>Bulk RNA-seq libraries were sequenced on HiSeq 4000 (Illumina).<br>scRNA-seq libraries were sequenced on an NovaSeq 6000 System (Illumina).<br>TCR-seq libraries were sequenced on MiSeq (Illumina). |
| Data analysis | Flow cytometry data were analyzed using FlowJo v10.6.1 (BD).<br>ELISA data were calculated with Gen5 3.02.2 (BioTek).<br>Statistical analyses of biological experiments were performed using Prism v7.0.<br>For the bulk RNA-seq experiment, STAR aligner v2.7.3a and Genome Analysis Toolkit v4.1.4.1 were used for alignment, R v4.0.2 was used for measuring the raw count of reads per gene, and the DESeq2 R package v1.28.1 was used to perform differential gene expression analysis.<br>For scRNA-seq, fastq files were processed using Cell Ranger v3.0 (10x Genomics), MAGIC v0.1.1 was used for imuptation, Palantir v1.0.0 was used for trajectory analysis, PhenoGraph v1.5.7 was used for clustering, and Scanpy v1.7.2 on Python v3.6.10 was used for calculating differentially enriched genes. Seurat v3.1.5 was used for calculating module scores.<br>TUNEL staining was quantified with ImageJ v2.0.0-rc-69/1.52p.<br>For TCR-seq, fastq files were aligned with MiXCR v3.0.13. The clonotypes were analyzed with VDJtools v1.2.1. |

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 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

All sequencing data generated in this study can be accessed at GEO under accession number GSE179710. The custom mouse genome used for scRNA-seq analysis, which was generated by adding the tdTomato sequence to GRCm38 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001635.20/), is also available at GEO. The following GSEA gene sets were used for data analysis: GO_CELL_CYCLE_G1_S_PHASE_TRANSITION, GO_CELL_CYCLE_G2_M_PHASE_TRANSITION, GO_DNA_REPLICATION, HALLMARK_APOPTOSIS, HALLMARK_FATTY_ACID_METABOLISM, HALLMARK_G2M_CHECKPOINT, HALLMARK_GLYCOLYSIS, HALLMARK_IL2_STAT5_SIGNALING, HALLMARK_OXIDATIVE_PHOSPHORYLATION, HALLMARK_PI3K_AKT_MTOR_SIGNALING, HALLMARK_REACTIVE_OXYGEN_SPECIES_PATHWAY, HALLMARK_TGF_BETA_SIGNALING, HALLMARK_WNT_BETA_CATENIN_SIGNALING, KEGG_CITRATE_CYCLE_TCA_CYCLE, KEGG_PURINE_METABOLISM, REACTOME_EUKARYOTIC_TRANSLATION_INITIATION.

# 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 sample size calculation was performed. Sample size was determined according to common practice in the field of immunology and prior experience. Whenever possible at least 2 independent biological replicates were analyzed. In vivo experiments were performed at least twice to ensure reproducibility. |
| Data exclusions | In the bulk RNA-seq experiment, three biological replicates of each cell type were generated but Treg “wannabes” ended up being represented by only two because the third replicate seemed to be an outlier based on principal component analysis. |
| Replication | All in vivo experiments were repeated at least twice and all attempts at replication were successful. |
| Randomization | Mice were grouped according to genotype and all experiments were performed with sex-matched littermates. |
| Blinding | For pathology scoring, the pathologist was blinded from the genotype and treatment of the mice. For other experiments, group allocation was not applicable because mice were grouped based on and compared across different genotypes. Blinding was not possible because mice of different genotypes have vastly distinct disease manifestation and cellularity, which need to be accounted for during the sample preparation process to ensure technical soundness. |

# 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 |
| ☒ | ☐ Human research participants |
| ☒ | ☐ Clinical data |
| ☒ | ☐ Dual use research of concern |

### Methods

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

## Antibodies

| | |
|---|---|
| Antibodies used | Flow cytometry (clone, vender, catalog number, dilution): anti-Siglec-F (E50-2440, BD, 562681, 400), anti-I-A/I-E (M5/114.15.2, BioLegend, 107641, 1000), anti-NK1.1 (PK136, ThermoFisher, 47-5941-82, 200), anti-CD45 (30-F11, BioLegend, 103136, 600), anti- |

CD11b (M1/70, BioLegend, 101257, 800), anti-CD3e (17A2, BioLegend, 100237, 500), anti-Ly-6C (HK1.4, BioLegend, 128037, 1000), anti-CD90.2 (30-H12, BioLegend, 105331, 1000), anti-Foxp3 (FJK-16s, ThermoFisher, 17-5773-82, 400), anti-CD19 (6D5, BD, 563557, 400), anti-Ly-6G (1A8, BioLegend, 127618, 500), anti-TCRb (H57-597, ThermoFisher, 61-5961-82, 400), anti-F4|80 (BM8, ThermoFisher, 61-4801-82, 200), anti-CD90.1 (HIS51, ThermoFisher, 17-0900-82, 200), anti-CD4 (RM4-5, BioLegend, 100553, 400), anti-CD8a (53-6.7, BioLegend, 100759, 400), anti-GITR (DTA-1, ThermoFisher, 48-5874-82, 400), anti-CD73 (TY/11.8, BioLegend, 127215, 400), anti-CD44 (IM7, BD, 563971, 400; ThermoFisher, 48-0441-82, 400; BioLegend, 103049, 400), anti-CD103 (M290, BD, 564322, 200), anti-CD62L (MEL-14, BioLegend, 104438, 1600; BioLegend, 104441, 400), anti-CTLA4 (UC10-4B9, BioLegend, 106314, 600), anti-Helios (22F6, BioLegend, 137218, 400), anti-Ki-67 (16A8, BioLegend, 652420, 400), anti-CD25 (PC61.5, ThermoFisher, 47-0251-82, 400; ThermoFisher, 17-0251-82, 400), anti-PD-1 (29F.1A12, BioLegend, 135225, 400), anti-ICOS (7E.17G9, ThermoFisher, 12-9942-82, 400), anti-CD45.1 (A20, BioLegend, 110738, 100), anti-CD45.2 (104, BD, 612778, 200), anti-IL-2 (JES6-5H4, BioLegend, 503818, 400), anti-IL-17A (17B7, ThermoFisher, 48-7177-82), anti-IFNg (XMG1.2, ThermoFisher, 17-7311-82, 500), anti-IL-4 (BVD6-24G2, ThermoFisher, 25-7042-82, 400), anti-GARP (YGIC86, ThermoFisher, 25-9891-82, 200), anti-CD119 (2E2, BD, 550482, 100; GR20, BD, 740032, 100), anti-EpCAM (G8.8, BioLegend, 118227, 100; BD, 740281, 100), anti-CD124 (mIL4R-M1, BD, 742172, 100), anti-TCR Vb5.1, 5.2 (MR9-4, BioLegend, 139508, 400), anti-TCR Vb6 (RR4-7, ThermoFisher, 46-5795-82, 300), anti-TCR Vb8 (F23.1, BD, 553860, 400), streptavidin (BioLegend, 405225, 4000; ThermoFisher, 61-4317-82, 4000), anti-CD45R/B220 (RA3-6B2, ThermoFisher, 47-0452-82, 400).
ELISA capture: purified anti-mouse IgE (R35-72, BD Pharmingen, 553413), Goat Anti-Mouse IgG1 (RRID: AB_2794408, SouthernBiotech, 1070-01), Goat Anti-Mouse IgG3 (RRID: AB_2794567, SouthernBiotech, 1100-01), Goat Anti-Mouse IgG2a (RRID: AB_2794475, SouthernBiotech, 1080-01), Goat Anti-Mouse IgG2b (RRID: AB_2794517, SouthernBiotech, 1090-01), Goat Anti-Mouse IgG2c (RRID: AB_2794464, SouthernBiotech, 1079-01), Goat Anti-Mouse IgA (RRID: AB_2314669, SouthernBiotech, 1040-01), Goat Anti-Mouse IgM (RRID: AB_2794197, SouthernBiotech, 1020-01), Mouse Pentraxin 2/SAP Antibody (R & D Systems, MAB2558).
ELISA detection: Goat Anti-Mouse Ig-HRP (RRID: AB_2728714, SouthernBiotech, 1010-05), Biotin Rat Anti-Mouse IgE (R35-118, BD Pharmingen, 553419), Biotinylated Pentraxin 2/SAP Antibody (R & D Systems, BAF2558).
ELISA standard curve: Purified Mouse IgE, kappa, Isotype Control (C38-2, BD Pharmingen, 557079), Purified Mouse IgA, kappa, Isotype Control (M18-254, BD Pharmingen, 553476), Purified Mouse IgG3, kappa, Isotype Control (A112-3, BD Pharmingen, 553486), Purified Mouse IgG1, kappa, Isotype Control (15H6, SouthernBiotech, 0102-01), Purified Mouse IgG2a, kappa, Isotype Control (UPC-10, Sigma, M5409), IgM Isotype Control from murine myeloma (MOPC 104E, Sigma, M5909), IgG2b Isotype Control from murine myeloma (MOPC-141, Sigma, M5534), Mouse IgG2c (6.3, RRID: AB_2794064, SouthernBiotech, 0122-01).

Validation

All above antibodies are well validated commercial clones or preps routinely QC'ed by the manufacturer. Please refer to the spec sheets on the respective vendors' websites for technical information and detail by searching with the catalog numbers provided.

## Animals and other organisms

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

Laboratory animals

Male or female mice on the C57/BL6 background housed under SPF condition at an average ambient temperature of 21.5C and an average humidity of 48% were used in this study. Male Foxp3-LSL and Foxp3-DTR-GFP mice was treated at 2 weeks of age, and monitored for up to 7 months afterwards. Female Foxp3-LSL/WT, Foxp3-LSL/DTR-GFP, and Foxp3-DTR-GFP/WT mice were used at 2 weeks or 8-10 weeks of age as described in the manuscript.

Wild animals

No wild animals were used in this study.

Field-collected samples

No field-collected samples were used in this study.

Ethics oversight

Experiments in this study were approved by the Sloan Kettering Institute (SKI) Institutional Animal Care and Use Committee under protocol 08-10-023.

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

Animals were perfused with a total of 20 mL PBS into both left and right ventricles immediately after euthanasia. Cells were retrieved from spleens, peripheral (brachial, axillary and inguinal) lymph nodes, mesenteric lymph nodes, thymuses, and livers by meshing the organs through a 100 um strainer (Corning, 07-201-432) with a syringe plunger. Cells in the colonic lamina propria were isolated as previously described (Campbell et al. Immunity 2018). Briefly, colons were cleaned by flushing the luminal content out with PBS using a syringe, defatted, opened up longitudinally and diced into 1-2 cm pieces. Tissues were then incubated in 25 mL IEL solution [1x PBS w/ 2% FBS (ThermoFisher, 35010CV), 10 mM HEPES buffer (ThermoFisher, MT 25–060-CI), 1% penicillin/streptomycin (ThermoFisher, MT 30–002-CI), 1% L-glutamine (ThermoFisher, MT 25–005-CI), plus 1 mM EDTA (Sigma-Aldrich, E4884) and 1 mM DTT (Sigma-Aldrich, D9779) added immediately before use] for 15 minutes at 37°C with vigorous shaking (250 rpm) to remove the epithelial fraction. Tissues were then retrieved,

washed extensively, and digested in 25 mL LPL solution [1x RPMI 1640 w/2% FBS, 10 mM HEPES buffer, 1% penicillin/streptomycin, 1% L-glutamine, 0.2 U/mL collagenase A (Sigma, 11088793001) and 1 U/mL DNase I (Sigma-Aldrich, 10104159001)] for 30 minutes at 37°C with vigorous shaking (250 rpm). ¼inch ceramic beads (MP Biomedicals, 116540034) were added during this step (3–4 per sample) to facilitate tissue dissociation. The digested samples were passed through a 100 um strainer, pelleted at 450 g for 5 minutes and washed extensively. Lungs were digested in the same fashion as the lamina propria fraction of the colons but for 45 minutes. Cells from non-lymphoid organs were centrifugated in 40% PBS-adjusted Percoll (v/v, ThermoFisher, 45–001-747) in PBS to remove debris. Erythrocytes in the spleen, lung, and liver samples were lysed with ACK lysis buffer [150 mM NH4Cl (Sigma-Aldrich, A9434), 10 mM KHCO3 (Sigma-Aldrich, P7682), 0.1 mM Na2EDTA, pH 7.4].
To measure cytokine production after ex vivo restimulation, single cell suspensions were incubated at 37°C for 3-4 hours with 5% CO2 in 96-well flat-bottom plates in the presence of 50 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, P8139) and 500 ng/mL ionomycin (Sigma-Aldrich, I0634) with 1 ug/mL brefeldin A (Sigma-Aldrich, B6542) and 2 uM monensin (Sigma-Aldrich, M5273) to inhibit ER and Golgi transport. Cells were then stained with Ghost Dye Violet 510 (Tonbo, 13-0870), Ghost Dye Red 780 (Tonbo, 13-0865), or Zombie NIR Flexible Viability Kit (Biolegend, 423106) in PBS for 10 minutes at 4°C to help identify dead cells and then with Purified Anti-Mouse CD16/CD32 (2.4G2, Tonbo, 70-0161) in staining buffer [0.5% (w/v) BSA, 2 mM EDTA, 10 mM HEPES, 0.02% NaN3 (Sigma-Aldrich, S2002) in 1x PBS] for 10 minutes at 4°C to block the Fc receptors. Samples were then incubated with fluorescently-conjugated antibodies against cell surface antigens in staining buffer for 25 minutes at 4°C and then washed extensively. For accessing intracellular antigens, cells were fixed and permeabilized with the BD Cytofix/Cytoperm Kit for measuring cytokine production, or with the ThermoFisher Transcription Factor Fix/Perm Kit for staining cytosolic and nuclear antigens, according to manufacturers' instructions.

Instrument

Samples were recorded on an LSR II cytometer (BD) or an Aurora cytometer (Cytek).

Software

Flow cytometry data were collected on an LSR II cytometer using FACS Diva v8.0 (BD) or an Aurora cytometer (Cytek) using SpectroFlo v2.2.0.3 (Cytek). Data were analyzed with FlowJo v10.6.1 (BD).

Cell population abundance

Cells for functional experiments were sorted once with >95% purity. Cells for bulk and scRNA-seq experiment were double sorted to achieve >99% purity.

Gating strategy

Cells were first gated based FSC-A and SSC-A to exclude debris. Doublets were excluded using FSC-W against FSC-H, and then SSC-W against SSC-H. Cells were then gated on CD45 positive and live-dead dye negative. Subsequently, CD4 T and CD8 T cells in the periphery were gated as CD4+ or CD8+, TCRb+Dump-(B220, NK1.1, CD11b, F4/80)MHCII-. Thymic CD4 and CD8 T cells were gated as CD4+CD8-TCRbhi or CD8+CD4-TCRbhi, Dump-(B220, NK1.1, CD11b, F4/80)MHCII-. Within the CD4 T cell gate, Treg cells were gated as GFP or Foxp3+ (when the Foxp3 reporter was not present), Treg "wannabes" were gated as Thy1.1+, and conventional T cells were gated as GFP- or Foxp3-, Thy1.1-. Eosinophils were gated as SSChi, CD11bhi, SiglecF+. Neutrophils were gated as CD11bhi, SiglecF-, Ly6G+. Monocytes were gated as SiglecF-Ly6G-CD11b+Ly6Chi.

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