TOX is a critical regulator of tumour-specific T cell differentiation

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Tumour-specific CD8 T cell dysfunction is a differentiation state that is distinct from the functional effector or memory T cell states1–6. Here we identify the nuclear factor TOX as a crucial regulator of the differentiation of tumour-specific T (TST) cells. We show that TOX is highly expressed in dysfunctional TST cells from tumours and in exhausted T cells during chronic viral infection. Expression of TOX is driven by chronic T cell receptor stimulation and NFAT activation. Ectopic expression of TOX in effector T cells in vitro induced a transcriptional program associated with T cell exhaustion. Conversely, deletion of TOX in TST cells abrogated the exhaustion program: TOX-deleted TST cells did not upregulate genes for inhibitory receptors (such as Pdcd1, Entpd1, Havcr2, Cd44 and Tigit), the chromatin of which remained largely inaccessible, and retained high expression of transcription factors such as TCF-1. Despite their normal, ‘non-exhausted’ immunophenotype, TOX-deleted TST cells remained dysfunctional, which suggests that the regulation of expression of inhibitory receptors is uncoupled from the loss of effector function. Notably, although TOX-deleted CD8 T cells differentiated normally to effector and memory states in response to acute infection, TOX-deleted TST cells failed to persist in tumours. We hypothesize that the TOX-induced exhaustion program serves to prevent the overstimulation of T cells and activation-induced cell death in settings of chronic antigen stimulation such as cancer.

Using an inducible model of autochthonous liver cancer in which SV40 large T antigen (TAAG) is the oncogenic driver and tumour-specific antigen7 (Fig. 1a and Extended Data Fig. 1a), we recently showed that CD8+ T cells expressing a restricted T cell receptor (TCR) specific for TAAG (hereafter referred to as TCHRAG cells) differentiate to an epigenetically encoded dysfunctional state, exhibiting hallmarks of TST cell dysfunction including the expression of inhibitory receptors and loss of effector cytokines5,23. Numerous transcription factors were dysregulated in dysfunctional TCHRAG cells (such as NFAT, TCF-1, LEF1, IRF4 and BLIMP1) compared with functional effector or memory TCHRAG cells generated during acute infection with Listeria (using a recombinant Listeria monocytogenes strain that expressed TAAG epitope 1 (LmTAAG))5. However, many of these transcription factors are also crucial for the development of normal effector and memory T cell8; thus, we set out to identify transcription factors that were specifically expressed in dysfunctional TCHRAG cells. We analysed our RNA sequencing (RNA-seq) data5 and found that the gene encoding the nuclear factor TOX was highly expressed in dysfunctional TCHRAG cells, but low in functional, effector and memory TCHRAG cells (Fig. 1b). TOX is a nuclear DNA-binding factor and a member of the high-mobility group superfamily that is thought to bind DNA in a sequence-independent but structure-dependent manner8. Although TOX is required during thymic development of CD4+ lineage cells, natural killer and innate lymphoid cells10–12, and in regulating CD8 T cell-mediated autoimmunity13, its role in tumour-induced T cell dysfunction is unknown.

To assess TOX expression during CD8 T cell differentiation in acute infection and tumorigenesis, congenically marked naive TCHRAG cells were transferred into (i) wild-type C57BL/6 (B6) mice immunized with LmTAAG, or (ii) tamoxifen-inducible liver cancer mice (AST x Cre-ER12; AST denotes albumin-floxStop-SV40 large T antigen) treated with tamoxifen (Fig. 1a and Extended Data Fig. 1a). TOX was expressed at low levels early after Listeria infection but declined to baseline levels (by day 5 after infection) and remained low in memory T cells (Fig. 1c and Extended Data Figs. 1c, 2). By contrast, during tumour progression, TOX expression increased in TCHRAG cells and remained high (Fig. 1c and Extended Data Figs. 1c, 2). High expression of TOX correlated with high expression of several inhibitory receptors and low expression of TCRP14 cells failed to produce the effector cytokines IFNγ and TNF after stimulation ex vivo with cognate peptide or phosphor myristate acetal (PMA) and ionomycin (Fig. 1e and Extended Data Fig. 1e–g).

Persistent antigen encounter or TCR stimulation drives expression of inhibitory receptors and T cell exhaustion during chronic infections14 and in tumours15. Therefore, we analysed the expression of TOX and inhibitory receptors in GP33 virus-specific CD8 T (TCRP14) cells during acute infection with lymphocytic choriomeningitis virus (LCMV) Armstrong and chronic infection with LCMV clone 13 (Extended Data Fig. 2). TOX was transiently expressed early during acute infection with LCMV Armstrong but declined to baseline by day 5 after infection. In chronic infection with LCMV clone 13, TOX expression progressively increased in TCHRAG cells, remained increased, and correlated with high expression of several inhibitory receptors (Extended Data Fig. 2).

We confirmed TOX expression in the mouse B16F10 (B16) melanoma model. B16 tumours overexpress two melanoma-associated proteins, TRP2 and PMEL, which are recognized by TRP2-specific (TCHRAG2) and PMEL-specific (TCHRAG3) CD8 T cells, respectively16,17. Naive transgenic TCHRAG2 or TCHRAG3 cells were adoptively transferred into B16 tumour-bearing mice, and again we found that dysfunctional, tumour-infiltrating TCHRAG2 and TCHRAG3 cells expressed high levels of
TOX and inhibitory receptors, and low levels of TCF-1 (Extended Data Fig. 3a–c). Thus, persistent upregulation of TOX in T cells is induced in settings of chronic antigen stimulation such as chronic infection and cancer.

Next, we examined the expression of TOX in human CD8+ tumour-infiltrating lymphocytes (TILs) and peripheral blood mononuclear cells (PBMCs) from patients with melanoma, breast, lung and ovarian cancer (Fig. 1f–h and Extended Data Fig. 3d–g). CD45RO+ PD-1 hi CD39 hi CD8+ TILs from the same tumour (Extended Data Fig. 3g). Thus, TOX is expressed higher levels of TOX, CD39, TIM-3 and LAG-3 than PD-1 lo or CD45RO− TILs in the same tumour (Extended Data Fig. 3g). ATAC-seq revealed that DEGs had associated changes in chromatin accessibility: Tox, Pdcd1 (encoding PD-1), Entpd1, Cd38 and Cd244 loci were more accessible in TCR TAG cells than in TCR OT1 cells, whereas the Tcf7 locus was less accessible (Fig. 2e, Extended Data Fig. 4d–f and Supplementary Table 2). Chromatin accessibility analysis of TILs from patients with melanoma and lung cancer5 showed that PD-1hi TILs uniquely gained several peaks of open chromatin in TOX and lost multiple peaks in TCF7 when compared with human naive CD45RA+CD8+ PBMCs, or central memory CD45RA−CD45RO−CD62LhiCD8+ PBMCs from healthy donors5 (Extended Data Fig. 5a).
NFAT is a crucial regulator of T cell exhaustion and dysfunction, and NFAT1-binding sites in genes encoding negative regulators and inhibitory receptors have increased chromatin accessibility in dysfunctional and exhausted T cells. Thus, we compared published NFAT1 chromatin immunoprecipitation with high-throughput sequencing (ChIP–seq) data with our published and newly generated ATAC-seq datasets (Fig. 2) and found evidence that NFAT1 bound to regions within the Tox locus with significantly increased chromatin accessibility in dysfunctional TCR TAG cells (Extended Data Fig. 5b). To inhibit NFAT, we treated AST×Cre mice adoptively transferred with TCR TAG cells with the calcineurin inhibitor FK506 as previously described. We found that TCR TAG cells from FK506-treated mice had decreased expression of TOX and PD-1, and increased levels of TCF-1 (Extended Data Fig. 5c), suggesting that NFAT regulates TOX expression.

To determine whether ectopic expression of TOX in effector CD8 T cells in vitro was sufficient to induce exhaustion in the absence of chronic antigen and TCR stimulation, we transduced effector TCR TAG cells generated in vitro with retroviral vectors encoding full-length TOX fused to green fluorescent protein (GFP) or GFP alone (Fig. 3a). After transduction, effector TCR TAG cells were cultured for 6 days with IL-2 (without any additional TCR stimulation) and sorted for GFP expression (Fig. 3b). Cytokines/cytotoxic molecules, transcription factors, and inhibitory receptors were analysed using intracellular cytokine staining and flow cytometry (Fig. 3c). The MA plot of the RNA-seq dataset showed significantly differentially expressed genes (DEGs) in TOX–GFP+ compared to GFP+ TCR TAG cells (Fig. 3d). The Heat map of RNA-seq expression (row-normalized log2(counts per million) for DEGs; false discovery rate (FDR) < 0.10) indicated that DEGs are coloured in red. The Heat map of RNA-seq expression (row-normalized log2(counts per million) for DEGs; false discovery rate (FDR) < 0.10) in TOX–GFP+ and GFP+ TCR TAG cells.
expression (Extended Data Fig. 6a). RNA-seq analysis revealed 849 DEGs between TOX–GFP + and GFP − T cells (Fig. 3b, Extended Data Fig. 6b and Supplementary Table 3). GSEA revealed that the transcriptional program of TOX–GFP + TCR TAG cells was significantly enriched for genes associated with chronic infections and tumours, with reduced expression of several genes encoding transcription factors (Tcf7, Left1 and Id3), and increased expression of genes encoding inhibitory receptors (Pdcd1, Cd244, Havcr2 and Entpd1) and transcription factors such as Ahr, Nfsl3, Prdm1 and Id2 (Fig. 3c, b and Extended Data Fig. 6c–g). Despite expressing numerous exhaustion-associated genes, TOX–GFP + TCR TAG cells remained highly functional and proliferative (Extended Data Fig. 6d–f).

Next, we examined how genetic deletion of Tox affected CD8 T cell differentiation during acute infection or in tumours. TCR TAG mice were crossed to TcrTag KO mice and mice expressing Cre-recombinase under the distal Lck promoter to generate TOX-knockout TCR TAG mice (Fig. 4a and Extended Data Fig. 7a). TCR TAG cells from TOX-knockout TCR TAG mice developed normally and similarly to littermate control mice (Extended Data Fig. 7b, c). Naive TOX-knockout and wild-type (Thy1.1 + ) TCR TAG cells expanded equally well after adoptive transfer into AST × Cre mice. Data are from three independent experiments. b. Wild-type and knockout donor TCR TAG cells 19 days after transfer into AST × Cre mice. TOX-knockout and wild-type TCR TAG cells expanded equally well (n = 6) and knockout (n = 6) TCR TAG cells isolated and flow-sorted from liver tumour lesions. Results from two independent experiments. Memory (Mem) TCR TAG cells are shown as a control.

g. Percentage of KI67-positive wild-type and knockout TCR TAG cells from malignant liver lesions 6–8 days after transfer into AST × Cre mice. Data are from three independent experiments. h. Wild-type and knockout donor TCR TAG cells 19 days after transfer into liver tumours (WT, n = 5; KO, n = 5). Data are representative of two independent experiments. In b–h, each symbol represents an individual mouse. 1, MA plot of RNA-seq data. Significantly DEGs are in red. j. Chromatin accessibility of wild-type and knockout TCR TAG cells. Each row represents one peak (differentially accessible between wild-type and knockout; FDR < 0.05) displayed over a 2-kb window centred on the peak summit; regions were clustered with k-means clustering. Genes associated with peaks within individual clusters are highlighted. k, ATAC-seq signal profiles across the Tox and Tcf7 loci. Peaks uniquely lost or gained in knockout TCR TAG cells are highlighted in red or blue, respectively. Data are mean ± s.e.m. ***P ≤ 0.01, ****P ≤ 0.001, ***P ≤ 0.001, two-sided Student’s t-test.
TCR\(_{\text{TAG}}\) cells equivalently infiltrated the liver (Fig. 4d), proliferated and upregulated CD44, CD69 and CD25 (Fig. 4e and Extended Data Fig. 7f). Notably, by 8–10 days after transfer, TOX-knockout TCR\(_{\text{TAG}}\) cells did not upregulate inhibitory receptors including PD-1, LAG-3, CD38, CD39 and 2B4, in contrast to wild-type TCR\(_{\text{TAG}}\) cells (Fig. 4e and Extended Data Fig. 7f). Nevertheless, TOX-knockout and wild-type TCR\(_{\text{TAG}}\) cells showed comparable reductions in the production of IFN-\(\gamma\) and TNF, the expression of CD107, granzyme B (GZMB), and the specific lysis of TAG-peptide-pulsed EL4 target cells (Fig. 4f and Extended Data Fig. 7g–i). Thus, despite their normal, 'non-exhausted' phenotype (Fig. 4e) and proliferative capacity (Fig. 4g), TOX-knockout TCR\(_{\text{TAG}}\) cells remained dysfunctional, revealing that the regulation of inhibitory receptors is uncoupled from T cell effector function. Notably, by 2–3 weeks after transfer, very few TOX-knockout TCR\(_{\text{TAG}}\) cells could be found in liver tumour lesions, whereas wild-type TOX TCR\(_{\text{TAG}}\) cells persisted throughout the course of tumour progression (Fig. 4h and Extended Data Fig. 8a). Indeed, TOX-knockout TCR\(_{\text{TAG}}\) cells had increased levels of active caspases 3 and 7, increased annexin V staining, and an enrichment of apoptosis genes, although the expression of pro- and anti-apoptotic proteins such as BIM, BCL-2 and BCL-xL was similar between knockout and wild-type TCR\(_{\text{TAG}}\) cells (Extended Data Fig. 8b–e).

We performed RNA-seq and ATAC-seq analyses from TOX-knockout and wild-type TCR\(_{\text{TAG}}\) cells isolated from liver tumours of AST\(\times\)Cre mice 8–9 days after adoptive transfer and identified 679 DEGs and 12,166 differentially accessible chromatin regions, respectively (Fig. 4i, j, Extended Data Fig. 9 and Supplementary Tables 1, 2). TOX-knockout TCR\(_{\text{TAG}}\) cells had low expression of genes encoding transcription factors and inhibitory receptors including Njil3, Pdcd1, Cish, Pdel1, Entpd1, Tigit, Haver2 and Cd38, and high expression of the transcription factors Tcf7, Lef1 and Id3. GSEA of DEGs between TOX-knockout and wild-type TCR\(_{\text{TAG}}\) cells revealed strong enrichment for genes and pathways associated with T cell exhaustion during chronic infection and tumorigenesis (Extended Data Fig. 9b). Transcriptional differences were associated with corresponding changes in chromatin accessibility patterns of the respective genes (Fig. 4) and Extended Data Fig. 9c–g). For example, the loci of Tox, Pdcd1, Cd38 and Entpd1 were less accessible in TOX-knockout TCR\(_{\text{TAG}}\) cells than in TOX wild-type TCR\(_{\text{TAG}}\) cells, whereas the loci of Tcf7, Cd28, Fyn and Il7r were more accessible (Fig. 4k and Extended Data Fig. 9e). More accessible regions in TOX-knockout TCR\(_{\text{TAG}}\) cells showed significant enrichment for Gene Ontology (GO) terms associated with (i) cytokine and chemokine receptor activity; (ii) chromatin binding and bending, regulatory region DNA binding; and (iii) \(\beta\)-catenin binding (Extended Data Fig. 9f). We also found enrichment of apoptosis pathways in TOX-knockout TCR\(_{\text{TAG}}\) cells and increased expression of genes associated with apoptosis such as Fas, Tnf, Gas2 and Tfrs25 (which encodes DR3) in TOX-knockout TCR\(_{\text{TAG}}\) cells (Extended Data Figs. 8e, 9e).

In summary, TOX is specifically required for T cell differentiation in settings of chronic antigen stimulation (such as tumours and chronic infection). A key finding of our study is that the regulation of inhibitory receptor expression is uncoupled from the loss of effector function in dysfunctional TST cells. Supporting this point is the notable phenotypic and transcriptional similarities between dysfunctional TOX-knockout TCR\(_{\text{TAG}}\) TILs (Fig. 4) and functional TOX-negative, bystander TCR\(_{\text{OT1}}\) TILs (Fig. 2 and Extended Data Figs. 10a, b). TOX-deficient TST cells failed to persist in tumours, and we hypothesize that the TOX-induced gene regulation of inhibitory receptors and other exhaustion-associated molecules serve as a physiologically negative feedback mechanism to prevent overstimulation of antigen-specific T cells and activation-induced cell death in settings of chronic antigen stimulation such as chronic infection and cancer (Extended Data Fig. 10c).

Online content Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-019-1324-y.

Received: 18 May 2018; Accepted: 4 June 2019;
Published online 17 June 2019.

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In summary, TOX is specifically required for T cell differentiation in settings of chronic antigen stimulation (such as tumours and chronic infection). A key finding of our study is that the regulation of inhibitory receptor expression is uncoupled from the loss of effector function in dysfunctional TST cells. Supporting this point is the notable phenotypic and transcriptional similarities between dysfunctional TOX-knockout TCR\(_{\text{TAG}}\) TILs (Fig. 4) and functional TOX-negative, bystander TCR\(_{\text{OT1}}\) TILs (Fig. 2 and Extended Data Figs. 10a, b). TOX-deficient TST cells failed to persist in tumours, and we hypothesize that the TOX-induced gene regulation of inhibitory receptors and other exhaustion-associated molecules serve as a physiologically negative feedback mechanism to prevent overstimulation of antigen-specific T cells and activation-induced cell death in settings of chronic antigen stimulation such as chronic infection and cancer (Extended Data Fig. 10c).
METHODS

Mice. AST (Albumin-flxStop-SV40 large T antigen (TAG)) mice were previously described3,5, TCRTAG transgenic mice (B6.Cg-Tg(TcraTcrb)1Ybtb/B6)4, Cre-ER2 (B6.129-Gt(Rosa)26Sortm1(CreERT2)Byj, Alb-Cre (B6.Cg-Tg(AlbCre)21Mgn/J), TCROT1 (C57BL/6-Tg(TcraTcrb)1100Mjb/J), Ly5.1 (B6.SJL-Ptprc<+>Pepcb<+>Boyj), B6.Cg-Tg(Lck-cre)3779Nkj (Lck-Cre) and C57BL/6 J Thy1.1 mice were purchased from The Jackson Laboratory. TCR678 (GIGD7; eBioscience), CTLA-4 (UC10-410-11; Tonbo Biosciences), TCF-1 (C63D9; Santa Cruz Biotechnology); BIM (C34C5; Cell Signaling Technology), CD8 (53-1.5), TIM-3 (RMT3-23), TNF (MP6-XT22), and 7-amino-actinomycin (7-AAD); from FlowJo (Tree Star).

Lentiviral infection. The L. monocytogenes (Lm) Δacta ΔulinB strain expressing the TAG epitope (I026-SANINQAKL-215, 2540 large T antigen) together with the OVA SIINFEKL epitope was generated by Aduro Biotech as previously described3,5. The Lm strain was constructed using the previously described strategy5. Experimental vaccination stocks were prepared by growing bacteria to early stationary phase, washing in PBS, formulated at approximately 1 × 10^6 CFU ml^-1, and stored at −80 °C. Mice were infected intraperitoneally with 5 × 10^5 CFU of LmTAG.

Cell isolation for subsequent analyses. Splenocytes were mechanically disrupted with the back of a 3-ml syringe, filtered through a 70-μm strainer, and red blood cells were lysed with ammonium chloride potassium buffer. Cells were washed twice with cold RPMI 1640 media supplemented with 2 μM glutamine, 100 U ml^-1 penicillin/streptomycin, and 5–10% FCS. Liver tumour and B16 tumour tissues were mechanically disrupted and dissociated with scissors (in 1–2 ml of cold complete RPMI). Dissociated tissue pieces were transferred into a 70-μm strainer (placed into a 60-mm dish with 1–2 ml of cold complete RPMI) and further dissociated with the back of a 3-ml syringe. Cell suspension was filtered through 70-μm strainers. Tumour homogenate was spun down at 400g for 5 min at 4 °C. Pigment cells (15–30% PCs in HBSS) containing melanin, and remaining cells were washed twice with 8.5 μl Percoll, mixed by several inversions, and spun at 500g for 10 min at 4 °C. Pellet was lysed with ammonium chloride potassium buffer and cells were further processed for downstream applications.

Human samples. PBMC and tumour samples were obtained from patients with cancer enrolled on a biospecimen procurement protocol approved by the MSKCC Institutional Review Board (IRB). Each patient signed an informed consent form and received a patient information form before participation. Human samples were analysed using an IRB-approved biospecimen utilization protocol. Breast cancer samples were selected from patients who had evidence of a dense mononuclear cell infiltrate on conventional haematoxylin and eosin and IHC (E8) staining. For human ovarian tumour samples (Extended Data Fig. 3) tumour samples were obtained as per protocols approved by the IRB. All patients provided informed consent to an IRB-approved correlative research protocol before the collection of tissue (Memorial Sloan Kettering Cancer Center IRB 00144 and 06-107). Human peripheral blood lymphocytes were obtained from the New York Blood Center or from patients where indicated. Human tumours were mechanically disrupted as described for solid mouse tumours, centrifuged on Percoll gradients and further processed by flow cytometric analysis.

FK506 studies. Naive TCRAC Thy1.1+ cells were transferred into AST × Cre-ER2 Thy1.1 mice, which were treated with tamoxifen 1 day later. On days 2–8, mice were treated with the calcineurin inhibitor FK506 (Prograf, 5 mg ml^-1) (2.5 mg per kg mouse intraperitoneally, once daily). Control mice were treated with PBS. All mice were analysed on day 10.

TOX overexpression experiments. Mouse Tox cDNA (accession number NM_145711.4) without the stop codon fused in-frame with the coding sequence of a monomeric form of green fluorescent protein (mGFP) was obtained from OriGene Technologies (MR208435L2). PCR cloning was used to amplify TOX–mGFP, which was then cloned into the pMIGR1 retroviral vector to generate pMIGR1 TOX–mGFP using the restriction enzymes EcoRI and PacI. pMIGR1 TOX–mGFP and control pMIGR1-GFP containing only mGFP were used for retroviral transduction of TCRAC CD8+ T cells as follows: on day 1, the retroviral packaging cell line Plat-E (Cell Biolabs) was transfected using Effectene (Qiagen) following the manufacturer’s instructions. On day 2, splenocytes from TCRAC mice were resuspended in the viral supernatant with PBS. On day 3, activated splenocytes were resuspended with 10^5 IL-2 and 5 × 10^5 μg ml^-1 Polybrene (Santa Cruz Biotechnology), transferred to 12-well plates, and spun at 1,000g for 90 min. This process was repeated the next day. Transduced T cells were cultured for six additional days, replacing media and adding fresh IL-2 (100 μl ml^-1) every other day. T cells were collected and flow-sorted for high GFP expression for downstream transcriptome analysis.

Intracellular cytokine and transcription factor staining. Intracellular cytokine staining was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) per manufacturer’s instructions. In brief, T cells were mixed with a fluorescently marked secondary antibody and incubated with TAG epitope 1 peptide (0.5 μg ml^-1) or OVA peptide (0.1 μg ml^-1) for 4–5 h at 37 °C in the presence of GolgiPlug (brefeldin A). Where indicated, naive splenocytes or APCs were activated either in vivo (single intraperitoneal injection of 50 μg lipopolysaccharide...
Active caspase-3/7 analysis. For the flow cytometric analysis of active caspase-3/7, cells were incubated with 500 nM CellEvent Caspase 3/7 Green Detection Reagent (Invitrogen; C10423) for 30 min at 37°C. Chromium release assay. Mouse EL4 lymphoma cells were loaded with 150 μCi of [51Cr]sodium chromate for 2 h. TAG epitope I peptide (SAIDNYAQLK) at a concentration of 1 μg ml−1 was added during last 30 min of incubation. Cr-labelled, TAG-1-pulsed EL4 cells were co-cultured with flow-sorted memory TCR.Tag T cells or wild-type or knockout TOX TCR.Tag T cells isolated and flow-sorted from liver tumours of AST×Cre mice (6–8 days after transfer) at a 5:1 (effector:target) ratio for 16 h. Medium alone or 2% Triton-X was added to set spontaneous or total lysis, respectively. Specific killing was calculated using following formula: percentage lysis = ([test counts per min − spontaneous counts per min])/(total counts per min − spontaneous counts per min)) × 100.

Sample preparation for ATAC-seq and RNA-seq. Replicate samples were isolated from spleens or livers and sorted as follows: (i) naïve TCR.Tag Thy1.1 T cells were sorted by flow cytometry (CD8+ from spleen of TCR.Tag Thy1.1 transgenic mice. (ii) Wild-type and knockout TOX TCR.Tag T cells were sorted from livers of established AST×Cre mice 8–9 days after transfer. Cells were gated on CD8+Thy1.1 PD-1hi/LACZ+CD39hi/bright. A small aliquot of sorted cell populations was used to confirm TOX expression (for wild-type) and TOX deficiency (for knockout). (iii) TCR.Tag T and TCR.Tag T cells were sorted from livers of transferred AST×Cre mice 20–21 days after transfer/Listeria infection. After flow-sorting, all samples for downstream ATAC-seq analysis were frozen in 10% FCS in DMEM and stored at −80°C. Samples for RNA-seq were directly sorted into Trizol and frozen and stored at −80°C.

Transcriptome sequencing. Samples for RNA-seq were sorted directly into TRIzol LS (Invitrogen). The volume was adjusted to 1 ml with PBS and samples frozen and stored at −80°C. RNA was extracted using RNeasy mini kit (Qiagen) per instructions provided by the manufacturer. After ribogreen quantification and quality control of Agilent Bioanalyzer, total RNA underwent amplification using the SMART-seq V4 (Clonetech) ultralow input RNA kit for sequencing (12 cycles of amplification for 2–10 ng of total RNA). Subsequently, 10 ng of amplified cDNA was used to prepare Illumina Hiseq libraries with the Kapa DNA library preparation chemistry (Kapa Biosystems) using 8 cycles of PCR. Samples were barcoded and run on a Hiseq 4000, in a 50-bp/50-bp paired-end run, using the TrueSeq SBS Kit v3 (Illumina).

ATAC-seq. Frozen 25,000–50,000 cells were thawed and washed in cold PBS and lysed. Transposition was performed at 42°C for 45 min. After purification of the DNA with the MinElute PCR purification kit (Qiagen), material was amplified for five cycles. Additional PCR cycles were evaluated by quantitative PCR. Final product was cleaned by Ampure Beads at a 1.5× ratio. Libraries were sequenced on a Hiseq 2500 IT in a 50-bp/50-bp paired-end run, using the TrueSeq SBS Kit v3 (Illumina).

Bioinformatics methods. The quality of the sequenced reads was assessed with FastQC and QoRTs (for RNA-seq samples (ref. 34 and Babraham Bioinformatics v0.11.7 http://www.bioinformatics.babraham.ac.uk/projects/fastqc (2010)). Unless stated otherwise, all plots involving high-throughput sequencing data were obtained with custom R scripts (see github.com/friedue/Scott2019 for the code; R: A Language and Environment for Statistical Computing https://www.R-project.org/ (2014); and ref. 35).

RNA-seq. DNA sequencing reads were aligned with default parameters to the mouse reference genome (GRCm38) using STAR.78 Gene expression estimates were obtained with featureCounts using composite gene models (union of the exons of all transcript isoforms per gene) from Gencode (version M17).77,78 DEGs. DEGs were determined with DESeq2. The q-value cut-offs for the final lists of DEG were as follows: (i) TOX–GFP versus GFP: 849 DEGs with q < 0.10; (ii) TAG versus OT1: 2,347 DEGs with q < 0.05; and (iii) wild-type versus knockout: 679 DEGs with q < 0.05.

Pathway and GO term enrichment analyses. Gene set enrichment analyses were done using GSEA48 on RPKM values against a gene set permutation (the seed was set to 1). Heat maps. Heat maps were created using log(counts per million) of genes identified as differentially expressed by DESeq2 (adjusted P < 0.05 unless otherwise noted). Rows were centred and scaled using z-scores.

ATAC-seq. ATAC-seq data2 were downloaded from GEO (accession GSE89308). These datasets were processed in the same manner as the newly generated datasets described in this study.

Alignement and identification of open chromatin regions. The data was processed following the recommendations of the ENCODE consortium (The ENCODE Consortium ATAC-seq Data Standards and Prototype Processing Pipeline https://www.encodeproject.org/atac-seq/). Reads were aligned to the mouse reference genome (version GRCm38) with BWA-backtrack.40 Post-alignment filtering was done with samtools and Picard tools to remove unmapped reads, improperly paired reads, non-unique reads, and duplicates (ref. 41 and Broad Institute Picard http://broadinstitute.github.io/picard/ (2015)). To identify regions of open chromatin represented by enrichments of reads, peak calling was performed with MACS2.45 For every replicate, the narrowpeak results of MACS2 were used after filtering for adjusted P < 0.01.

Differentially accessible regions. Regions where the chromatin accessibility changed between different conditions were identified with diffBind (DiffBind: Differential Binding Analysis of Chip-Seq Peak Data https://bioconductor.org/packages/release/bioc/html/DiffBind.html (2011)) with the following options: minOverlap = 4, bUseSummaryOverlaps = t, minMembers = 2, bFullLibrarySize = TRUE. A total of 12,166 differentially accessible peaks were identified between wild-type and knockout TCR.Tag T cells (see Fig. 4); 19,071 differentially accessible peaks were identified between TCR.Tag and TCR.Tag T cells (see Fig. 2).

Coverage files. Individual coverage files for replicate normalized for differences in sequencing depths between the different samples were generated with bamCoverage of the deepTools suite46 using the following parameters: -bs 10 --normalization-unit GC --genomeSize 2150570000 --blackListFileName mm10.blacklist --ignoreForNormalization chrX chrY --ignoreDuplicates --minFragmentLength 40 -p 1. To create merged coverage files of replicates of the same condition, we used multiBigwigSummary to obtain the sequencing-depth-normalized coverage values for 10 bp bins along the entire genome, that is, for every condition, we obtained a table with the coverage values in every replicate within the same bin. Subsequently, we chose the mean value for every bin to represent the coverage in the resulting ‘merged’ file (see github.com/friedue/Scott2019 for the code that was used). Merged coverage files were used for display in IGV and for heatmaps.

Heat maps. Heat maps displaying the sequencing-depth-normalized coverage from different ATAC-seq samples were generated with computeMatrix and plotHeatmap of the deepTools suite41.

Every row corresponds to a single region that was determined to be differentially accessible when comparing either TCR.Tag (TAG) to TCR.Tag OT1 T cells or wild-type to TOX-knockout TCR.Tag T cells. The plots display the centre of each differentially accessible peak region ± 1 kb; the colour corresponds to the average normalized coverage across all replicates of the respective condition. Gene labels indicate genes that overlapped with a given differentially accessible region (arguing directly with the given gene locus for the gene).

Combining RNA-seq and ATAC-seq data. The relationship between RNA-seq and ATAC-seq was explored via ‘diamond’ plots for select genes detected as differentially expressed via DESeq2. Each gene was represented by a stack of diamond-shaped points coloured by the associated chromatin state of the gene (blue indicating closing and red indicating opening). The bottom-most point in each stack corresponds to the log2-fold-transformed fold change in expression for that gene. NFAT1 ChIP seq (publicly available). NFAT1 ChIP-seq samples were generated as previously described32 from cells expressing endogenous NFAT1 (wild type) or lacking NFAT1 (knockout). Cells lacking endogenous NFAT1 were transfected with an empty GFP vector (mock) or with a vector containing a mutated form of NFAT (CA-RIT-RV). Either cell type was either left resting (none) or stimulated with PMA and ionomycin (P + 1) for 1 h.

We downloaded the sequencing results (fastq files generated by SOLID sequencing technology) from the Sequence Read Archive (GEO series GSE64407); see Supplementary Table 4 for further details. SOLID adapters had to be trimmed off, which we did with cutadapt44 specifying –format=raw-fasta –minimum-length 15 –colorspace and the sample specific adapter sequences via –a and –g (see https://ars.els-cdn.com/content/image/1-s2.0-S1076741315003021-mmcx1b.xbl for the sample-specific adapters). The trimmed reads were subsequently aligned to the mouse genome version GRCm38 with bowtie1 using the colorspace option45. Coverage tracks normalized for differences in sequencing depths were be generated with bamCoverage of the deepTools suite (v.3.1.0)42 using the following parameters: -bs 10 --normalizeUsing RPGC --effectiveGenomeSize 2150570000 --blackListFileName mm10.blacklist --ignoreForNormalization chrX chrY --ignoreDuplicates --minFragmentLength 40 -p 1. Blacklisted regions were downloaded from https://sites.google.com/site/anandandujai/projects/blacklists/.

Regions of statistically significant read enrichments in the ChIP samples compared with the corresponding input samples (peaks) were identified with MACS2 (2.1.1.20160309)42 using ChIP and corresponding input files and the following

(37.0°C for 4 h. After staining for cell-surface molecules, the cells were fixed, permeabilized and stained with antibodies to IFNγ, TNF and GZMB. Intracellular transcription factor staining was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) as per the manufacturer’s instructions.

Annexin V staining. Apoptosis was assessed by flow cytometry using V450 Annexin V (BD Biosciences; 560506) and 7-AAD following the manufacturer’s instructions.
parameters: \( g \approx 1.87 \pm 0.01 \) – keep-up-dall all. For final peak files, the narrowpeak outputs of MACS2 were used, keeping only peaks with adjusted \( P \) values below 0.01.

**Digital droplet PCR.** TOX–GFP-overexpressing and GFP-overexpressing TCR\( \text{RAG} \) T cells were sorted directly into TRIZol (Invitrogen). RNA was extracted with chloroform. Isoopropanol and linear acrylamide were added, and the RNA was precipitated with 75% ethanol. Samples were resuspended in RNase-free water. Quantity was assessed by PicoGreen (ThermoFisher) and quality by BioAnalyzer (Agilent). Droplet generation was performed on a QX200 ddPCR system (Bio-Rad; 864001) using cDNA generated from 100 pg total RNA with the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad; 1864021) according to the manufacturer’s protocol with reverse transcription at 42°C and annealing/extension at 55°C. Each sample was evaluated in technical duplicates. Reactions were partitioned into a mixture of approximately 30,000 droplets per well. Plates were read and analysed with the QuantaSoft software to assess the number of droplets positive for the gene of interest, reference gene (Gapdh; dMmuCPE5195283), both, or neither. PrimePCR ddPCR Expression Probe Assays were ordered through Bio-Rad for the following genes of interest: Lag3 (dMmuCPE5122546), Id2 (dMmuCPE5094108), Pdml1 (dMmuCPE5113738), Prf1 (dMmuCPE5112024), and GzmB (dMmuCPE509396).

**Data reporting.** No statistical methods were used to predetermine sample size. The investigators were not blinded to allocation during experiments and outcome assessment, and experiments were not randomized.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

All data generated and supporting the findings of this study are available within the paper. The RNA-seq and ATAC-seq data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE126974. Source Data is provided with the online version of the paper. Additional information and materials will be made available upon request.

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**Acknowledgements**

We thank the members of the Schietinger laboratory, S. Reiner and M. Li for discussions and technical help; R. Nadler for technical assistance; A. Hurwitz and N. Restifo for providing TRP2 transgenic mice; MSKCC Flow Cytometry Core, especially R. Gardner. Integrated Genomics Operation Core, especially A. Viale, N. Mohibullah, A. Farina and R. Patel. We thank the J. Sun laboratory and B. Moltedo (Rudensky laboratory) for providing LCMV strains. D.Z., A. Schietinger, M.D.H. and C.A.K. are members of the Parker Institute for Cancer Immunotherapy, which supports the MSKCC Cancer Immunotherapy Program. This work was supported by NIH-NCI grants DP2 CA252212, R00 CA172371 (to A. Schietinger), U54 CA209975, V Foundation for Cancer Research (to A. Schietinger), the Anna Fuller Foundation (to A. Schietinger), the William and Ella Owens Medical Research Foundation (to A. Schietinger), the Josie Robertson Young Investigator Award (to A. Schietinger), NIH-NCI grant K08 CA158069 (to M.P.), V Foundation Scholar Award (to M.P.), Serodino Family Adventure Allie Fund (to M.P.), the Parker Institute for Cancer Immunotherapy (C.A.K. and A. Schietinger), the Weill Cornell Medicine Core Laboratories Center (P.Z., F.D., D.B.), Francois Wallace Monahan Fellowship (to O.L.), NIH-NIAID U19 AI11143 (to M.S.G.), NIH-NIAID R01 A054977 (to J.K.), the von Arx Rynjen Cancer Research Foundation O-96-18 (to C.A.K.), NCI R33 CA25239 (to C.A.K.), the Manhasset Women’s Coalition Against Breast Cancer (to C.A.K.), the MSK Cancer Center Core Grant P30 CA007848. The Integrated Genomics Operation Core was supported by Cycle for Survival and the Marie-Josée and Henry R. Kravis Center for Molecular Oncology.

**Author contributions**

A.C.S., M.P. and A. Schietinger conceived and designed the study. A.C.S., M.P., D.B., F.D., P.Z. and A. Schietinger conceived the computational analyses; D.B., F.D. and P.Z. performed all of the computational analyses. A.C.S., M.P., P.T., L.M., M.S., H.A. and S.S.C. carried out experiments. A.C.S., M.P., F.D., P.Z., D.B., S.S.C., C.A.K. and A. Schietinger interpreted data. S.C. and H.A. assisted with mouse breeding; T.W., A. Snyder, D.Z., M.D.H., M.R.T., F.A.C., H.Y.W. and C.A.K. provided human samples; N.A., Y.L. and N.K.A. contributed to the analysis of human samples. O.L. and M.S.G. provided help in establishing the knockout model. O.L., M.S.G. and J.K. provided mice. P.L. provided Listeria strains. A.C.S., M.P., F.D., P.Z. and A. Schietinger wrote the manuscript, with all authors contributing to writing and providing feedback.

**Competing interests**

C.A.K. is a consultant and/or advisor to Aleta Biotherapeutics, Bellicum Pharmaceuticals, Bristol-Myers Squibb, Cell Design Labs, G1 Therapeutics, Kius Pharma, Obsidian Therapeutics and Rxi Therapeutics. A.C.S. receives research funding unrelated to this work from Kite/Gilead. M.D.H. has received research funding from Bristol-Myers Squibb; is paid as a consultant to Merck, Bristol-Myers Squibb, AstraZeneca, Genentech/Roche, Janssen, Nektar, Syndet, MiraCure, and has received travel support/honoraria from AstraZeneca and BMS; a patent has been filed by MSK related to the use of tumour mutation burden to predict response to immunotherapy (PCT/US2015/062208), which has received licensing fees from PGGdx. A. Snyder is a current employee and owns stock in Merck. D.Z. reports grants from Merck, and consulting fees from Merck, Synlogic Therapeutics, Trieza Therapeutics, and Tesaro. D.Z. owns a patent in Merck. D.Z. has received research funding from Bristol-Myers Squibb, Genentech/Roche, Ceres, Kite/Gilead, Janssen, Genentech, Nektar, Genentech/Roche, and has received travel support/honoraria from Pfizer and Novartis; and receives research funding from Genentech/Roche unrelated to this work.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41586-019-1324-y.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | T cell differentiation during tumorigenesis.

a, Scheme of autochthonous liver cancer model to investigate tumour-specific CD8 T cell differentiation and dysfunction. AST × Cre liver cancer model. Cre-mediated deletion of the flox-stop cassette leads to TAG expression and tumour initiation. TAG-specific CD8 T cells isolated from TCRTAG transgenic mice recognize TAG epitope I (shown in red) on major histocompatibility complex (MHC) class I H-2D^b. Tamoxifen-inducible Cre-ER^{T2} (AST × Cre-ER^{T2}) or constitutive Alb-Cre (AST × Alb-Cre) mouse strains are used as indicated.

b, Top, scheme of Listeria infection. Bottom, phenotypic characterization of Thy1.1^+ effector and memory TCRTAG cells isolated from spleens 7 and more than 35 days after transfer into B6 mice followed by Listeria infection. Gating strategy is shown. KLRG1, CD127, CD44 and CD62L expression levels are shown.

c, Naive congenically marked (Thy1.1^+) TCR TAG cells were adoptively transferred into (Thy1.2^+) AST × Alb-Cre mice. T cells were isolated 7 or more than 20 days after transfer from either spleens (for effector and memory T cells after Listeria infection) or liver tumour lesions of AST × Alb-Cre mice. TOX expression was assessed by flow cytometry. TOX isotype is shown as a control for each sample. Naive TCRTAG cells are shown in grey as a control.

d, Flow cytometric analysis of TCRTAG cells isolated from liver lesions of AST × Cre-ER^{T2} mice more than 20 days after transfer (red). TOX expression with PD-1, LAG-3, 2B4, CD39, TIGIT, TIM-3, CD101, CD38, CTLA4 and TCF-1 expression levels are shown. Naive TCRTAG cells are shown in grey as a control.

e–g, Intracellular IFNγ and TNF production of TCR TAG cells isolated at days 7–10 and day 60 after transfer into AST × Cre-ER^{T2} mice after 4-h ex vivo peptide stimulation with antigen-presenting cells (APCs) (from B6 spleens) (e), or peptide stimulation with in vitro (f, top) or in vivo (f, bottom) LPS-activated splenocytes (f), or stimulation with PMA and ionomycin (g). LPS-mediated activation of APCs was confirmed by flow cytometric analysis assessing the upregulation of MHC-II, CD80, CD86 and CD40 on CD11c^+ APCs, CD11b^+ APCs and CD19^+ B cells (splenocytes). Memory TCRTAG cells are shown as controls. Gates are set based on no-peptide controls. All FACS plots are gated on CD8^+ Thy1.1^+ TCRTAG cells (experiments in f and g are repeated twice). These data are representative of more than ten independent experiments.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Antigen-specific CD8 T cell differentiation during acute and chronic viral LCMV infections, acute *Listeria* infection, and during tumorigenesis. a, Top, experimental scheme for acute *L. monocytogenes* (expressing TAG epitope I) infection (green) and AST × Cre-ER<sup>T2</sup> liver tumorigenesis after treatment with tamoxifen (red). Bottom, experimental scheme for acute (Armstrong; blue) and chronic (clone 13; orange) infection with LCMV. b, Expression profiles of TOX, PD-1, LAG-3 and TCF-1 at various time points after infection or tamoxifen treatment. Relative MFI values are shown normalized to naive transgenic TCR<sub>P14</sub> T cells (specific for the LCMV epitope GP33) or naive TCR<sub>TAG</sub> T cells (dashed grey line). c, Top, flow cytometric analysis of TOX, TCF-1, PD-1, LAG-3, 2B4, TIM-3, CD39, TIGIT, CD38 and CTLA4 expression levels of TCR<sub>TAG</sub> T cells after *Listeria* infection (green) or tamoxifen treatment (red). Bottom, flow cytometric analysis of TOX, TCF-1, PD-1, LAG-3, 2B4, TIM-3 and CD39 expression levels of GP33-specific T cells at indicated time points after infections with LCMV Armstrong (blue) and LCMV clone 13 (orange). Naive T cells are shown in grey as a control. Data are mean ± s.d. and are representative of two independent experiments with *n* = 2 (*Listeria*) and *n* = 2–3 (AST × Cre-ER<sup>T2</sup>; LCMV Armstrong; LCMV clone 13) mice per time point.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3  | Phenotypic and functional characterization of TILs from mouse and human tumours. a–c, TCR_{TRP2} (TRP2) and TCR_{PMEL} (PMEL) TILs in mouse B16 melanoma tumours. a, TOX expression and TCF-1, PD-1, LAG-3, CD39, 2B4 and TIM-3 expression levels of TRP2 (Thy1.1^+) TILs (red; top) and PMEL (Thy1.1^+) TILs (red; bottom) isolated more than 15 days after adoptive transfer from established B16 melanoma tumours growing subcutaneously in B6 (Thy1.2^+) mice. Naive CD8 T cells are shown in grey as a control. T cells are gated on CD8^+Thy1.1^+ cells. b, Intracellular IFNγ and TNF production of TRP2 and PMEL TILs after 4-h peptide stimulation ex vivo. c, Relative MFI values of TOX, TCF-1 and PD-1 of the indicated tumour models and TIL specificities shown on a log 10 scale. Each symbol represents an individual mouse. Data are mean ± s.e.m of n = 2 (PMEL); n = 4 (TRP2); and n = 5 (TAG) mice, and are representative of two independent experiments. d–g, Phenotypic characterization and TOX expression profiles of human TILs and PBMCs isolated from patients with melanoma, lung, breast and ovarian cancer. d, Flow cytometric analysis of PBMCs and TILs of patients with breast cancer. TOX expression of TILs and matched PBMC CD8^+ T cells. Gating strategy is shown. CD45RO^−PD-1^hiCD39^hi (TILs; red), CD45RO^−PD-1^lo (PBMCs; blue), CD45RO^+PD-1^hi (PBMCs; green), and CD45RA^−CD45RO^− (naive PBMCs; grey). TOX isotypes are shown as controls for each sample. e, Top, TOX expression in human CD45RO^+PD-1^hiCD39^lo (dark blue) and CD45RO^+PD-1^hiCD39^hi (red) TILs isolated from human primary melanoma. Isotypes are shown and data correspond to Fig. 1f. Bottom, TOX expression of TILs and matched PBMC CD8^+ T cells from patients with melanoma. CD45RO^+PD-1^hi (TIL; red; n = 4), CD45RO^+PD-1^hi (PBMCs; blue, n = 4). TOX isotypes are shown as controls for each sample/patient. Bar plot shows MFI values for TOX. Each symbol represents an individual TIL and PBMC matched pair. f, TOX expression in human CD8^+PD-1^hi TILs isolated from human primary ovarian tumours. Flow plots are gated on CD8^+CD45RO^+PD-1^hi T cells (red). CD8^+CD45RO^− T cells from healthy donors are shown in grey. Gating strategy is shown. Each symbol represents a patient or healthy donor sample. g, TOX, CD39, TIM-3 and LAG-3 expression of CD8^+CD45RO^+PD-1^hi (red) and CD8^+CD45RO^+PD-1^lo (blue) TILs from human melanoma (n = 5), breast (n = 5) and lung (n = 6) tumours. Each symbol represents an individual matched PD-1^hi/PD-1^lo patient sample. Data are mean ± s.e.m. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, two-sided Student’s t-test. ns, not significant.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Phenotypic, functional, transcriptional and epigenetic characterization of TCR_{TAG} and TCR_{OT1} cells in liver tumours. a, Approximately $3 \times 10^4$ TCR_{TAG} (TAG, red; Thy1.1^+) and TCR_{OT1} (OT1, black; Ly5.1^+) T cells were transferred into wild-type B6 mice or liver tumour-bearing AST \times Alb-Cre mice and immunized with $5 \times 10^6$ CFU of *Listeria Lm^{TAG-I-OVA}*. Three to four weeks after immunization, livers from AST \times Alb-Cre mice and spleens from B6 mice were analysed for the presence of donor TAG and OT1 T cells by FACS; the percentages of CD8 T cells are shown. Expression of CD62L, CD44, CD69 and Ki67 of TAG and OT1 T cells. Naive T cells are shown in grey as a control. CD107 expression after 4-h TAG or OVA peptide stimulation of TAG and OT1 TILs isolated 3–4 weeks after transfer. Flow plots are gated on CD8^+Thy1.1^+ and CD8^+Ly5.1^+ cells. Data are representative of three independent experiments. b, Heat map of RNA-seq-normalized expression values (log2(counts per million)) across all samples (colour corresponds to z-scores) for genes differentially expressed between TAG and OT1 T cells (FDR < 0.05). c, GSEA of RNA-seq data generated from TAG and OT1 T cells isolated from AST \times Cre liver lesions 3 weeks after adoptive transfer and *Listeria* infection. Gene sets used: T cell exhaustion during chronic viral infection^{20} (GEO accession GSE30962) and mutant/constitutively-active form of NFAT1-overexpressing CD8 T cells^{21}. NES, normalized enrichment score. d, Venn diagrams showing the numbers and percentage of significantly opening (left) and closing (right) peaks between TAG and OT1 T cells (FDR < 0.05, log2-transformed fold change > 2). e, Genome browser view of ATAC-seq signal intensities of TAG and OT1 T cells at *Pdcd1, Entpd1, Cd38* and *Cd244* loci. Red or blue boxes indicate peaks that become significantly more accessible or inaccessible in TAG versus OT1 T cells, respectively. ATAC-seq peaks from naive TAG T cells are shown in grey as a control. f, Chromatin accessibility heat map for TAG and OT1 T cells. Each row represents one peak (differentially accessible between TAG and OT1 T cells; FDR < 0.05) displayed over a 2-kb window centred on the peak summit; regions were clustered using k-means clustering. Genes associated with individual clusters are highlighted.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Chromatin accessibility of the mouse and human Tox locus. a, Accessibility of TOX and TCF7 loci in human tumour-infiltrating PD-1hiCD8+ T cells. ATAC-seq signal profiles of TOX (left) and TCF7 (right) in naive CD8+ CD45RA+ (grey), CD8+ CD45RO+ CD62L+ central memory T cells (green) and CD8+ CD45RO+ PD-1hi TILs isolated from patients with melanoma and lung cancer (red). Red or blue boxes, respectively, indicate peaks that become accessible or inaccessible in PD-1hi TILs as compared to naive or memory T cells. Naive and memory T cells were isolated from PBMCs of healthy donors. b, c, NFAT1 binds to differentially accessible regions in the Tox locus in mice and pharmacological targeting of NFAT1 reduces TOX expression. b, Genome browser view of the Tox locus and numerous ATAC-seq and ChIP-seq tracks. On top, ATAC-seq signals of naive (N; grey), effector (E5, E7; green), memory (M; green), dysfunctional liver tumour-infiltrating TCR.Tag cells (blue series, with D indicating the days after transfer when T cells were isolated from liver lesions) are shown. These data are from ref. 5. These are followed by newly generated ATAC-seq data from TCR.Tag (TAG; orange) and TCR.OT1 (OT1; green) cells from ASTM Cre liver lesions (as described in Fig. 2) as well as NFAT1 ChIP-seq tracks generated previously22 representing wild-type NFAT1 (blue) and mutant/constitutive active NFAT1-overexpressing T cells (red) (with and without stimulation). The vertical bars at the bottom of the plot represent statistically significantly enriched NFAT1-binding sites (peaks) as well as regions with statistically significantly changing accessibility between ATAC-seq of OT1 and TAG T cells. Red stars and pink boxes highlight NFAT1-binding sites that overlap with regions of increased chromatin accessibility in dysfunctional TCR.Tag compared to TCR.OT1 cells. c, Pharmacological targeting of NFAT signalling decreases TOX expression in vivo. Naive TCR.Tag (Thy1.1+) cells were transferred into AST×Cre;Er22 (Thy1.2+) mice, which were treated with tamoxifen (Tam) 1 day later. At days 2–9, mice were treated with the calcineurin inhibitor FK506 (2.5 mg per kg per mouse; blue, n = 3) or PBS (control group; black, n = 3). At day 10, TCR.Tag cells were isolated from livers and assessed for expression of CD44, TOX, PD-1 and TCF-1. Linear regression analysis of MFI values are shown. Naive TCR.Tag cells are shown in grey as a control (n = 1). Each symbol represents an individual mouse. $R^2 = 0.6886$ (TOX/TCF-1); $R^2 = 0.947$ (TOX/PD-1); data are representative of two independent experiments. Dotted lines represent 95% confidence interval.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Ectopic expression of TOX in T cells in vitro induces a molecular signature of T cell exhaustion. a, Gating strategy for TOX–GFP-expressing (blue) and GFP-expressing (green) TCR<sub>TAG</sub> cells, and their corresponding TOX expression levels. TOX isotypes are shown for each sample. Naive TCR<sub>TAG</sub> cells adoptively transferred into AST × Cre mice and isolated from liver tumours after transfer (red), and naive TCR<sub>TAG</sub> cells (grey) are shown as controls. Inset numbers show MFI values. b, Heat map of RNA-seq expression values (row normalized log<sub>2</sub>(counts per million)) for genes differentially expressed between TOX–GFP and GFP TCR<sub>TAG</sub> cells (FDR < 0.10). c, Relative expression of selected genes as determined by digital droplet PCR. Data show raw droplet counts normalized to the housekeeping gene, Gapdh; n = 2 (TOX–GFP, GFP). d, Flow cytometric analysis of PD-1, 2B4, CD160, CD39 and TIM-3 expression levels of TOX–GFP (n = 3) or GFP (n = 3)-expressing TCR<sub>TAG</sub> cells. e, FACS analysis of TOX expression (left) on day 6 after spinfection of TCR<sub>TAG</sub> cells transduced with TOX–GFP (n = 2) or GFP (n = 2), and cytokine production (right) after 4-h peptide stimulation. f, Percentage of Ki67<sup>+</sup> cells (top), and GZMB<sup>+</sup> cells (with or without 4-h peptide stimulation) (bottom) in TCR<sub>TAG</sub> cells transduced with TOX–GFP (blue, n = 3) or GFP (green, n = 3). Naive TCR<sub>TAG</sub> cells are shown in grey as a control (n = 1). Data are mean ± s.e.m and representative of two independent experiments (n = 3 per experiment, with n representing a biological replicate/individual transduced spleen). *P ≤ 0.05, **P ≤ 0.01, two-sided Student's t-test. g, GSEA of TCR<sub>TAG</sub> cells transduced with TOX–GFP or GFP. T cell exhaustion gene sets used: tumour-specific T cell dysfunction<sup>5</sup> (left), and T cell exhaustion during chronic viral infection<sup>20</sup> (GEO accession GSE30962) (right). Corresponding heat maps with selected genes with significant enrichment scores are shown below.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Phenotypic and functional characterization of TOX wild-type and knockout TCRTAG mice. a, Mouse strains generated and used in this study. We define wild type as littermate controls TCRTAG;DLck-Cre;Tox+/+ or TCRTAG;Tox+/+. We define knockout as TOX-deficient T cells from TCRTAG;DLck-Cre;Tox/fl/fl mice. b, Thymocytes and peripheral CD8 T cells from knockout mice develop normally. CD4 and CD8 flow staining of thymocytes isolated from knockout (red, n = 5) or littermate controls (grey, n = 3). TCR Vβ7 and CD44 expression, and enumeration of single-positive CD8+ thymocytes from knockout and wild-type mice. c, Enumeration of total splenocytes (n = 5) and CD8+ splenocytes (n = 4) of knockout and wild-type mice. d, e, TOX is not required for effector and memory CD8 T cell differentiation during acute Listeria infection. d, Approximately 1 × 10^5 congenically marked naive wild-type and knockout TCRTAG cells were adoptively transferred into B6 mice, and infected with Listeria 1 day later. Flow cytometric analysis of CD44, CD62L, CD127 and KLRG1 expression directly ex vivo (inset numbers show percentage in respective quadrants) of wild-type and knockout effector TCRTAG cells isolated from spleens of LmTAG-immunized B6 mice 7 days after immunization. e, Flow cytometric analysis of CD44, CD62L, CD127 and KLRG1 expression of wild-type and knockout TCRTAG cells isolated from spleens of LmTAG-immunized B6 mice 3 weeks after immunization. Right, intracellular IFNγ and TNF production after 4-h ex vivo TAG peptide stimulation of wild-type (n = 4) and knockout (n = 4) memory TCRTAG T cells. Flow plots are gated on CD8+Thy1.1+ cells. Data are representative of at least three independent experiments. f-i, Phenotypic and functional characterization of TOX wild-type and knockout TCRTAG cells differentiating in developing liver tumours of AST×Cre mice. f, Top, CD44, CD69, CD25 and PD-1 expression and CellTrace Violet (CTV) dilution of adoptively transferred, CTV-labelled naive wild-type (black) or knockout (red) TCRTAG cells isolated from livers of AST×Cre mice 3 days after transfer. Data are representative of three independent experiments. Middle, expression of CD44 and proliferation (CTV dilution) of wild-type (black) or knockout (red) TCRTAG cells isolated from AST×Cre liver lesions 5 days after transfer. CTV-labelled TCRTAG cells transferred into B6 control mice are shown in grey as controls transferred and isolated at the same time points. Bottom, PD-1 and LAG-3 expression together with TOX expression of wild-type and knockout TCRTAG cells isolated from the livers of AST×Cre mice 8 days after transfer. All FACS plots are gated on CD8+ and Thy1.1+. g, Flow cytometric analysis of intracellular IFNγ and TNF production (top), CD107 degranulation (middle), and GZMB expression (bottom) of day 7–10 wild-type (black) or knockout (red) TCRTAG cells after 4-h peptide stimulation. h, i, PMA and ionomycin stimulation (h) or 4-h peptide stimulation using in vivo LPS-activated APCs (i). Each sample is gated on its respective no-peptide control. All flow plots are gated on CD8+Thy1.1+ T cells. Data are representative of three independent experiment and shown as mean ± s.e.m. P values determined by two sided Student’s t-test.
Extended Data Fig. 8 | TOX wild-type and knockout TCR\textsubscript{TAG} cells reveal differences in genes and proteins associated with apoptosis.

**a**, Flow cytometric analysis of PD-1 \((n = 3 \text{ (KO)}; n = 5 \text{ (WT)})\), LAG-3 \((n = 4 \text{ (KO)}; n = 5 \text{ (WT)})\), CD38 \((n = 4 \text{ (KO)}; n = 5 \text{ (WT)})\), 2B4 \((n = 2 \text{ (KO)}; n = 3 \text{ (WT)})\), and TCF-1 \((n = 4 \text{ (KO)}; n = 5 \text{ (WT)})\), expression levels in wild-type (black) or knockout (red) TCR\textsubscript{TAG} cells isolated from liver lesions approximately 3 weeks after adoptive transfer. Data are representative of at least three independent experiments.

**b**, Flow cytometric analysis of TOX wild-type (black) and knockout (red) TCR\textsubscript{TAG} cells isolated 7–10 days after transfer from AST\textsubscript{×}Cre liver lesions. BIM, BCL-2, and BCL-xL expression levels were assessed directly ex vivo. Each pair of symbols represents an individual mouse \((n = 4)\). Data are representative of two independent experiments.

**c**, Flow cytometric analysis of active caspases 3 and 7 in TOX wild-type (black) and knockout (red) TCR\textsubscript{TAG} cells. These data are combined results of two experiments \((n = 11)\). Each pair of symbols represents an individual mouse.

**d**, Representative histograms and quantification of annexin V\(^+\) wild-type (black, \(n = 3\)) and knockout (red, \(n = 3\)) TCR\textsubscript{TAG} cells isolated 7–10 days after transfer from AST\textsubscript{×}Cre liver lesions. Data are mean ± s.e.m. \(*P \leq 0.05\), \(**P \leq 0.01\), \(***P \leq 0.001\), two-sided Student’s t-test.

**e**, GSEA of DEGs between TOX wild-type and knockout T cells. ‘Hallmark\_apoptosis’ and ‘wikipathways\_MM\_apoptosis\_WP254’ gene sets show normalized enrichment score (NES) of \(-1.52\) and \(-1.1\), respectively, and the corresponding heat maps of genes with significant enrichment scores are shown. Data are mean ± s.e.m.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | TOX wild-type and knockout TCR_{TAG} cells reveal transcriptional and chromatin accessibility changes. a, Heat map of RNA-seq expression (row normalized log_{2}(counts per million)) for genes differentially expressed between TOX wild-type and knockout TCR_{TAG} cells (FDR < 0.05). b, GSEA between wild-type and knockout TCR_{TAG} T cell exhaustion gene sets used: tumour-specific T cell dysfunction{superscript}5 (left) and T cell exhaustion during chronic viral infection{superscript}20 (GEO accession GSE30962) (right). Selected genes with significant enrichment score are listed. c, Pie chart showing the proportions of reproducible ATAC-seq peaks in indicated regions for all peaks within the atlas. d, Venn diagrams showing the numbers and percentages of significantly opening (top) and closing (bottom) peaks between TOX wild-type and knockout TCR_{TAG} cells (FDR < 0.05, log_{2}-transformed fold change > 2). e, Gains and losses of regulatory elements for the top 100 most DEGs between TOX wild-type and knockout TCR_{TAG} cells that were part of the gene set of tumour-specific T cell dysfunction{superscript}5. The plot is divided into top and bottom 50 genes with the highest and lowest respective log_{2}-transformed fold change of gene expression. Each gene is illustrated by a stack of diamonds, in which each diamond represents a region of high chromatin accessibility (peak) overlapping with the locus of the respective gene. Red diamonds denote peaks that are more accessible in wild-type (and less accessible in TOX KO) T cells; blue diamonds denote peaks that are more accessible in TOX knockout T cells. f, Molecular function (GO terms) enriched in genes associated with peaks that are more accessible in TOX knockout versus wild-type T cells. g, ATAC-seq signal profiles across the Pdcd1 and Entpd1 loci. Peaks less accessible in knockout TCR_{TAG} cells are highlighted in red.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Comparison of functional TOX+/+ OT1 and dysfunctional TOX knockout T cells in tumours with proposed model on the role of TOX in tumour-specific CD8 T cell exhaustion and dysfunction. a, DEGs of the TAG versus OT1 comparison (see Fig. 2) were compared with DEGs of the wild-type versus TOX-knockout comparison (see Fig. 4). There were 389 genes identified to be significantly differentially expressed in both (WT vs KO and TAG vs OT1). b, Heat map of normalized expression values (log2(counts per million)) across all samples (colour corresponds to z-scores) for these 389 genes. Selected genes of interest are highlighted. c, Proposed model on the role of TOX in tumour-specific CD8 T cell exhaustion and dysfunction. Top, antigen-specific T cells in solid tumours are continuously triggered with tumour antigen. Chronic TCR stimulation leads to NFAT-mediated expression of TOX. TOX induces a transcriptional and epigenetic program and phenotype associated with exhaustion, including the expression of numerous inhibitory receptors (for example, PD-1, LAG-3, 2B4, CD39 and CD38) and downregulation of transcription factors (such as TCF-1). The TOX-mediated exhaustion program prevents T cells from overactivation or overstimulation and activation-induced cell death. Bottom, TOX-deficient T cells do not upregulate inhibitory receptors, become overstimulated or overactivated, and eventually undergo activation-induced cell death. Despite their non-exhausted phenotype, TOX-deficient T cells are dysfunctional.
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.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of 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 statistics 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 possible.
- 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
- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection
- STAR v2.6.0c
- featureCounts v1.6.2
- FastQC v0.11.7
- QoRTs v1.3.0
- DESeq v1.18.1
- BWA v0.7.17
- picard v2.18.9
- macs2 2.1.1.20160309
- DiffBind v2.10.0
- bamCoverage v3.1.3
- multiFgerwigSummary v3.1.3
- Homer v4.9.1.5

Data analysis
- RNA-seq data was aligned with STAR default parameters to the mouse reference genome (GRCm38). Gene expression estimates were obtained with featureCounts using composite gene models (union of the exons of all transcript isoforms per gene) from Gencode (version M17). The quality of the sequenced reads was assessed with FastQC and QoRTs. Differentially expressed genes (DEG) were determined with DESeq2. The q-value cut-offs for the final lists of DEG were as follows: TOX-GFP vs. GFP: 849 DEG with q-value smaller than 0.10, TAG vs. OTI: 2347 DEG with q-value smaller than 0.05, and WT vs. TOX KO: 679 DEG with q-value smaller than 0.05.
ATAC-seq data was processed following the recommendations of the ENCODE consortium [The ENCODE Consortium, n.d.]: Reads were aligned to the mouse reference genome (version GRCm38) with BWA-backtrack. Post-alignment filtering was done with samtools and Picard tools to remove unmapped reads, improperly paired reads, non-unique reads, and duplicates. To identify regions of open chromatin represented by enrichments of reads, peak calling was performed with MACS2. For every replicate, the narrowpeak results of MACS2 were used after filtering for adjusted p-values smaller than 0.01. Regions where the chromatin accessibility changed between different conditions were identified with diffBind; with the following options: minOverlap=4, bUseSummarizeOverlaps=T, minMembers=2, bFullLibrarySize=TRUE. 12,166 differentially accessible peaks were identified between WT and KO TCR TAG, 19,071 differentially accessible peaks were identified between TCR-TAG and TCR-OT1. Coverage files individual coverage files per replicate normalized for differences in sequencing depths between the different samples were generated with bamCoverage of the deepTools suite [Ramirez et al. 2016] using the following parameters: --bs 10 --normalizeUsingRPGC --effectiveGenomeSize 2150570000 --blacklistFileName mm10 blacklist --ignoreForNormalization chrX chrY --ignoreDuplicates --minFragmentLength 40 -p 1. To create merged coverage files of replicates of the same condition, we used multiBigWigSummary to obtain the sequencing-depth-normalized coverage values for 10 bp bins along the entire genome, i.e., for every condition, we obtained a table with the coverage values in every replicate within the same bin. Subsequently, we chose the mean value for every bin to represent the coverage in the resulting “merged” file (see github.com/friedue/Scott2019 for the actual code that was used).

Flow cytometry: FlowJo (v10.4.1); Treestar[], FACSDiva (8.0.2; BD) Graphs: Prism (v7); Graphpad
Figure Layout: Powerpoint 2016 (Microsoft) ddPCR: QuantaSoft (Bio-Rad)
All custom scripts are deposited at www.github.com/friedue/Scott2019

For manuscripts utilizing custom algorithms or software that are not yet described in published literature, software must be made available to editors/reviewers upon request. 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 data generated and supporting the findings of this study are available within the paper. The RNA-Seq and ATAC-seq data have been deposited in the Gene Expression Omnibus (GSE126974). Source Data are provided with the online version of the paper. Additional information and materials will be made available upon request.

Field-specific reporting

Please select 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/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were not predetermined. The group sizes of mice, samples including human samples were chosen based on the numbers we used for previous publications to generate statistically significant results. |
|-------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Flow Cytometry: Samples from mice or human patients were excluded if CDB T cell population of interest could not be identified/found within sample/tissue by flow cytometric analysis. This was a pre-established criteria. |
| | TOX-overexpressing T cells RNA-seq: We excluded one sample from each condition (TOX-GFP and GFP control) because they clustered distinctly from all other samples (in PCA plot). The excluded samples were generated in a separate experiment and sorted on a different day on a different flow sorter machine. We do not know the factors that were causing the outlier. This was not a pre-established criteria. |
| | ATAC-seq: Two samples were excluded from analysis, because they revealed low FRIP scores (which is a standard analysis recommended by ENCODE) compared to the other samples. Low FRIP score could be due to suboptimal sample preparation techniques. This was a pre-established criteria. |
| Replication | All data was reliably reproduced. The precise number of repeats and sample sizes are provided in each figure legend. |
| Randomization | Mice used in this study were sex- and age-matched and then randomly assigned to experimental groups. |
| Blinding | Investigators were not blinded to group allocation during experimental setup, data collection and/or analysis. No blinding was performed due |
Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ | Unique biological materials |
| ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☒ | Palaeontology |
| ☒ | Animals and other organisms |
| ☒ | Human research participants |

Methods

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

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

All unique materials will be made available by authors upon request.

Antibodies

Antibodies used

For Flow Cytometry:

All antibodies were used at a 1:200 dilution unless otherwise noted.

Mouse Antibodies

Name Clone Vendor Fluorophore Catalog No. Lot No. Dil.

2B4 m2B4 Biolegend FITC 133504 B256991

Annexin V N/A BD V450 565056 7345547 1:100

BCL-2 BCL-1OC4 Biolegend PE-Cy7 633512 B252888

BCL-xL H-S Santa Cruz APC sc-8392 12815

BIM C345C Cell Signalling FITC 948055 1

CD101 Moushi101 eBioscience PE-Cy7 25-1011-82 4347264

CD107a 1D4B BD FITC 553793 B232618

CD107b ABL-93 BD FITC 558758 7146708

CD11c N418 Biolegend PE-Cy7 113738 B222652

CD127 A7R34 Biolegend APC-Cy7 135040 B242148

CD19 G05 Biolegend PerCP-Cy5.5 115549 B267443

CD25 PC61.5 Torbo PE-Cy7 60-0251-U025 C0251060118602

CD3 145-2C11 Biolegend PerCP-Cy5.5 100328 B249753

CD8 BD Biolegend FITC 102705 B255499

CD39 DuHa59 Biolegend PE-Cy7 143806 B262675

CD40 3/23 Biolegend APC 124612 B264579

CD44 IT7 Torbo FITC 35-0441-U100 CD41031618353

CD62L MEL-14 Biolegend APC-Cy7 104428 B233851

CD69 H1.2F3 Torbo FITC 35-0691-U100 C0691050118353

CD70 FR70 Biolegend PE 104606 B254477

CD8 53-6.7 Biolegend BV650 100472 B261468

CD8 53-6.7 eBioscience APC-e780 47-0081-82 4322567 1:800

CD80 16-10H1 Biolegend BV650 104732 B247737

CD86 GL-1 Biolegend FITC 105066 B243722

CD90.1 Hi5S1 eBioscience APC-e780 47-0900-82 4340103 1:500

CD90.1 OK-7 Biolegend BV650 202533 B276510 1:500

CD90.2 30-H12 Biolegend BV421 105341 B240026 1:400

CD90.2 53-2.1 Biolegend APC 140312 B241503 1:400

CD95 jo2 BD FITC 554257 50233857

CTLA-4 UC10-F410-11 Torbo PE-Cy7 50-1522-U100 C1522031918603

CXR5 L138D7 Biolegend PE 145503 B248537

Eomes Dam11mag eBioscience PE-Cy7 46-4873-82 4336373 1:100

F4/80 BM8 Biolegend BV421 123331 B258771

GZMB G811 Biolegend AF647 515406 B205770 1:100

IFNγ XMG1.2 Biolegend BV421 505829 B269386 1:400

IL-2 JES6-5H4 Biolegend BV421 1503826 B242779

K67 B65 BD FITC 556026 S112714 1:100
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  Plat-E were purchased from Cell Biolabs, B16-F10 and EL4 were purchased from ATCC.

Authentication  All cell lines were purchased directly from the manufacturer. They were not authenticated but had phenotypic and functional characteristics as previously reported.

Mycoplasma contamination  B16 and EL4 cell lines were tested for Mycoplasma contamination and test confirmed that these cell lines are negative for Mycoplasma. Plat-E were not tested for mycoplasma.

Commonly misidentified lines  No commonly misidentified cell lines were used.

Animals and other organisms

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

Laboratory animals  AST [Albumin-floxStop-SV40 large T antigen (TAG)] mice were previously described.31 TCRαTg transgenic mice [B6.Cg-Tg(TcraTcrb)1A1G6revj/J]32, Cre-ERT2 (B6.D2-129-Gt[ROSA]26Sortm1(CreERT2)Tg/J), AlsCre (B6.Cg-Tg(AlsCre)Z1Mgn/J), TCRαTg (C57BL/6-Tg[TcraTcrb]1HdbMub/J), Ly5.1 (B6.129-Plp1rca PepcPbsm), B6.Cg-Tg(Lck-Ires-tdTomato77/9Niv/I) (B6.Cg-Tg(Lck-Ires-tdTomato77/9Niv/I)), and C57BL/6] Thy1.1 mice were purchased from The Jackson Laboratory. TOXlox/Flox mice were previously described, and obtained from M. Glickman, MSKCC, with the permission from J. Kaye. TOXlox/Flox mice were crossed to TCRαTg and Lck-Cre to obtain TCRαTg TOXlox/Flox mice. TCRαTg and Lck-Cre mice were crossed to Thy1.1 mice to generate TCRTRP and TCRαTg Thy1.1 mice, respectively. TCRαTg and Lck-Cre mice were crossed to Ly5.1 mice to generate TCRαTg ly5.1 mice. AST mice were crossed to Cre-ERT2 or AlsCre mice to obtain ASTCre-ERT2 and ASTAlsCre mice, respectively. TCRPMEL and TCR14 mice were purchased from The Jackson Laboratory. AST mice were also crossed to Thy1.1 mice to generate ASTCre-ERT2 Thy1.1 mice. All mice were bred and maintained in the animal facility at Memorial Sloan Kettering Cancer Center (MSKCC). Experiments were performed in compliance with the MSKCC Institutional Animal Care and Use Committee regulations. For any additional information please contact the corresponding author.

Wild animals  This study did not involve wild animals.

Field-collected samples  This study did not involve samples collected from the field.

Human research participants

Policy information about studies involving human research participants

Population characteristics  N/A (as per rules and regulations, patient information is not disclosed for this study).

Recruitment  Peripheral blood mononuclear cells (PBMC) and tumor samples were obtained from cancer patients enrolled on a biospecimen
Recruitment

procurement protocol approved by the MSKCC institutional review board (IRB). Each patient signed an informed consent form and received a patient information form prior to participation. Human samples were analyzed using an IRB approved biospecimen utilization protocol. Breast cancer samples were selected from patients who had evidence of a dense mononuclear cell infiltrate on conventional H&E staining. For human ovarian tumor samples (Extended Data Figure 3): tumor samples were obtained as per protocols approved by the IRB. All patients provided informed consent to an IRB-approved correlative research protocol before the collection of tissue (Memorial Sloan Kettering Cancer Center IRB #00–144 and 06–107). Human peripheral blood lymphocytes were obtained from the New York Blood Center and from patients where indicated.

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
The sample preparation procedure is detailed in "METHODS SUMMARY: Cell isolation for subsequent analyses".

Instrument
The cytometers used were: BD LSRII Fortessa and BD FACSAria.

Software
FACSDiva (BD) was used for sample collection. FlowJo (Treestar) was used for data analysis.

Cell population abundance
Samples were sorted directly into Trizol (for RNA-seq) and 100% FCS (for ATAC-seq) and thus post sort purity was confirmed using a separate aliquot of sample.

Gating strategy
Gating strategies are shown in the figure section and explained in the "METHODS SUMMARY: adoptive transfer studies during acute Listeria infection and ASTxCre tumor models" and "sample preparation for ATAC-seq and RNA-seq". Further gating strategies will be provided upon request.

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