The expression of co-inhibitory receptors, such as CTLA-4 and PD-1, on effector T cells is a key mechanism for ensuring immune homeostasis. Dysregulated expression of co-inhibitory receptors on CD4+ T cells promotes autoimmunity, whereas sustained overexpression on CD8+ T cells promotes T cell dysfunction or exhaustion, leading to impaired ability to clear chronic viral infections and diseases such as cancer1,2. Here, using RNA and protein expression profiling at single-cell resolution in mouse cells, we identify a module of co-inhibitory receptors that includes not only several known co-inhibitory receptors (PD-1, TIM-3, LAG-3 and TIGIT) but also many new surface receptors. We functionally validated two new co-inhibitory receptors, activated protein C receptor (PROCR) and podoplanin (PDPN). The module of co-inhibitory receptors is co-expressed in both CD4+ and CD8+ T cells and is part of a larger co-inhibitory gene program that is shared by non-responsive T cells in several physiological contexts and is driven by the immunoregulatory cytokine IL-27. Computational analysis identified the transcription factors PRDM1 and c-MAF as cooperative regulators of the co-inhibitory module, and this was validated experimentally. This molecular circuit underlies the co-expression of co-inhibitory receptors in T cells and identifies regulators of T cell function with the potential to control autoimmunity and tumour immunity.

We used single-cell RNA sequencing (scRNA-seq) to analyse co-inhibitory and co-stimulatory receptor expression in 588 CD8+ and 316 CD4+ tumour-infiltrating lymphocytes (TILs) from B16F10 mouse melanoma. We found that the expression of Pdcd1 (also known as PD-1), Tim3 (Havcr2), Lag3, Clda4, 4-1BB (Tnfrsf9) and Tigit strongly co-vari in CD8+ TILs. CD4+ TILs showed a similar pattern with the additional co-expression of Eos, Gitr (also known as Tnfrsf18) and Oxo40 (Tnfrsf4) (Fig. 1a, top). Single-cell mass cytometry (cytometry by time of flight, CyTOF) confirmed the surface co-expression of these receptors (Fig. 1a, bottom, Supplementary Information 1). The expression of PD-1, LAG-3, TIM-3 and TIGIT was tightly correlated on both CD8+ and CD4+ TILs (Fig. 1a, bottom). Clustering analysis (t-stochastic neighbourhood embedding (t-SNE)5, Methods) showed two groups of CD8+ TILs (clusters 1 and 2) (Fig. 1b, Extended Data Fig. 1a, c), with PD-1, LAG-3, TIM-3 and TIGIT mainly expressed in cluster 1 cells (Fig. 1b, Extended Data Fig. 1c), in addition to LILRB4 (Extended Data Fig. 1a) and co-stimulatory receptors of the TNF receptor family, 4-1BB, OX40 and GITR. By contrast, ICOS and CD226 were less restricted to cluster 1 (Extended Data Fig. 1a). We further observed two discrete clusters of CD4+ TILs (clusters 3 and 4), with co-expression of PD-1, TIM-3, LAG-3 and TIGIT restricted to cluster 3 (Fig. 1b, Extended Data Fig. 1c).

The co-expression of co-inhibitory receptors on CD8+ and CD4+ T cells suggests a common trigger. One candidate is IL-27, a heterodimeric member of the IL-12 cytokine family that suppresses autoimmunity6, induces IL-10-secreting type 1 regulatory T (Treg) cells6,7 and induces expression of TIM-3 and PD-L1 on CD4+ and CD8+ T cells8,9. Activation of CD4+ and CD8+ T cells in the presence of IL-27 induced the expression of TIM-3, LAG-3 and TIGIT at both the mRNA (Fig. 1c) and protein levels (Extended Data Fig. 2a). mRNA expression of Tim3 (Havcr2), Lag3 and Tigit was reduced in IL-27-RA-deficient T cells, whereas Pdcd1 expression was unaffected by IL-27 in vitro (Fig. 1c, Extended Data Fig. 2a).

CyTOF analysis showed that the loss of IL-27RA resulted in the loss of cells in cluster 1 of CD8+ TILs and cluster 3 of CD4+ TILs (Fig. 1d, P = 5 × 10−23 and 6.8 × 10−7 for CD8+ and CD4+, respectively, hypergeometric test; Extended Data Fig. 2b–d), indicating a key role for IL-27 in driving co-inhibitory receptor co-expression in both CD4+ and CD8+ T cells in vivo. Although PD-1 expression was not dependent on IL-27 in vitro, it was dependent on IL-27RA signaling in vivo. Consistent with the induction of IL-10 by IL-275–7, we observed reduced IL-10 in IL-27RA-knockout CD8+ TILs (Extended Data Fig. 2b).

scRNA-seq of CD8+ and CD4+ TILs from wild-type and IL-27RA-knockout mice (Fig. 1e, Extended Data Fig. 3a, b, Methods) revealed distinct clusters of CD8+ (cluster 5) and CD4+ (cluster 4) TILs that highly expressed the co-inhibitory receptors Pdcd1, Tim3, Lag3 and Tigit. The expression of these genes was decreased in CD8+ TILs from IL-27RA-knockout mice, whereas the expression of only Tim3 and Lag3 was decreased in CD4+ TILs from IL-27RA-knockout mice (Fig. 1e). Thus, IL-27 drives a module of co-inhibitory receptors that are strongly co-expressed in vivo together with IL-10.

The co-inhibitory receptor module could be part of a larger IL-27-driven inhibitory gene program. We analysed the mRNA profiles of CD4+ and CD8+ T cells stimulated in the presence or absence of IL-27. IL-27 induced similar expression programs in CD4+ and CD8+ T cells (Extended Data Fig. 4a, b). We identified 1,201 genes with IL-27-dependent expression (Methods). We compared the IL-27-driven gene program to the gene signatures for four different states of T cell non-responsiveness: CD8+ T cell exhaustion in both cancer5 and chronic viral infection10, and antigen-specific11 and non-specific (anti-CD3 antibody12) CD4+ T cell tolerance. We found a significant overlap with all of these signatures (Methods, Extended Data Fig. 4c–f).

Projection of the IL-27 and CD8+ cancer T cell exhaustion overlap signature onto the single-cell profiles of CD8+ TILs marked a distinct subset of cells (Fig. 2a, panel I). This subset scored highly for the overlap signatures between the IL-27-driven gene program and each of the
Fig. 1 | Several co-inhibitory receptors are expressed as a module on CD4+ and CD8+ T cells. a, CD4+ and CD8+ TILs were obtained from wild-type (WT) mice bearing B16F10 melanoma tumours. Top, co-expression analysis of co-inhibitory and co-stimulatory receptor mRNA expression as determined by scRNA-seq for 316 CD4+ and 588 CD8+ TILs. Bottom, protein expression by CyTOF for 23,656 CD4+ and 36,486 CD8+ TILs. Spearman correlation, followed by dendrogram ordering of the matrix using Euclidian distance, is shown. Data are from biologically independent experiments. H vem is also known as Tnfrsf14. b, TILs from wild-type mice bearing B16F10 melanoma were analysed using CyTOF with a custom panel of antibodies against co-inhibitory and co-stimulatory cell-surface receptors24 (Supplementary Table 1). Data were analysed using viSNE. Polygons indicating clusters 1 and 2 (in CD4+ T cells), and 3 and 4 (in CD4+ T cells) are shown. Individual panels show expression of the indicated markers. c, Naïve T cells from either wild-type or IL-27RA-knockout (KO) mice were stimulated with anti-CD3/CD28 in the presence or absence of IL-27. The indicated expression of co-inhibitory receptors was examined by quantitative PCR (qPCR) at 96 h (CD4) and 72 h (CD8). Data are mean ± s.e.m. from biologically independent animals. d, viSNE plot showing wild-type (red) and IL-27RA-knockout (blue) cells. e, scRNA-seq of TILs from mice bearing B16F10 melanoma. Data were analysed using t-SNE. Polygons indicating clusters 4 (in CD4+ T cells, orange) and 5 (in CD8+ T cells, blue) are shown. Individual panels show expression of the indicated markers. Bar graphs show the mean signal intensity for indicated co-inhibitory receptors from: WT (CD4+ n = 849); CD8+ (n = 1752); WT IL-27RA KO (CD4+ n = 628); CD8+ (n = 541) TILs for CyTOF (d) or WT (CD4+ n = 707); CD8+ (n = 825) and IL-27RA-KO (CD4+ n = 376); CD8+ (n = 394) TILs for scRNA-seq (e). Error bars indicate s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001, two-sided t-test.
and PDPN, were highly expressed in the setting of cancer (Fig. 2c). Activation of naive CD4+ and CD8+ T cells in vivo in the presence of IL-27 induced the expression of PROCR and PDPN (Extended Data Fig. 5a). In vivo, PROCR and PDPN exhibited IL-27-dependent co-expression with PD-1 and TIM-3 on CD8+ TILs (Extended Data Fig. 5b).

PROCR+ CD8+ TILs exhibited an exhausted phenotype, producing less TNF and IL-2 and more IL-10 than PROCR- CD8+ TILs (Extended Data Fig. 5c). The growth of B16F10 melanoma was inhibited in PROCR hypomorph (Procrd/d, hereafter Procrd) mice (Fig. 2d), and Procrd/d CD8+ TILs mice exhibited enhanced production of TNF, but no difference in the production of IL-2, IFN-γ or IL-10 (Fig. 2e). Procrd/d TILs exhibited a decreased frequency of TIM-3hi and PD-1hi CD8+ T cells, suggesting that PROCR signalling promotes a severely exhausted phenotype in CD8+ T cells14 (Fig. 2f). Adoptive transfer of CD8+ T cells that lack PROCR revealed a T cell-specific role for PROCR in constraining tumour growth (Extended Data Fig. 5d).

Although PDPN can limit CD4+ T cell survival in inflamed tissues15, its role in T cell exhaustion is unknown. We observed a significant delay in B16F10 tumour growth in mice with PDPN deficiency in T cells (PDPN conditional knockout (cKO)) (Fig. 2g). PDPN-deficient CD8+ TILs exhibited enhanced TNF production but no significant difference in IL-2, IFN-γ or IL-10 (Fig. 2h). The frequency of TIM-3hi and PD-1hi CD8+ T cells was decreased, indicating a reduced accumulation of T cells with a severely exhausted phenotype in PDPN cKO mice14 (Fig. 2i). Consistent with previous data15, PDPN-deficient PD-1+ TIM-3hi CD8+ TILs had higher expression of IL-7RA, indicating that PDPN may limit the survival of CD8+ TILs in the tumour microenvironment (Extended Data Fig. 5e, f).

We identified the transcription factor PRDM1 as a candidate regulator of the co-inhibitory module. PRDM1 is induced in vivo by IL-27 in CD4+ and CD8+ T cells (Extended Data Fig. 6a), is enriched in TILs with high expression of the IL-27 co-inhibitory module (Extended Data Figs. 3c–f, 6b, c, Methods), and is overexpressed in exhausted CD8+ TILs (P = 0.0004, t-test, Extended Data Fig. 6d). Network analysis based on profiling of naive CD8+ T cells from mice with a T cell-specific deletion of PRDM1 (PRDM1 cKO) stimulated with IL-27, showed that PRDM1 regulates several genes in the IL-27 co-inhibitory module (Extended Data Fig. 6e, F = 2.32 × 10−12;
Data are mean ± s.e.m. from biologically independent animals. *P < 0.05, **P < 0.001, two-sided t-test. b, Wild-type (n = 5) and PRDM1 cKO (n = 5) mice were implanted with B16F10 melanoma. Data are mean ± s.e.m. from three biologically independent experiments. c, Left, gene expression in CD8+ TILs from wild-type and PRDM1 cKO mice bearing B16F10 melanoma was analysed by nCounter codeset (Supplementary Table 3). Differentially expressed genes are shown as a heat map. Right, expression of Maf in CD8+ TILs from wild-type and PRDM1 cKO mice as determined by qPCR. Data are mean ± s.e.m. from biologically independent animals. *P = 0.03, two-sided t-test. d, Summary data of co-inhibitory receptor protein expression on CD8+ TILs from wild-type and c-MAF cKO. Data are mean ± s.e.m. from biologically independent animals. *P < 0.05, two-sided t-test. e, Frequency of co-inhibitory receptor expression of PRDM1 cKO (grey bar) and c-MAF cKO (open bar) CD8+ TILs relative to wild type (filled bar). Data are mean ± s.e.m., calculated based on data from a and d with wild-type set to 100%. f, Left, wild-type (n = 8) and c-MAF cKO (n = 5) mice were implanted with B16F10 melanoma. Data are mean ± s.e.m. from two biologically independent experiments. Right, expression of Prdm1 in CD8+ TILs from wild-type and c-MAF cKO mice as determined by qPCR (expression levels relative to Actb).

Mice with a T cell-specific deletion in both PRDM1 and c-MAF (PRDM1/c-MAF conditional double-knockout (cDKO)) showed normal development of CD4+ and CD8+ T cells in terms of frequency and expression of memory or activation markers, although the frequency of FOXP3+ Treg cells was increased (Extended Data Fig. 8a). CD4+ and CD8+ TILs from cDKO mice bearing B16F10 melanomas exhibited a near absence of PD-1, TIM-3, LAG-3, TIGIT, PDPN and PROCR expression (Fig. 4b, Extended Data Fig. 8b). Moreover, cDKO CD8+ TILs exhibited enhanced IL-2 and TNF production (Extended Data Fig. 8c). In contrast to singly deficient mice, cDKO mice showed significant control of B16F10 tumour growth despite the increased frequency of Treg cells (Fig. 4c). We addressed whether PRDM1 and c-MAF have a cell-intrinsic role in CD8+ and CD4+ T cells in controlling tumour growth by using an adoptive transfer model. Although CD8+ T cells from cDKO were able to inhibit tumour growth with decreased expression of co-inhibitory molecules, these effects were stronger when PRDM1 and c-MAF were lacking in both CD4+ and CD8+ T cells (Fig. 4d, Extended Data Fig. 8d). We examined the roles of PRDM1 and c-MAF in tumour antigen-specific T cell responses using the MC38-OVA tumour model. We observed a significant reduction in tumour growth in mice receiving cDKO T cells as compared to mice receiving wild-type T cells (Extended Data Fig. 8e). We also observed a strong increase in ovalbumin (OVA)-specific T cells in the tumour draining lymph nodes and in OVA-specific IFN-γ- and TNF-producing CD8+ T cells in both the tumour infiltrate and the periphery in mice receiving double-knockout T cells (Fig. 4e, f, Extended Data Fig. 8f). Lastly, we observed an increase in CD8+ K67+ T cells in the periphery of mice receiving double-knockout T cells (Fig. 4f).
Fig. 4 | PRDM1 and c-MAF together regulate a co-inhibitory gene module that determines anti-tumour immunity. a, Network model based on coupling RNA-seq gene expression data of naïve CD8\(^+\) T cells from PRDM1 cKO or c-MAF cKO mice stimulated in the presence of IL-27 and PRDM1 and c-MAF ChIP–seq data. Upregulated genes (green arrows), downregulated genes (red arrows), and c-MAF or PRDM1 binding events (grey arrows) are shown. b, Summary data of indicated co-inhibitory receptors expression on CD8\(^+\) TILs from wild-type and PRDM1/c-MAF cDKO mice bearing B16F10 melanoma. Data are mean ± s.e.m. from biologically independent experiments. c, d, Tumour size. Data are mean ± s.e.m. \(*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001\), repeated measures ANOVA, Sidak’s multiple comparisons test. e, f, T cells were obtained from RAG1-knockout mice that received an adoptive transfer of CD4\(^+\) and CD8\(^+\) T cells from wild-type or cDKO mice (2:1 ratio of CD4:CD8) followed by subcutaneous injection of MC38-OVA (Extended Data Fig. 8e). e, The frequency of IFN-γ and TNF CD8\(^+\) TILs after OVA-peptide stimulation. Data are mean ± s.e.m. from biologically independent animals. \(*P < 0.05, **P < 0.01, ***P < 0.001\), two-sided t-test. g, Nine hundred and forty differentially expressed genes between CD8\(^+\) TILs from wild-type and cDKO mice bearing B16F10 melanoma. Adjusted \(P < 0.05\), likelihood ratio test and FDR correction (top) and their corresponding expression pattern in PD-1\(^-\)TIM-3\(^-\)CD8\(^+\), PD-1\(^+\)TIM-3\(^-\)CD8\(^+\), and PD-1\(^+\)TIM-3\(^+\)CD8\(^+\) TILs.

We tested for non-additive effects between PRDM1 and c-MAF by using a binomial generalized linear model to compare the effect of single knockouts to the cDKO, and found that 149 out of 940 differentially expressed genes (adjusted \(P < 0.05\), likelihood ratio test and false discovery rate (FDR) correction) between wild-type and cDKO CD8\(^+\) TILs have non-additive (that is, synergistic) effects (Extended Data Fig. 9, Methods).

Examination of the transcriptional signatures of cDKO CD8\(^+\) TILs showed significant overlap with those of CD8\(^+\) TIM-3\(^-\) PD-1\(^-\) TILs (Fig. 4g, \(P = 2.8 \times 10^{-7}\), one-sample Kolmogorov–Smirnov test; Extended Data Fig. 10a–c), suggesting that the loss of both c-MAF and PRDM1 increases the proportion of non-exhausted CD8\(^+\) effectors that exist normally in tumours. We scored the individual scRNA-seq profiles of CD8\(^+\) TILs for the cDKO 940 gene signature and found that the expression of the cDKO gene signature and the co-inhibitory gene module signature mark mutually exclusive populations of TILs (Extended Data Fig. 10e). The cDKO signature showed significant overlap with PD-1\(^-\)CXCR5\(^+\)CD8\(^+\) T cells,
which may represent precursors for functional effectors in chronic lymphocytic choriomeningitis virus (LCMV) infection\textsuperscript{23} (Extended Data Fig. 10d, e, \(P = 1 \times 10^{-13}\), one-sample Kolmogorov–Smirnov test). Furthermore, the IL-27RA-knockout TIL signature also showed significant overlap with this PD-1\(^+\)CXCR5\(^+\)CD8\(^+\) T cell signature (\(P < 2.2 \times 10^{-16}\), one-sample Kolmogorov–Smirnov test; Fig. 2a, Extended Data Fig. 10c). Collectively, our data indicate that the loss of c-MAF and PRDM1 preferentially results in loss of the co-inhibitory gene module expression and acquisition of a more responsive effector T cell state.

In conclusion, we identified a co-inhibitory gene module, which is expressed in several settings of both CD4\(^+\) and CD8\(^+\) T cell non-responsiveness, along with its transcriptional regulators. The discovery of this module provides a basis for the identification of novel co-inhibitory and co-stimulatory receptors that may have an important role in T cell regulation.

Online content
Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586-018-0206-z.

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Competing interests A.C.A. is a member of the SAB for Potenza Therapeutics and Tizona Therapeutics. V.K.K. has an ownership interest and is a member of the SAB for Potenza Therapeutics and Tizona Therapeutics. A.C.A.’s and V.K.K.’s interests were reviewed and managed by the Brigham and Women’s Hospital and Partners Healthcare in accordance with their conflict of interest policies. A.R. is an SAB member for Thermo Fisher and Syros Pharmaceuticals and is a consultant for Driver Group.

Additional information
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Mice. C57BL/6 wild-type, IL-27RA-knockout, and Prdm15 floxed mice were obtained from the Laboratory of Maffl (CD4+), Pdpn1 floxed mice and ProcGfl floxed mice were previously described. Pdpn1 floxed mice were initially obtained from C. Buckley and crossed to CD4-Cre mice to obtain conditional deletion in T cells. CD4-Cre mice were purchased from Taconic. Prdm15 floxed and Maffl floxed mice were crossed to CD4-Cre mice to generate doubly deficient T cell conditional knockout mice. All experiments were performed in accordance with the guidelines outlined by the Harvard Medical Area Standing Committee on Animals.

Tumour experiments. B16F10 melanoma cells (ATCC) (5 × 10^6) were implanted into the right flank of C57BL/6 mice. Tumour size was measured in two dimensions using a caliper. TILs were isolated by dissociating tumour tissue in the presence of 2.5 mg ml⁻¹ collagenase D for 20 min before centrifugation on a discontinuous Percoll gradient (GE Healthcare). Isolated cells were then used in various assays of T cell function. For antigen specific analysis, we applied adoptive transfer tumour experiments using T cells from Prdm15-c-Maf CDKO mice, CD4⁻ or CD8⁻ T cells sorted from CD8⁺ mice or literate controls were transferred into RAG1−/− knockout mice at a 2:1 ratio (CD4: 1 million per mouse and CD8: 0.5 million per mouse) 2 days before subcutaneous injection of B16-OVA or MC38-OVA tumour. B16-OVA was a gift from K. Wucherpfennig, and MC38-OVA was a gift from M. Smyth. For adoptive transfer tumour experiments using T cells from ProcGfl mice, CD4⁺ T cells from wild-type and CD8⁻ T cells from wild-type or ProcGfl mice were isolated by cell sorting (BD FACSAria) and transferred into RAG1−/− recipient mice at a 2:1 ratio (WT CD4⁺: 1 million per mouse and WT or ProcGfl CD8⁻: 0.5 million per mouse) 2 days before tumour implantation. Although we did not perform any immunofluorescence experimental approach to label these animals of target gene knockout and control mice were used to adequately power biological validation experiments throughout the article. All mice used are C57BL/6 background, both male and female, 6–12 weeks of age, 15–25 g. Each experiment was performed using age- and sex-matched controls (Supplementary Table 5).

CyTOF. Antibodies were labelled using MaxPar Metal Labelling Kits (DVS) by The Longwood Medical Area CyTOF Antibody Resource and Core. In some experiments, TILs were enriched using Dynabeads FlowMap Mouse Pan T (CD90.2) Kit (Invitrogen). Cells were washed and resuspended in CyTOF PBS (PBS plus 0.05% sodium azide and 0.5% BSA) and stained viability marker Rhodium (DVS) following the cocktail of antibodies against cell-surface molecules for 30 min. Cells were washed again and resuspended in CyTOF PBS with 4% paraformaldehyde. After 10 min fixation, cells were washed and barcoded with Cell-ID intercalators (DVS). Before analysis, cells were resuspended in water with beads and loaded to the CyTOF Mass Cytometer (DVS). CyTOF data were recorded in dual-count according to Fluidigm’s recommended settings that calibrated on the fly, combining pulse-count and intensity information. Data obtained as mass peaks for the channels are processed according to cell event selection criteria. These criteria include exclusion of dead cells (alt7, Prdm15⁻/⁻ cells). Single-cell selection (Intercalator-Ir), and barcoding selection (Pt194 and Pt198) to identify single-cell events from wild-type and knockout TILs for further analysis.

To obtain clusters of cells similar in their protein expression patterns, cells were clustered using k-means algorithm. Optimal cluster number was estimated using the within groups sum of squared error (SSE) plot followed by gap statistics with bootstrapping and first standard error max method. These methods suggested 9 clusters as optimal in the multidimensional space. Applying k-means clustering with k = 9 on our CyTOF data, resulted in clear distinction between cluster 1 and 2 of the CD4⁺ TILs and cluster 3 and 4 of the CD4⁻ TILs. This separation could be further visualized by two-dimensional nonlinear embedding of the protein expression profiles using t-SNE. The t-SNE plot can then be overlaid by k-means clustering results reflecting a non-biased approach to the clusters or with intensity of the different markers.

Flow cytometry. Single-cell suspensions were stained with antibodies against CD4 (RM4-5), CD8 (33-6.7), PD-1 (RMP1-30), LAG-3 (C9B7W), TIGIT (GID7), TIM-3 (S1D1), PROC (cBio1560) and PDPN (8.1.1) obtained from BioLegend. Fixable viability dye eFluor 560 (eBioscience) was used to exclude dead cells. For intracellular cytokine (ICC) staining, cells were stimulated with phorbol myristate acetate (50 ng ml⁻¹) and ionomycin (1 μg ml⁻¹) or with OVA 323-339 peptide for antigen specific experiments. Permeabilized cells were then stained with antibodies against IL-2, TNF, IFN-γ or IL-10. All data were collected on a BD LSR II (BD Biosciences) and analysed with FlowJo software (Tree Star). In brief, data were analysed by FSC/SSC gates of starting cell population (the gating strategy is exemplified in Supplementary Fig. 1). Positive gates were set based on fluorescence minus one (FMO) controls in each setting for cell surface molecules and based on unsupplemented sample for ICC staining.

In vitro stimulation. CD4⁺ and CD8⁻ T cells were purified by spleen and lymph nodes using anti-CD4 microbeads and anti-CD8α microbeads (Miltenyi Biotec) then stained in PBS with 0.5% BSA for 15 min on ice with anti-CD4, anti-CD8, anti-CD62L, and anti-CD44 antibodies (all from Biolegend). Naïve CD4⁺ or CD8⁺ CD62L⁺CD44⁻ T cells were sorted using the BD FACSAria cell sorter. Sorted cells were activated with plate-bound anti-CD3 (2 μg ml⁻¹ for CD4 and 1 μg ml⁻¹ for CD8) and anti-CD28 (2 μg ml⁻¹) in the presence of recombinant mouse IL-27 (25 ng ml⁻¹) (eBioscience). Cells were collected at various time points for RNA, intracellular cytokine staining and flow cytometry.

qPCR. Total RNA was extracted using RNeasy columns (Qiagen). Reverse transcription of mRNA was performed in a thermal cycler (Bio-Rad) using iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed in the Viia Real-Time PCR system (Applied Biosystems) using the primers for Tgαn gene expression (Applied Biosystems). Data were normalized to the expression of Actb.

Nanostring analysis. Gene expression profiling of TILs. We analysed gene expression in CD8⁻ TILs from Prdm15 or c-Maf CDKO mice bearing B16F10 melanoma collected on day 14 after tumour implantation, using a custom nanostring codelset of 397 genes representing both the IL-27-driven gene signature (245 genes) and the dysfunctional CD8⁻ TIL gene signature (245 genes) (Supplementary Table 3). Expression values were normalized by first adjusting each sample based on its relative value to all samples. This was followed by subtracting the calculated background (mean±2sd) from each sample with additional normalization by housekeeping geometric mean, in which housekeeping genes were defined as: Hprt, Gapdh, Actb and Tubb5. Differentially expressed genes were defined using the function that fits multiple linear models from the Bioconductor package limma in R with P < 0.05.

Microarray processing and analysis. Naïve CD4⁺ and CD8⁻ T cells were isolated from wild-type or IL-27RA-knockout mice, and differentiated in vitro with or without IL-27. Cells were collected at 72 h for CD4⁺ and 96 h for CD8⁻, and Affymetrix Mouse430-2.0 Arrays were used to detect the mRNA levels at these time points. Individual CEL files were RNA normalized and merged to an expression matrix using the ExpressionFileCreator of GenePattern with default parameters. Gene-specific intensities were then computed by taking the mean of each gene and sample i the maximal probe value observed for that gene. Samples were then transferred to log-space by taking log(intensity). Differentially expressed genes were annotated as genes with FDR-corrected ANOVA < 0.05 computed between the CD4 with or without IL-27 stimulation (CD4⁺ IL-27 and T helper type 0 (Th0) subpopulations, 1,202 genes). A total of 468 genes were differentially expressed between wild-type CD8⁻ T cells stimulated in the presence or absence of IL-27 (P < 0.05). Two hundred and thirty-four genes were shared between these two differentially expressed gene lists (P = 2.25 × 10⁻³⁷, hypergeometric test, background = 16,618 (union of genes expressed)). A list of 972 cell surface/cytokines genes of interest that include: cytokines, adhesion, aggregation, chemotaxis and other cell-surface molecules (Supplementary Table 4) composed using Gene Ontology (GO) annotation in Biomart was used to generate the gene subset in Fig. 2b and c.

RNA-seq gene expression profiling of tumour infiltrating cells. Tumour-infiltrating cells were isolated from wild-type or IL-27RA-knockout mice bearing B16 melanoma as sorted cells were collected within 96–well plates with 5 μl lysis buffer comprising of buffer TGL (Qiagen) plus 1% 2-mercaptoethanol (Sigma). Plates were then spun down for 1 min at 3,000 r.p.m. and immediately frozen at −80°C. Cells were thawed and RNA was isolated with 2.2 x RNAclean SPRI beads (Beckman Coulter Genomics) without final elution. The beads were then air-dried and processed immediately for cDNA synthesis. Samples were then processed using the Smart-seq2 protocol, with minor modifications applied to the reverse transcription step (M.S.K. and A.R., in preparation). This was followed by making a 25 μl reaction mix for each PCR and performing 21 cycles for cDNA amplification. Then 0.25 ng cDNA from each cell and 0.25 of the standard Illumina NexteraXT reaction mix for PCR amplification and Illumina sequencing steps. Finally, libraries were pooled and sequenced (50 × 25 paired-end reads) using a single kit on the NextSeq500 5 instrument. All CD4⁺ TIL (wild-type and IL-27RA-knockout) scRNA-seq data were generated as part of this study.
CD8+ TIL single-cell data include wild-type CD8+ TIL data from Singer et al. and wild-type and IL-27RA-knockout CD8+ single-cell data generated as part of this study. scRNA-seq data preprocessing and expression. Initial preprocessing was performed as previously described. In brief, paired reads were mapped to mouse annotation mm10 using Bowtie2 (allowing a maximum of one mismatch in seed alignment, and suppressing reads that had more than 10 valid alignments) and TPMs were computed using RSEM34, and log2(TPM + 1) values were used for subsequent analyses.

Next, we filtered out low-quality cells and cell doublets, maintaining for subsequence analysis the cells that had (1) 1,000–4,000 detected genes (defined by at least one mapped read), (2) at least 200,000 reads mapped to the transcriptome, and (3) at least 50% of the reads mapped to the transcriptome, ending with a total of 707 CD4+ and 825 CD8+ wild-type TILs and 376 CD4+ and 394 CD8+ IL-27RA-knockout TILs. We restricted the genes considered in subsequent analyses to be the genes expressed at log2(TPM + 1) ≥ 2 in at least 20% of the cells.

After removal of low-quality cells, the data were normalized using quantile normalization followed by principal component analysis (PCA). Principal components 1–10 were chosen for subsequent analysis owing to a drop in the proportion of variance explained following principal component 10. We used t-SNE35 to visualize single-cells in a two-dimensional nonlinear embedding.

scRNA-seq clustering and differential expression analysis. For the coupled data set of wild-type and IL-27RA-knockout TILs, we followed the analysis previously described. We performed batch correction using ComBat36 and the batch-corrected expression matrix was then reduced using PCA; principal components 1–13 were chosen for subsequent analysis owing to a drop in the proportion of variance explained following principal component 13. Next, we cluster the cells based on their principal component scores using the Louvain–Jaccard method using 40 nearest neighbours, and the 13 principal components, 11 clusters were detected. We then compared the composition of each cluster in terms of total number and percentage of wild-type and IL-27RA-knockout cells and found cluster 5 to be enriched for wild-type CD8 TILs (P = 0.0357, one sample t-test, Extended Data Fig. 3c, d). Projecting the IL-27 co-inhibitory gene module onto the scRNA-seq data highlighted clusters 4 and 5 (CD4 and CD8, respectively) (Extended Data Fig. 3e), further showing that in addition to the decrease in the expression of the co-inhibitory receptors PD-1, TIM-3, LAG-3, and TIMT (Fig. 1e), a significant decrease in the total IL-27 co-inhibitory gene module signature score is observed with lack of IL-27 signalling (P = 0.01, t-test, Extended Data Fig. 3f). Last, we searched for differentially expressed genes between clusters 4 and 5 and the rest of the clusters using a nonparametric binomial test.

Signature analysis of other states of T cell non-responsiveness. Given that orthogonal approaches were used to generate the various signatures, we first addressed the robustness of each signature before the comparative analysis. First, to address some of the concerns regarding the definition of these signatures, we sub-sampled the genes in each of the signatures and observed the resulting changes by projection on the single-cell data. These changes were quantified by randomly selecting decreasing subsets of genes from each signature (100%, 90%, ... 30%) and calculating the average silhouette width of the cells that scored high for the different generated signatures, based on Euclidian distance between the principal component values used to generate the t-SNE plot. This analysis shows that the signatures are relatively resilient to this procedure up to 60% of the original signature (Extended Data Fig. 4e).

Second, we calculated a signature P value per cell. The P value is calculated by generating random sets of signatures that are composed of genes with a similar average and variance expression levels as the original signature. This was followed by comparing the generated scores to the score obtained from the original signature. Cells that had a statistically significant score (adjusted P < 0.05) were marked by a plus symbol ‘+’ (Extended Data Fig. 4f).

For viral exhaustion, a microarray dataset37 was downloaded, followed by RMA. A signature of viral exhaustion was defined as the genes that are differentially expressed between chronic and acute viral infection on day 15 and day 30. Genes were ranked based on a t-test statistic and fold change, each gene rank was then adjusted for multiple hypotheses testing using FDR. A threshold of fold change ≥ 1.1 and FDR < 0.2 was applied.

For antigen-specific tolerance, data38 were downloaded. Two groups were defined, group 1 that includes the PBS and 0.008 μg treated samples (treatment number 1) versus group 2–80 μg (treatment number 5 and 6). After log2 transformation and quantile normalization, the Limma package was used to estimate the fold changes and standard errors by fitting a linear model for each gene for the assessment of differential expression. Genes with P < 0.05 were selected: 1,845 genes were upregulated of which 88 were defined as cytokine and cell surface molecules.

For antigen non-specific tolerance, data39 were downloaded. Robust multi-array average (RMA) and quantile normalization were applied for background correction and normalization using the ExpressionFileCreator module of GenePatterns.

Differentially expressed genes were defined using signal-to-noise ratio, following FDR correction. Differentially expressed genes were identified as genes having a FDR < 0.2 between mRNA expression profiles of naïve CD4+ or CD4+ GFP/IL-10+ T cells isolated from the spleen or central lymph nodes of B6NODF1–IL-10–GFP mice following nasal treatment with anti-CD3, which attenuates the progressive phase of experimental autoimmune encephalomyelitis.

For cancer, data1 were obtained. In brief, mRNA samples from CD8+ TIM-3+ PD-1- (double negative) TILs, CD8+ TIM-3+ PD-1+ (single positive), and CD8+ TIM-3- PD-1- (double positive) TILs were measured using Affymetrix GeneChip Mouse Genome 430 2.0 Arrays. Expression values were RNA normalized, corrected for batch effects using ComBat36 and gene-specific intensities were then computed by using the maximal prob intensity per gene, values were transferred to log-space by taking log(1 + intensity). Differentially expressed genes were defined as genes with either an FDR-corrected t-test P value smaller or equal to 0.2 computed between the double negative and double positive subpopulations and a fold-change of at least 1.5 between the two subpopulations.

The IL-27–Inhibitory gene module was defined as a union of the overlap between the IL-27–driven gene program (1,201 genes; see ‘Microarray processing and analysis’ section) and each of the four different states of T cell non-responsiveness mentioned above (272 genes, Supplementary Table 2).

For the IL-27RA-knockout signature, mRNA samples from FACs sorted CD8+ TILs from wild-type and IL-27RA-knockout mice bearing B16 melanomas were measured an adaptation of the SMART-Seq 2 protocol39 (see ‘RNA expression profiling of tumour infiltrating cells’ section). Differentially expressed genes were defined as genes with either an FDR-corrected t-test P value smaller or equal to 0.2 computed between the wild-type and IL-27RA-knockout, or a fold-change of at least 1.5 between the two subpopulations. IL-27RA-knockout signature was defined as 929 differentially expressed genes in IL-27RA-knockout CD8+ TILs compared to wild-type CD8+ TILs.

Single-cell gene signature computation. As an initial step, the data were scaled (z-score across each gene) to remove bias towards highly expressed genes. Given a gene signature (list of genes), a cell-specific signature score was computed by first sorting the normalized scaled gene expression values for each cell followed by summing up the indices (ranks) of the signature genes. For gene-signatures consisting of an upregulated and downregulated set of genes, two ranking scores were obtained separately, and the downregulated associated signature score was subtracted from the upregulated generated signature score. A contour plot was added on top of the t-SNE space, which takes into account only those cells that have a signature score above the mean to further emphasize the region of highly scored cells.

Network construction. Networks were generated using Cytoscape version 3.2.140. The network model is based on coupling in vitro RNA-seq gene expression data of naïve CD8+ T cells from knockout (PRDM1 or c-MAF) and wild-type controls and an in vivo RNA-seq data set of PRDM1 and c-MAF in vivo for c-MAF and predicted PRDM1-binding sites by motif scan. More specifically, differentially expressed genes between wild-type control and knockout were defined using the function that fits multiple linear models from the Bioconductor package limma in R36 with FDR < 0.05. We used published c-MAF ChIP-seq data19 and PRDM1 ChIP-seq data40. In addition, potential PRDM1-binding sites were detected using FIMO (MEME suite; http://meme-suite.org/doc/fimo.html). Association to gene promoters was based on the following thresholds (upstream = 5,000, downstream = 500 of transcription start site) and the overlap with the co-inhibitory module was found to be significant (P = 0.009 hypergeometric, background of 20,000 genes). In the network presentation, we visualize all the genes that are part of the IL-27 inhibitory module (Fig. 4a, Extended Data Fig. 6a).

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

Data availability. Sequence data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) with the accession code GSE13968. All other data are available from the corresponding authors upon reasonable request.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | CyTOF analysis of co-inhibitory and co-stimulatory receptor co-expression in TILs. a, TILs were collected from B16F10 melanoma tumour-bearing wild-type and IL-27RA-knockout mice from Fig. 1b and analysed using CyTOF (5,000 cells from each). CyTOF data were analysed using viSNE. Applying $k$-means clustering with $k = 9$ on the CyTOF data resulted in a clear distinction between clusters 1, 2, 3 and 4. Polygons indicating clusters 1 and 2 (in CD8$^+$ T cells), and 3 and 4 (in CD4$^+$ T cells) are shown. Individual panels show expression of the indicated markers. b, Pie charts show the distribution of wild-type or IL-27RA-knockout CD8$^+$ and CD4$^+$ TILs in clusters 1 and 2 (C1 and C2) of CD8$^+$ TILs and clusters 3 and 4 (C3 and C4) of CD4$^+$ TILs as defined in Fig. 1d. c, Independent data of wild-type and IL-27RA-knockout TILs samples from that shown in Fig. 1 (5,000 cells from each). Applying $k$-means clustering with $k = 7$ on the CyTOF data resulted in a clear distinction between clusters 1, 2, 3 and 4. Polygons indicating clusters 1 and 2 (in CD8$^+$ T cells), and 3 and 4 (in CD4$^+$ T cells) are shown. d, viSNE plot highlighting the distribution of cells from wild-type (blue) and IL-27RA-knockout (red) mice in CD8$^+$ TILs clusters 1 and 2 and CD4$^+$ TILs clusters 3 and 4. Pie charts show the distribution of wild-type or IL-27RA-knockout CD8$^+$ and CD4$^+$ TILs in each cluster.
Extended Data Fig. 2  |  IL-27 induces multiple co-inhibitory receptors on CD4⁺ and CD8⁺ T cells. a, Naive T cells from wild-type or IL-27RA-knockout mice were stimulated in vitro with anti-CD3/CD28 in the presence or absence of IL-27. Expression of co-inhibitory receptors was determined by flow cytometry. Representative data of three biologically independent experiments are shown. b, Expression of PD-1, TIM-3, LAG-3, TIGIT and IL-10 on CD8⁺ TILs obtained from wild-type and IL-27RA-knockout mice bearing B16F10 melanoma was determined by flow cytometry. Thy1.1-IL-10 reporter mice crossed with wild-type and IL-27RA-knockout mice were used for IL-10 expression analysis. Representative data of three biologically independent experiments are shown.
Extended Data Fig. 3 | scRNA-seq expression analysis of wild-type and IL-27RA-knockout TILs. a, TILs were obtained from B16F10 melanoma tumour-bearing wild-type (707 and 825 for CD4+ and CD8+, respectively) and IL-27RA-knockout (376 and 394 for CD4+ and CD8+, respectively) mice as in Fig. 1e. t-SNE plot shows the presence of wild-type and IL-27RA-knockout CD4+ and CD8+ TILs as indicated. b, Clustering using the Louvain–Jaccard method (40 nearest neighbours and 13 principal components37). c, The composition of each cluster in terms of total number (c) and percentage (d) of wild-type (red) and IL-27RA-knockout (blue) cells. *P < 0.05, **P < 0.01, ***P < 0.001, one sample t-test.

e, Projection of the IL-27 co-inhibitory module signature on the scRNA-seq data. The contour plot marks the region of highly expressing cells by taking into account only those cells that have an expression value above the mean. f, Violin and box plots displaying the distribution of the IL-27 co-inhibitory module signature score compared between wild-type (72 and 98 for CD4+ and CD8+, respectively) and IL-27RA-knockout (85 and 77 for CD4+ and CD8+, respectively) cells in clusters 4 and 5 (CD4+ and CD8+, respectively). *P = 0.01, one-sided t-test. The top and bottom hinges in the boxplot correspond to the first and third quartiles, and the horizontal line denotes the median.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Overlap of the IL-27-induced gene program with signatures from four states of T cell impairment, tolerance and dysfunction. a, Pearson correlation between wild-type CD4+ and CD8+ T cells for the 1,201 genes that were differentially expressed between wild-type CD4+ T cells stimulated in the presence or absence of IL-27 (FDR < 0.05). b, Expression profile of 118 differentially expressed genes (from a) encoding cell-surface receptors and cytokines are shown as a heat map. c, The IL-27-induced gene program (1,201 genes) was compared to T cell signatures obtained from four states of T cell non-responsiveness. Number of overlapping genes between the IL-27 gene program and each signature is depicted. ***P < 0.001, hypergeometric test: nasal anti-CD3 4.7 × 10^{-21}; cancer 1.2 × 10^{-33}; antigen-specific tolerance 4 × 10^{-14}; and viral exhaustion 1.7 × 10^{-26}. d, P-value statistics for the significance of the overlap between the IL-27-induced gene program (1,201) and genes induced in other states of T cell non-responsiveness using WilcoxonGST and camera. e, Gene signatures from c were sub-sampled and projected onto the CD8+ single-cell TIL data. Changes were quantified by randomly selecting decreasing subsets of genes from each signature and calculating the average silhouette width of cells that scored high for the different generated signatures based on Euclidian distance between the principal component values used to generate the t-SNE plot. The top and bottom hinges in the boxplot correspond to the first and third quartiles, and the horizontal line denotes the median (Methods). f, Panels I–V, t-SNE plots of the 588 CD8+ single-cell TILs (dots) obtained from wild-type mice bearing B16F10 melanoma tumour. Cells are coloured by their signature score. The score reflects the relative average expression of the genes in the overlap of the IL-27 gene signature with the signatures for each of the indicated states of T cell non-responsiveness. Panel VI is a projection of a signature of the differentially expressed genes between CD8+ TILs from wild-type and IL-27RA-knockout mice bearing B16F10 melanomas (Methods). The contour plot marks the region of highly scored cells by taking into account only those cells that have a signature score above the mean score. Cells that had a statistically significant score (adjusted P < 0.05) are marked by a plus symbol (Methods).
Extended Data Fig. 5 | Characterization of the role of PDPN and PROCR in CD8⁺ TILs. a, PDPN and PROCR protein and mRNA expression was determined in T cells from wild-type and IL-27RA-knockout mice stimulated with anti-CD3/CD28 in the presence or absence of IL-27. CD4⁺ cells were analysed at 96 h and CD8⁺ cells at 72 h. Data are mean ± s.e.m. from representative flow cytometry and qPCR data from biologically independent animals. b, Representative flow cytometry data of three independent experiments showing PDPN and PROCR expression in PD-1⁺TIM-3⁺CD8⁺ and PD-1⁺TIM-3⁻CD8⁺ TILs obtained from wild-type and IL-27RA-knockout mice bearing B16F10 melanoma. c, TILs from wild-type mice bearing B16F10 melanoma were stimulated with phorbol myristate acetate and ionomycin. Cytokine production in PROCR⁺ or PROCR⁻ CD8⁺ TILs is shown. Thy1.1-IL-10 reporter mice were used for IL-10 expression analysis. Data are mean ± s.e.m. from biologically independent animals. *P < 0.05, **P < 0.01, paired t-test. d, CD8⁺ T cells (5 × 10⁵) from wild-type or Procr⁻/- mice were transferred along with 1 × 10⁶ wild-type CD4⁺ T cells to RAG1-knockout mice (n = 5). On day 2, 5 × 10⁵ B16F10 cells were implanted. Data are mean ± s.e.m. *P < 0.05, repeated measures ANOVA, Sidak's multiple comparisons test. e, TILs were obtained from wild-type and PDPN cKO mice bearing B16F10 melanoma and stained for the expression of IL-7RA. Representative flow cytometry data from three independent animals. f, Summary data of IL-7RA expression are from biologically independent animals. Data are mean ± s.e.m. *P < 0.05, one-sided t-test.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | PRDM1 is a candidate regulator of the co-inhibitory module. a, The log2 fold change in RNA levels between naive CD4⁺ or CD8⁺ T cells simulated with or without IL-27. Data are from two independent experiments. Transcription factors that are part of the IL-27 co-inhibitory module are shown (differentially expressed transcription factors were annotated as genes with FDR-corrected ANOVA < 0.05). b, Transcription factors that are both in the IL-27 co-inhibitory module and are also overexpressed in clusters 4 and 5 in the single-cell data (clusters that were enriched for the IL-27 signature; Extended Data Fig. 3e, f). Differentially expressed genes between clusters 4 and 5 and the rest of the clusters were determined using binomcount.test (binomial distribution, Methods). The log effect corresponds to log proportion of expressing cells and the P value is calculated by the probability of finding n or more cells positive for the gene in clusters 4 and 5 given the fraction in the rest of the clusters. c, t-SNE plot of Fig. 1e showing the expression of PRDM1 in wild-type (707 and 825 for CD4⁺ and CD8⁺, respectively) and IL-27RA-knockout (376 and 394 for CD4⁺ and CD8⁺, respectively) cells. d, Normalized RNA expression levels of PRDM1 in PD-1⁺TIM-3⁺ (n = 3) and PD-1⁻TIM-3⁻ (n = 3) CD8⁺ TILs. Data are mean ± s.e.m. ***P = 0.0004, two-sided t-test. e, Network model based on RNA-seq gene expression data of naive CD8⁺ T cells from Prdm1fl/fl (WT) or Cd4crePrdm1fl/fl (PRDM1 cKO) mice stimulated in the presence of IL-27 and actual binding events (ChIP–seq data for PRDM1)⁹. Green arrows designate genes upregulated by PRDM1, red arrows designate genes downregulated by PRDM1, and dashed grey arrows denote binding events.
Extended Data Fig. 7 | Genomic tracks surrounding the co-inhibitory molecules. a–d, LAG-3 (a), PD-1 (b), TIGIT (c) and TIM-3 (d) with overlay of ChIP–seq data of PRDM16 and c-MAF19 and ATAC-seq data of naive CD4+ cells induced with IL-27 for 72 h and ATAC-seq data of CD8+ T cells 27 days after chronic viral infection22. Regions of binding sites common to both PRDM1 and c-MAF are indicated by the dotted rectangles. e, Luciferase activity in 293T cells transfected with pGL4.23 luciferase reporters for depicted enhancers of TIM-3 together with empty vector (control), constructs encoding PRDM1, c-MAF or both. Firefly luciferase activity was measured 48 h after transfection and is presented relative to constitutive Renilla luciferase activity. Data are mean ± s.e.m. from biologically independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, one-way ANOVA and Tukey’s multiple comparisons test.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Immune characterization of PRDM1 cKO, c-MAF cKO and PRDM1/c-MAF cDKO before and after tumour challenge. a, Analysis of steady-state immune system in wild-type, c-MAF cKO, PRDM1 cKO and PRDM1/c-MAF cDKO. Summary data for CD4, CD8, FOXP3, CD44, CD62L and CD69 expression in spleen from wild-type, c-MAF cKO, PRDM1 cKO and PRDM1/c-MAF cDKO mice. Data are mean ± s.e.m. from biologically independent animals. *P < 0.05, **P < 0.01, ****P < 0.0001, one-way ANOVA and Tukey’s multiple comparisons test. b, Co-inhibitory receptor expression in CD4+ TILs from PRDM1/c-MAF cDKO mice. Top, representative flow cytometry data from three independent experiments for TILs from wild-type and PRDM1/c-MAF cDKO stained for PD-1, TIM-3, TIGIT, PDPN and PROCR expression. Bottom, summary data. Data are mean ± s.e.m. from biologically independent animals. *P < 0.05, two-sided t-test. c, Top, representative flow cytometry data from three independent experiments showing cytokine production from CD8+ TILs from wild-type and cDKO bearing B16F10 melanoma. Bottom, summary data. Data are mean ± s.e.m. from biologically independent animals. *P < 0.05, two-sided t-test. d, Co-inhibitory receptor expression on CD8+ TILs sorted from B16-OVA-bearing RAG1-knockout mice that were transferred with PRDM1/c-MAF cDKO (n = 4) or wild-type (n = 4) CD4+ and CD8+ T cells as indicated. Data are mean ± s.e.m. from biologically independent animals. *P < 0.05, one way ANOVA and Tukey’s multiple comparisons test. e, RAG1-knockout mice were transferred with either wild-type or cDKO CD4+ and CD8+ (2:1 CD4:CD8 ratio), followed by subcutaneous injection of MC38-OVA. Data are mean ± s.e.m. ****P < 0.0001, repeated measures ANOVA, Sidak’s multiple comparisons test. On day 14 after tumour implantation, mice were euthanized and TILs, spleen and draining lymph nodes were obtained. f, The frequency of antigen-specific CD8+ T cells in the draining lymph nodes of mice in e.
Extended Data Fig. 9 | Examination of additive and non-additive (synergistic) effects of PRDM1 and c-MAF. a, A heat map showing all 940 differentially expressed genes between wild-type (n = 5) and cDKO (PRDM1/c-MAF, n = 4) mice, and their expression in single knockout (PRDM1 control n = 7, PRDM1 knockout n = 3, c-MAF control n = 4 and c-MAF-knockout n = 3) mice. The red markings at the top indicate genes on expression of which the two knockouts have a statistically significant (P < 0.05) non-additive effect in the cDKO (149 out of 940 differentially expressed genes). b, Volcano plot of the analysis in a for global gene expression. Genes whose expression in the two single knockouts have a statistically significant (P < 0.05) non-additive effect in the cDKO (1,144 out of 12,906 genes) and had an absolute coefficient value > 1 (779 out of 1,144) are shown in orange.
Extended Data Fig. 10 | Comparison of gene expression between PRDM1/c-MAF cDKO TILs and CD8+ TILs populations from wild-type mice. a, Barcode enrichment plot displaying two gene sets in a ranked gene list. The ranked gene list was defined as fold change in gene expression between PRDM1/c-MAF cDKO and wild-type CD8+ TILs. Three gene sets consist of differentially expressed genes between: PD-1−TIM-3−CD8+ (n = 3) and PD-1−TIM-3−CD8+ (n = 3) TILs, PD-1+TIM-3−CD8+ (n = 3) TILs and memory CD8+ (n = 3), and PD-1+TIM-3−CD8+ (n = 3) and PD-1+TIM-3−CD8+ TILs. This analysis was followed by four statistical tests (one-sample Kolmogorov–Smirnov test, mean-rank gene set test (Wilcoxon GST), hypergeometric, and competitive gene set test accounting for inter-gene correlation) for enrichment of these signatures in the cDKO expression profile.

b, Table showing the results of the statistical tests. The table includes the number of up-regulated and down-regulated genes, the p-values of the tests, and the correlation coefficients.

c, Wild-type versus cDKO volcano plot. Green indicates genes that were upregulated in the PD-1−TIM-3−CD8+ (double negative) TILs and red indicates genes that were upregulated in the PD-1+TIM3+CD8+ (double positive) TILs. d, Wild-type versus cDKO volcano plot. Red indicates genes that were upregulated in PD-1+CXCR5+CD8+ T cells and green indicates genes that were upregulated in PD-1−CXCR5−CD8+ T cells in chronic LCMV infection. e, A t-SNE plot of the 588 CD8+ TILs obtained from wild-type mice bearing B16F10 melanoma tumours, coloured by the relative signature score for the co-inhibitory module (272 genes, Supplementary Table 2), the cDKO signature (shown in g), and the PD-1+CXCR5+CD8+ T cell signature from chronic virus infection. The contour plot marks the region of highly scored cells by taking into account only those cells that have a signature score above the mean.
Experimental design

1. Sample size
   Describe how sample size was determined.
   At least 5 animals of target gene knock out and control mice were used to adequately power biological validation experiments throughout the article. Statistical differences provide the rationale for sufficiency of the sample sizes.

2. Data exclusions
   Describe any data exclusions.
   None

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   Experiments were repeated multiple times to ensure reproducibility of results and confirmed findings were reliably reproduced.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   No randomization was used as animals were genotyped prior to use.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Since mice were genotyped before the experiments, no randomization was used. However, investigators injected tumor randomly and tumor-size was assessed randomly to avoid any bias as much as possible. We will indicate this in the method section in the revised version.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a Confirmed
   □ □ The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   □ □ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   □ □ A statement indicating how many times each experiment was replicated
   □ □ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   □ □ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   □ □ The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted
   □ □ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   □ □ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

FlowJo software (Tree Star) was used for flow cytometry analysis. Prism software (GraphPad) was used for statistic analysis of biological experiments. R software was used for RNA-seq, ATAC-seq, ChIP-seq, gene expression analysis.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All unique materials used are readily available from the authors or from standard commercial sources.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

CD4 (RM4-5), CD8 (53-6.7), PD-1 (RMP1-30), Lag-3 (C9B7W), TIGIT (GIGD7), and Tim-3 (5D12), and Pdpn (8.1.1.) were obtained from BioLegend (San Diego, CA). Reproducibility is indicated in https://www.biolegend.com/reproducibility. Procr (eBio1560) and Fixable viability dye eF506 (eBioscience). Reproducibility is indicated in https://www.thermofisher.com/jp/ja/home/life-science/antibodies/invitrogen-antibody-validation.html. Antibodies of CyTOF is validated by comparing each molecule expression with Flow Cytometry.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

We obtained cell line; B16F10 from ATCC (CRL-6475), B16F10-Ova from Kai Wucherpfennig (Dana-Farber Cancer Institute, Boston, MA) and MC38-Ova from Mark Smyth (QIMR Berghofer, Queensland Institute of Medical Research, Brisbane Australia). This is indicated in method section.

b. Describe the method of cell line authentication used.

Morphology check by microscope and growth curve analysis were performed periodically.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines tested negative for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

None of the cell lines used are listed in the ICLAC database.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

All mice used are C57BL/6 background, both male and female, 6-12 weeks of age, 15-25g. Each experiment was performed using age, sex matched controls.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

☑ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☑ 2. 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).

☑ 3. All plots are contour plots with outliers or pseudocolor plots.

☑ 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation. Yes, it is indicated in method section (page #43)

6. Identify the instrument used for data collection. We used BD LSR2™ for FACS analysis and BD FACSAria™ for cell sorting

7. Describe the software used to collect and analyze the flow cytometry data. We used FlowJo software (Tree Star)

8. Describe the abundance of the relevant cell populations within post-sort fractions. The cell purity was confirmed as >95% by occasionally re-running the obtained samples using BD FACSAria™

9. Describe the gating strategy used. Obtained data were analyzed by FSC/SSC gates of starting cell population (a figure exemplifying the gating strategy is provided as a supplementary information). Positive gates were set based on fluorescence minus one (FMO) controls in each setting for cell surface molecules and based on unstimulated sample for ICC staining. This is standard for analysis of flow-cytometric data.

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