T helper 17 (TH17) cells are critically involved in host defence, inflammation, and autoimmunity. Transforming growth factor β (TGFβ) is instrumental in TH17 cell differentiation by cooperating with interleukin-6 (refs 6, 7). Yet, the mechanism by which TGFβ enables TH17 cell differentiation remains elusive. Here we reveal that TGFβ enables TH17 cell differentiation by reversing SKI–SMAD4-mediated suppression of the expression of the retinoic acid receptor (RAR)-related orphan receptor γt (RORγt). We found that, unlike wild-type T cells, SMAD4-deficient T cells differentiate into TH17 cells in the absence of TGFβ signalling in a RORγt-dependent manner. Ectopic SMAD4 expression suppresses RORγt expression and TH17 cell differentiation of SMAD4-deficient T cells. However, TGFβ neutralizes SMAD4-mediated suppression without affecting SMAD4 binding to the Rorc locus. Proteomic analysis revealed that SMAD4 interacts with SKI, a transcriptional repressor that is degraded upon TGFβ stimulation. SKI controls histone acetylation and deacetylation of the Rorc locus and TH17 cell differentiation via SMAD4: ectopic SKI expression inhibits H3K9 acetylation of the Rorc locus, Rorc expression, and TH17 cell differentiation in a SMAD4-dependent manner. Therefore, TGFβ-induced disruption of SKI reverses SKI–SMAD4-mediated suppression of RORγt to enable TH17 cell differentiation. This study reveals a critical mechanism by which TGFβ controls TH17 cell differentiation and uncovers the SKI–SMAD4 axis as a potential therapeutic target for treating TH17-related diseases.

We studied the mechanisms underlying the important role of TGFβ signalling in TH17 cell differentiation6–10. We found that CD4+ T cells from wild-type and Cd4-crc;Smad4fl/fl (S4 knockout) mice11 differentiated into TH17 cells comparably in the presence of interleukin (IL)-6 and TGFβ, as reported previously12. However, in stark contrast to wild-type T cells, SMAD4-deficient T cells consistently differentiated into TH17 cells when provided with IL-6 alone without TGFβ (Fig. 1a). This observation prompted us to hypothesize that SMAD4 deletion may result in TH17 cell differentiation in the absence of TGFβ signalling. To test this hypothesis, we blocked TGFβ signalling by using a pharmacological inhibitor of TGFβ receptor (TGFβR) kinase activity. Whereas wild-type T cells did not become TH17 cells, SMAD4-deficient T cells readily differentiated into TH17 cells and expressed TH17-related genes when TGFβ signalling was inhibited (Fig. 1a, b and Extended Data Fig. 1a). To confirm this finding and exclude the potential off-target effect of the inhibitor, we generated Cd4-crc;Smad4fl/fl;Tgfbr2fl/fl (S4–RII double-knockout) mice, in which both SMAD4 and TGFβ receptor II (TGFβRII) were deleted specifically in T cells11,13–15. In agreement with the results of the experiments using the TGFβR inhibitor, S4–RII double-knockout CD4+ T cells differentiated into TH17 cells when only IL-6 was provided (Fig. 1c, d).

Intrigued by the findings obtained in vitro, we further investigated whether SMAD4-deficient T cells could differentiate into TH17 cells in the absence of TGFβ signalling in vivo. Under steady state, S4–RII double-knockout mice had comparable, if not slightly higher, percentages of TH17 cells than wild-type mice, while virtually no TH17 cells were detected in Cd4-crc;Tgfbr2fl/fl (RII knockout) mice (Extended Data Fig. 1b). We then addressed whether S4–RII double-knockout T cells could differentiate into TH17 cells during development of experimental autoimmune encephalomyelitis (EAE). S4–RII double-knockout T cells differentiated into TH17 cells as effectively as wild-type T cells (Fig. 1e) upon elicitation of EAE. S4–RII double-knockout mice developed EAE and associated pathology (Fig. 1f, g). In addition, by using a tamoxifen-inducible Cre recombinase and oestrogen receptor (Cre-ER) fusion system16, we found that acute deletion of SMAD4 enabled TH17 cell differentiation in the absence of TGFβRRII, although TGFβRII-deficient T cells failed to differentiate into TH17 cells under the same conditions (Fig. 1h). These findings therefore demonstrate that SMAD4-deficient T cells can differentiate into pathological TH17 cells in the absence of TGFβ signalling.

We next investigated how SMAD4 deletion affected the molecular program controlling TH17 cell differentiation. The expression of many TH17-related genes was markedly increased in SMAD4-deficient T cells compared with wild-type T cells, as early as 1 day after activation when only IL-6 was provided (Extended Data Fig. 2a). To identify the primary target of SMAD4 for TH17 cell differentiation, we compared gene expression in wild-type and SMAD4-deficient T cells at very early time points. Whereas the expression of many TH17-related genes was undetectable or showed insignificant difference between wild-type and SMAD4-deficient T cells, Rorc expression was prominently increased in SMAD4-deficient T cells within 12 h of activation in the presence of IL-6 and TGFβRRII inhibitor (Fig. 2a and Extended Data Fig. 2b, c). Such an elevated Rorc expression was similarly observed in S4–RII double-knockout T cells (Extended Data Fig. 2d). RORγt protein expression was consistent with Rorc mRNA expression in both S4 knockout and S4–RII double-knockout T cells (Extended Data Fig. 2e, f). These results strongly suggest an involvement of RORγt in SMAD4-controlled TH17 cell differentiation. Indeed, deletion of RORγt in SMAD4-deficient T cells abolished their TH17 cell differentiation in the absence of TGFβ3 signalling (Fig. 2b).

We then addressed whether adding back SMAD4 into SMAD4-deficient T cells could restore their function to that of wild-type cells. Indeed, retrovirus-mediated ectopic SMAD4 expression inhibited the
T17 cell differentiation of SMAD4-deficient T cells in the absence of TGFβ3 signalling (Fig. 2c, d). SMAD4 suppressed the expression of Rorc before other T17-related genes (Fig. 2e). In addition, Rorc was a functionally critical SMAD4 target because ectopic RORγt expression overcame SMAD4–suppressed T17 cell differentiation in the absence of TGFβ3 signalling (Fig. 2f). SMAD4 seemed to suppress T17 cell differentiation directly by influencing Rorc expression, because SMAD4 bound to multiple sites in the Rorc locus, including the promoter region (Fig. 2g and Extended Data Fig. 2g), but not to Il17a or Il17f loci (Extended Data Fig. 2h).

On the basis of the findings described above, one may further predict that ectopic SMAD4 expression will also suppress RORγt expression and T17 cell differentiation in the presence of both IL-6 and TGFβ3 (the classic T17 cell polarizing condition). On the contrary, however, addition of TGFβ3 abolished the ability of SMAD4 to suppress T17 cell differentiation (Fig. 3a). The findings suggest that one important mechanism through which TGFβ3 enables T17 cell differentiation is reversing SMAD4-mediated suppression. TGFβ3 may do so by dislodging SMAD4 from the Rorc locus. However, this was not the case: SMAD4 remained bound to the Rorc locus regardless of the presence of TGFβ3 (Fig. 3b). Another possibility is that TGFβ3 signalling alters the interaction of SMAD4 with other proteins, because associating with different factors is important for SMAD4 function. We developed a screening strategy based on quantitative proteomics to identify proteins that preferentially bind to SMAD4 in the absence, but not in the presence, of TGFβ3 signalling in activated T cells. SKI, a factor whose deregulation is closely associated with tumorigenesis, is a direct SMAD4 and SKI was validated by immunoprecipitation assays (Fig. 3c). SKI is degraded upon TGFβ3 signalling in cancer cells. In T cells, very low doses of TGFβ3 stimulation during T17 cell differentiation induced a marked SKI protein downregulation that was partly Smad2- and Smad3-dependent (Fig. 3d and Extended Data Fig. 3e). We then investigated whether SKI–SMAD4 interaction is important for SMAD4-mediated suppression of T17 cell differentiation. Indeed, SMAD4 mutants that are defective in interacting with SKI failed to suppress T17 cell differentiation of SMAD4-deficient T cells in the absence of TGFβ3 signalling (Fig. 3f).
These findings prompted us to test how SKI expression affects T_{H}17 cell differentiation. SKI was ectopically expressed to maintain its expression in the presence of both IL-6 and TGFβ3. SKI expression strongly suppressed T_{H}17 cell differentiation (Fig. 4a) with a prompt suppression of Rorc expression in vitro (Fig. 4b). Similarly, in vivo, T cells that ectopically expressed SKI were defective in differentiating into T_{H}17 cells during development of EAE (Fig. 4c). In addition, disruption of SKI expression enabled CD4+ T cells to differentiate into T_{H}17 cells in the absence of TGFβ3 signalling (Extended Data Fig. 4a). These findings suggest that SKI functions downstream of TGFβ3 to inhibit Rorc expression and T_{H}17 cell differentiation. Indeed, ectopic RORγt expression restored the T_{H}17 cell differentiation of SKI-expressing cells (Extended Data Fig. 4b). SMAD4 is critical for mediating SKI function, as demonstrated by ectopic SKI expression failing to suppress the T_{H}17 cell differentiation of cells that were SMAD4-deficient (Fig. 4d). Therefore, SKI and SMAD4 interact and cooperate to suppress Rorc expression and to restrain T_{H}17 cell differentiation.

We further investigated the mechanism through which the SKI–SMAD4 axis suppresses Rorc expression. Because SKI recruits histone deacetylases to repress gene expression24, we studied how the SKI–SMAD4 axis may regulate the histone acetylation of the Rorc locus. As expected, H3K9 acetylation in the Rorc promoter region was substantially elevated under T_{H}17 cell polarizing conditions in a TGFβ3-dependent manner in wild-type T cells (Fig. 4e). Nonetheless, SMAD4 deletion led to H3K9 acetylation of the Rorc promoter region in the absence of TGFβ3 signalling (Fig. 4e). Importantly, ectopic SKI expression suppressed the acetylation of H3K9 in the Rorc promoter region in wild-type but not in SMAD4-deficient T cells (Fig. 4f). In addition, we found that SKI bound to the Rorc promoter at the same site as SMAD4 in a SMAD4- and TGFβ3-dependent manner (Extended Data Fig. 4c). These findings suggest that the SKI–SMAD4 complex regulates the histone acetylation of the Rorc locus directly to suppress RORγt expression.

These findings collectively suggest that, in the absence of TGFβ3 signalling, the SKI–SMAD4 complex prevents Rorc expression by acetylation of histone tails at the Rorc locus. Therefore, constitutive Rorc expression is blocked, which results in the failure of T_{H}17 cell differentiation. This mechanism can be explained by the histone acetylation/deacetylation changes at the Rorc locus, which are mediated by the SKI–SMAD4 complex.

**Figure 2** | SMAD4 controls T_{H}17 cell program by directly suppressing Rorc expression. a. Differential gene expression of SMAD4 knockout/wild-type cells cultured with IL-6 + TGFβ3 inhibitor by RNA-seq. b–d, f. Flow cytometry of cells without (b) or with (c, d, f) retrovirus (RV) transduction (n = 5 experiments in b, d and f; n = 6 experiments in c). S4–RORγt DKO, SMAD4 and RORγt double knockout. e, qRT–PCR of SMAD4 knockout cells cultured with IL-6 + TGFβ3 inhibitor, 18 h after retrovirus transduction (n = 3 experiments, mean ± s.d.). g. Chromatin immunoprecipitation followed by sequencing (ChIP–seq) analysis of SMAD4 binding at the Rorc locus in cells cultured with IL-6 + TGFβ3 inhibitor for 24 h (n = 2 experiments). (**P < 0.01, ***P < 0.001; two-sided t-test; centres indicate mean values.)
suppressed activin-promoted Th17 cell differentiation (Fig. 4i). In addition, SMAD4 was required for SKI-mediated suppression of Th17 cell differentiation induced by activin (Fig. 4i). Therefore, SKI–SMAD4 complexes relay diverse upstream signals to regulate Th17 cell differentiation.

Given the vital roles for Th17 cells in normal physiology and in many diseases, it is important to understand how these cells are generated.

IL-6 and TGFβ signalling are both instrumental in the induction of Th17 cells.6,7 Although IL-6/STAT3 signalling potentiates RORγt expression,26–28 it is insufficient for Th17 cell differentiation; TGFβ signalling is also required.6,7 In the current study, we have revealed SKI as a Th17 suppressor whose function relies on SMAD4 to restrain RORγt expression. Importantly, we have discovered that a central function of TGFβ in Th17 cell differentiation is to degrade SKI, thereby

Figure 3 | TGFβ3 signalling disrupts SKI–SMAD4 complex to enable Th17 cell differentiation. a, g, Flow cytometry of cells transduced with retrovirus-expressing wild-type or mutant SMAD4 (n = 7 experiments in a; n = 5 experiments in g). b, ChIP of SMAD4 binding at the Rorc promoter (n = 3 samples, mean ± s.d.). c–f, Immunoprecipitation and immunoblotting of SMAD4 knockout (c, f) or wild-type (d, e) cells treated as indicated (n = 3 experiments). In e, cells were pre-cultured with IL-6 for 24 h. (NS, not significant; **P < 0.001; two-sided t-test; centres indicate mean values.)

Figure 4 | SKI suppresses Th17 cell differentiation in a SMAD4-dependent manner. a, d–j, Flow cytometry of cultured cells (n = 7 experiments in a, n = 8 in d, and n = 6 in h–j). b, qRT–PCR of wild-type cells cultured with IL-6 + TGFβ, 18 h after retrovirus transduction (n = 3 experiments, mean ± s.d.). c, Flow cytometry of spinal-cord-infiltrating, transferred cells in EAE mice (n = 5). e, f, ChIP of H3K9 acetylation at the Rorc promoter (n = 3 experiments). g, Immunoblotting of wild-type cells cultured for 24 h (n = 3 experiments). (NS, not significant; **P < 0.01; ***P < 0.001; two-sided t-test; centres indicate mean values.)
reversing SKI–SMAD4-mediated Rorc expression and enabling T$_{H}$$\gamma$$\delta$ cell differentiation. These findings not only shed light on how T$_{H}$$\gamma$$\delta$ cell differentiation is controlled, but also uncover the SKI–SMAD4 axis as a critical molecular target that interferes with T$_{H}$$\gamma$$\delta$ cell function for treating related immune diseases.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** S.Z. contributed to the design and implementation of the cellular, molecular, biochemical, and animal experiments, and the writing of the manuscript. M.T., X.X., S.Y.T., P.A.W., and D.N.C. contributed to ChIP-seq and RNA-seq experiments and bioinformatic analysis. L.Z., Q.K., and X.C. contributed to proteomic and biochemical experiments and data analysis. A.D.G. contributed to the in vitro assays. W.C. and J.P.T. contributed to the EAE experiments. G.Z. contributed to ChIP analysis. B.W. contributed to qRT–PCR analysis. J.S.S. contributed critical reagents. Y.Y.W. conceived the project, designed experiments, and wrote the manuscript.

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Mice. Smad4fl/fl, Tgβr2fl/fl, Cda-cre, Er-cre, Rag1−/−, B6.C.B-H2-129/Ola-H-2d (B6.H-2d), CD4-cre, Cre-dependent-Cas9, and Cre-ER-expressing T cells of different genotypes or background. Littermates were used unless stated otherwise. All mice were housed and bred in specific pathogen-free conditions in the animal facility at the University of North Carolina at Chapel Hill. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. We complied with all relevant ethical regulations.

Flow cytometry and cell sorting. Lymphocytes were isolated from various organs of 6- to 18-week-old, age- and sex-matched mice. Cells were cultured in RPMI medium with 10% FBS and 1% antibiotics unless specifically indicated. Cells were cultured in the presence of 20 μg ml−1 anti-CD3 (145-2C11, BioXCell) and 10 μg ml−1 anti-CD28 (37.51, BioXCell) or by soluble 20 μg ml−1 anti-CD3 and irradiated (3,000 Gy) T-cell-depleted splenocytes. Cells were cultured in the presence of 20 ng ml−1 recombinant IL-2, 20 μg ml−1 recombinant IL-4, 10 ng ml−1 recombinant IL-7, 2 μg ml−1 recombinant IL-15, 1 μg ml−1 anti-IFN-γ, and 20 μg ml−1 anti-IL-4. Recombinant human activin A (100 ng ml−1) was added to the indicated conditions. TGFβR inhibitor SB253344 (10 μM; Selleckchem) was added into the culture medium with the indication ‘+ SB’ for the indication of receptor inhibition. For retroviral transduction, CD4+ T cells were isolated and cultured under various conditions on day 0 and then spin inoculated with indicated retroviruses at 1,500 g for 30°C for 1.5 h on day 1. Cells were harvested and analysed by flow cytometry on day 4 unless stated otherwise in the figure legends.

Elicitation of EAE in mice. MOG peptide (50 μg) and CFA were emulsified and subcutaneously injected into mice on day 0. Pertussis toxin (200 μg) was intraperitoneally injected on day 0 and day 2. Mice were monitored and euthanized at appropriate times. The EAE clinical scores were recorded on the basis of the following criteria: 0, no signs; 1, limp tail; 2, poor righting ability and/or partial hind-limb paralysis; 3, total hind-limb paralysis; 4, hind-limb paralysis + 75% of body paralysis; 5, moribund; 6, dead. Seventeen to 18 days after EAE induction, diseased mice were euthanized and the spinal cords were subjected to pathological analysis using Luxol fast blue staining. Lesions were indicated by the arrows in the figures. T cells from spinal cords were collected and subjected to immunological analysis. For EAE experiments with T-cell adoptive transfer, CD4+ T cells of different genotypes or treatment were transferred into Rag1−/− or irradiated (3,000 Gy) wild-type recipient mice on day 1. EAE was then elicited in the recipient mice on day 0. Tamoxifen was injected into the recipient mice on day 0 and day 2 and 4 to delete floxed alleles in Cre-ER-expressing T cells. Spinal cord lymphocytes were isolated and transferred cells were analysed by flow cytometry on day 17 after EAE elicitation.

Immunoblotting, immunoprecipitation, and mass spectrometry. For immunoblotting, protein extracts were resolved by AnySDS PAGE gel (Bio-Rad), transferred to a polyvinylidene fluoride membrane (Millipore), and analysed by immunoblotting with the following antibodies: anti-SKI (H-329, Santa Cruz), anti-ROCK1 (13D4G10, Abcam), anti-SMAD4 (D3M6U, CST), anti-pSmad3 (C25A9, CST), and anti-3-actin (I-19, Santa Cruz). For gel source data, see Supplementary Fig. 1

For immunoprecipitation analysis, cells were lysed with extract buffer (20 mM HEPES, pH 7.9, with 1.5 mM MgCl2, 0.2 mM EDTA, 25% (v/v) glycerol, 1% Triton X-100) containing protease inhibitor mixture (Roche Applied Sciences), diluted with three volumes of dilution buffer (20 mM HEPES, pH 7.9, with 1.5 mM MgCl2, 0.2 mM EDTA, 10 mM KCl, and 25% (v/v) glycerol) and sonicated with Bioruptor Pico. The supernatant was incubated with magnetic beads (Bio-Rad) that had been conjugated with indicated antibody or anti-Flag M2 magnetic beads (Sigma) in a cold room overnight. The immunocomplex was washed six times with washing buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, and 150 mM NaCl) containing 0.1% NP40. Associated proteins were eluted by adding 2× Laemmli sample buffer (Bio-Rad) and incubated at 95°C for 5 min. The eluted proteins were resolved by SDS–PAGE gel for subsequence immunoblotting or mass spectrometry.

For MS analysis, CD4+ T cells were cultured in SILAC/AACT L media (RPMI1640 media depletion of lysine and arginine were supplemented with normal l-lysine (K)/l-arginine (R)), or SILAC/AACT H media (RPMI1640 media depletion of lysine and arginine were supplemented with H3K6N2-lysine (K)/H3K6N2-arginine (R)). After immunoprecipitation, eluted proteins were resolved in SDS–PAGE gel and subjected to in-gel digestion with trypsin (Promega) at 37°C. Mass spectrometry analyses of the immunoprecipitation products were performed on a LTQ Orbitrap Velos (Thermo Scientific) coupled with a nano-LC-Ultra system (Eksigent). Samples were re-suspended in 20 μl HPLC buffer A (0.1% formic acid in water) and 5 μl was loaded onto an IntegraFrit column (C18, New Objective). The peptides were eluted at a flow rate of 250 nl min−1 with a multi-step gradient, 5–40% buffer B (0.1% formic acid in acetonitrile) for 70 min, 40–80% buffer B for 5 min, 80% buffer B for 5 min, 80–5% buffer B for 5 min, and 5% buffer B for 5 min. The mass spectrometry spectra were acquired in a data-dependent and positive ion mode at a spray voltage of 2.1 kV by Xcalibur software (version 2.1, Thermo Scientific). Orbitrap analyser was used to allow the survey scan at a resolution of 60,000 and a mass range between m/z 500 and 1,800. The top 11 most intense ions in every cycle were subjected to collision-induced dissociation fragmentation in the LTQ Orbitrap with normalized collision energy at 35% and activation Q 0.25. Dynamic exclusion was enabled. Mass spectrometry raw files were analysed by MaxQuant (version 1.5.0.25). They were searched with Andromeda against the Uniprot mouse database (release date 30 November 2010; 20,248 entries). A database containing contamination proteins was also used to exclude peptides that matched this database. Searches were performed with a precursor ion mass tolerance of 7.5 p.p.m. and tandem mass spectrometry tolerance at 0.5 Da. Peptide and protein identifications were filtered to a maximum 1% and 5% false discovery rate, respectively. Up to two missed cleavages were allowed. Only peptides with a minimum length of seven amino acids were considered for identification. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD007172.

ChiP assay. The ChiP assay was done according to the protocol of Upstate Biotechnology. Cells were cross-linked with 1% formaldehyde and lysed in lysis buffer. Lysates were sonicated with Bioruptor PICO or Covaris S220 to shear genomic DNA. Chromatin was subjected to immunoprecipitation overnight at 4°C with anti-H3K9ac (ab4441, Abcam), anti-IGF (sc-2027, Santa Cruz), anti-SMAD4 (EP618Y, Abcam), or anti-SKI (H-329, Santa Cruz). Quantitative real-time PCR was performed to determine the relative abundance of target genomic DNA. Specific PCR primers to detect Ror promoters were GGGGAGAGCTTTGTGCAGAT and AGTTAGGTAGCCCAAGCAG.

ChiP-seq. The sequencing libraries were prepared by a NEbNext Rapid DNAseq kit (BioXcell Scientific Corporation) and sequenced on NextSeq 500 (Illumina) at the National Institute of Environmental Health Sciences Epigenomics Core Facility. For primary data processing, data were collected using 36 paired-end reads on a NextSeq 500 platform (Illumina). Raw reads (36 million to 72 million reads per sample) were first cleaned for adapter sequences using Trim Galore with default parameters. Cleaned reads were aligned to mm10 using Bowtie2 with the parallel option enabled. These parameters ensured that fragments up to 1,500 base pairs (-X1500) and mismatch up to 2 (-v2) were allowed to align, and that only unique aligned reads were collected (-m1). For all data files, duplicates were removed using Picard. The ChiP-seq data are available in the Gene Expression Omnibus repository at the National Center for Biotechnology Information under accession number GSE101593.

RNA-seq. Total RNA was extracted from cells by lysis with TriZol (Thermo Fisher Scientific), and purified with an RNeasy mini kit (Qiagen). RNA-seq libraries were generated and poly(A) enriched using 1 μg of RNA as input with a TrueSeq RNA Sample Prep Kit (Illumina, San Diego, California, USA). Indexed samples were sequenced on the 50 base pair paired-end protocol via the HiSeq 2500 (Illumina) according to the manufacturer’s protocol. Reads (32 million to 45 million reads per sample) were aligned to the University of California, Santa Cruz (UCSC) mm10 reference genome using STAR with default parameters. The quantification results from featureCounts were then analysed with the Bioconductor package DESeq2, which fits a negative binomial distribution to estimate technical and biological variability. A gene was considered differentially expressed if the P value for differential expression was less than 0.05. The RNA-seq data are available in the Gene Expression Omnibus at the National Center for Biotechnology Information under accession number GSE101527.
**Statistical analysis and reproducibility.** No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. Statistical analysis was performed by two-tailed sided Student’s t-tests. \( P < 0.05 \) (confidence interval of 95%) was considered statistically significant. In the figures, asterisks are used to indicate \( P \) values as follows: \( *P < 0.05 \), \( **P < 0.01 \), and \( ***P < 0.001 \). The exact \( P \) values are shown in the Source Data. The sample sizes \( n \) are stated in the figure legends to indicate biologically independent replicates used for statistical analysis, except for ChIP assays (Figs 3b and 4e, f and Extended Data Figs 2g and 4c), for which statistical analysis used independent samples done in one of three independent experiments. Representative results shown in Figs 1g, 3c–f and 4g and Extended Data Figs 2e, f and 3b, d were from three biologically independent experiments with similar results.

**Data availability.** The RNA-seq data supporting the findings of this study have been deposited in the Gene Expression Omnibus at the National Center for Biotechnology Information under accession number GSE101527. Figure 2a and Extended Data Fig. 2b contain RNA-seq-related data. The ChIP–seq data supporting the findings of this study have been deposited in Gene Expression Omnibus under accession number GSE101593. Figure 2g and Extended Data Fig. 2h contain ChIP–seq-related data. The IP-MS proteomics data supporting the findings of this study have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD007172. Extended Data Fig. 3a contains IP-MS-related data. All other relevant data are available in the manuscript and or from the corresponding author on request.

29. Platt, R. J. et al. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* **159**, 440–455 (2014).

30. Labun, K., Montague, T. G., Gagnon, J. A., Thyme, S. B. & Valen, E. CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Res.* **44**, W272–W276 (2016).
Extended Data Figure 1 | Th17 cell differentiation in the absence of SMAD4. **a,** Naive CD4+ T cells isolated from wild-type and Cd4-cre;Smad4fl/fl (S4 KO) mice were activated in the presence of TGFβR inhibitor (i), IL-6+TGFβR inhibitor (IL-6+inhibitor), or IL-6+TGFβR (IL-6+TGFβ). IL-17A+ cells were assessed by flow cytometry 1 and 2 days later. Representative results (left) and statistical analysis (right) of five experiments are shown. **b,** The percentages of IL-17A+CD4+ and IFN-γ+CD4+ cells in the peripheral lymph nodes (pLN) and spleens from wild-type, Cd4-cre;Smad4fl/fl (S4 KO), Cd4-cre;Tgfbr2fl/fl (RII KO), and Cd4-cre;Smad4fl/fl;Tgfbr2fl/fl (S4–RII DKO) mice under steady state were assessed by flow cytometry. Representative results (left) and statistics from eight mice (right) are shown. (***P < 0.001; two-sided t-test; NS, not significant; centres indicate mean values.)
Extended Data Figure 2 | SMAD4 suppresses RORγt expression.

a, CD4+ T cells from wild-type and Cd4-cre;Smad4fl/fl (S4 KO) mice were activated in the presence of IL-6 and TGFβ3R inhibitor. The mRNA expression of TGFβ17-related genes was analysed at the indicated time points after activation by qRT–PCR. Means ± s.d. of three experiments are shown. b, Naive CD4+ T cells from wild-type and Cd4-cre;Smad4fl/fl (S4 KO) mice were sorted and activated in the presence of IL-6 and TGFβ3R inhibitor for 3 and 12 h. Total RNA was then collected for RNA-seq analysis. All genes were analysed and presented as volcano plots based on the log2(fold change) of SMAD4 knockout versus wild type and −log10(P value). Differentially expressed genes (P < 0.05) are highlighted in red. c, Naive CD4+ T cells from wild-type and Cd4-cre;Smad4fl/fl (S4 KO) mice were sorted and activated in the presence of IL-6 and TGFβ3R inhibitor. The mRNA expression of TGFβ17-related genes was analysed at the indicated time points after activation by qRT–PCR. Means ± s.d. of three experiments are shown. d, Naive CD4+ T cells from wild-type and Cd4-cre;Smad4fl/fl;Tgfbr2fl/fl;Smad4fl/fl (S4–RII DKO) mice were sorted and activated in the presence of IL-6. The mRNA expression of TGFβ17-related genes was analysed at the indicated time points after activation by qRT–PCR. Means ± s.d. of three experiments are shown. e, Naive CD4+ T cells from wild-type and Cd4-cre;Smad4fl/fl (S4 KO) mice were sorted and activated in the presence of IL-6 and TGFβ3R inhibitor. The RORγt protein expression was assessed by immunoblotting 1 and 4 days after activation. Results are representative of three experiments with similar results. f, CD4+ T cells from wild-type and Cd4-cre;Tgfbr2fl/fl;Smad4fl/fl (S4–RII DKO) mice were activated in the presence of IL-6. The RORγt protein expression was assessed by immunoblotting 1 day after activation. Results are representative of three experiments with similar results. g, CD4+ T cells from wild-type and Cd4-cre;Smad4fl/fl (S4 KO) mice were activated in the presence of IL-6 and TGFβ3R inhibitor. Cells were harvested after 12 h. ChIP assay was performed with control IgG antibody and SMAD4 antibody. The enrichment of SMAD4 binding to the Rorc promoter was determined. Means ± s.d. of three samples in one experiment of three are shown. h, CD4+ T cells from wild-type and Cd4-cre;Smad4fl/fl (S4 KO) mice were activated in the presence of IL-6 and TGFβ3R inhibitor. Cells were harvested after 12 h. ChIP–seq assay was performed with SMAD4 antibody. The enrichment of SMAD4 binding to the Il17a and Il17f loci was determined by the mapped read coverage of SMAD4 ChIP–seq data. The results of two independent experiments were show as ‘#1’ and ‘#2’. (*P < 0.05, **P < 0.01, ***P < 0.001; two-sided t-test; NS, not significant).
Extended Data Figure 3 | SKI identification and its degradation upon low doses of TGFβ. a, Schematic of quantitative immunoprecipitation and mass spectrometry proteomic strategy to identify SMAD4-binding proteins under different conditions. In one scheme, to identify SMAD4-binding protein in the absence of TGFβ signalling, CD4+ T cells from Cd4-cre;Smad4fl/fl (S4 KO) mice were sorted and activated in the presence of IL-6 + inhibitor in the SILAC/AACT medium provided either with amino acids containing light (L) isotopes or with amino acids containing heavy (H) isotopes. Cells were then transduced with retroviruses expressing either Flag tag (RV Flag) or Flag tag and SMAD4 fusion protein (RV Flag-S4). Cells were harvested and mixed 4 days after activation. Immunoprecipitation was performed using anti-Flag. Immunoprecipitated proteins were processed and subjected to quantitative mass spectrometry analysis. Proteins with a heavy/light ratio of more than 2 were identified. In the other scheme, to identify the proteins whose SMAD4 interaction was perturbed upon TGFβ stimulation, CD4+ T cells from wild-type mice were sorted and activated either in the presence of IL-6 + inhibitor in the SILAC/AACT medium provided either with amino acids containing light isotopes or with amino acids containing heavy (H) isotopes. Cells were then transduced with retroviruses expressing either Flag tag (RV Flag) or Flag tag and SMAD4 fusion protein (RV Flag-S4). Cells were harvested and mixed 4 days after activation. Immunoprecipitation was performed using anti-Flag. Immunoprecipitated proteins were processed and subjected to quantitative mass spectrometry analysis. Proteins with a heavy/light ratio of less than 1 were identified. The commonly identified protein SKI in the two experiments was subjected to further investigation. 

b, CD4+ T cells from wild-type mice were activated in the presence of IL-6 and the indicated doses of TGFβ. Cells were harvested after 24 h. SKI protein expression was detected by immunoblotting. Results are representative of three experiments with similar results.

c, CD4+ T cells from wild-type mice were activated in the presence of IL-6 and the indicated doses of TGFβ. IL-17A+ and Foxp3+ cells were assessed by flow cytometry on day 4. Representative results (top) and statistical analysis (bottom) of five experiments are shown (centres indicate mean values).

d, CD4+ T cells from wild-type or Cd4-cre;Smad2fl/fl (S2 KO) mice were activated in the presence of IL-6 for 24 h. Cells were then stimulated with the indicated conditions for an additional 1 h with or without 10 μM SIS3 (specific inhibitor of Smad3 phosphorylation). SKI protein expression and Smad3 phosphorylation were assessed by immunoblotting. Results are representative of three experiments with similar results.
Extended Data Figure 4 | SKI and SMAD4 cooperatively suppress T<sub>H</sub>17 cell differentiation. a, Bone marrow cells were isolated from the femur bones of sex- and age-matched Cd4-cre;CdC(Cas9, CD45.2<sup>+</sup>) mice and wild-type (wild type, CD45.1<sup>+</sup>) mice. Cells were mixed, and transduced with two different gRNA-expressing viruses (as indicated) and transferred into sublethally irradiated (400 cGy) Bag<sup>−/−</sup> recipient mice. CD4<sup>+</sup> T cells isolated from lymph nodes and spleen of generated bone marrow chimaeric mice were activated in the presence of IL-6 and TGFβR inhibitor. Cells transduced with gRNA in wild-type donors are indicated as wild type. Cells transduced with gRNA in Cd4-cre;CdC donor are indicated as Ski knockout. IL-17A<sup>+</sup> cells were assessed by flow cytometry on day 4. Representative results (left) and statistical analysis (right) of five experiments are shown.

b, CD4<sup>+</sup> T cells from wild-type mice were activated in the presence of IL-6 and TGFβ, and then transduced with MSCV-IRES–GFP (RV), MSCV-SKI-IRES–GFP (RV SKI), or co-transduced with MSCV-SKI-IRES–GFP and MSCV-RORγt-IRES–Thy1.1 (RV SKI+RORγt) retroviruses. IL-17A expression of transduced (GFP<sup>+</sup>) or co-transduced (GFP<sup>+</sup> Thy1.1<sup>+</sup>) T cells was assessed by flow cytometry. Representative results (left) and statistical analysis (right) of five experiments are shown.

c, CD4<sup>+</sup> T cells from wild-type and Cd4-cre;Smad4<sup>fl/fl</sup> (S4 KO) mice were activated in the presence of IL-6+TGFβR inhibitor (i) or IL-6+TGFβ. Cells were harvested 3 days later. ChIP assay was performed with control IgG antibody or SKI antibody. The relative enrichment of SKI binding to the Rorc locus was determined. Means ± s.d. of three samples in one experiment of three are shown. (***<i>P</i> < 0.001; two-sided t-test; NS, not significant; centres indicate mean values.)
Extended Data Figure 5 | TGFβ superfamily signalling overcomes SKI–SMAD4 complex-mediated suppression of RORγt expression in activated T cells to enable Th17 cell differentiation. a, RORγt expression is potentiated by IL-6–STAT3 signalling but restrained by the histone deacetylase activity-containing SKI–SMAD4 complex that associates with and deacetylates the Rorc locus. b, Additional TGFβ or activin signalling triggers SKI degradation. The disruption of SKI–SMAD4 complex dissociates histone deacetylase activity from the Rorc locus and enables RORγt expression and Th17 cell differentiation.
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work we publish. This form is published with all life science papers and is intended to promote consistency and transparency in reporting. All life sciences submissions use this form; while some list items might not apply to an individual manuscript, all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size
   
   Describe how sample size was determined.
   
   The sample sizes are determined based on the prevailing and widely accepted practise and methods. In addition, the sample sizes for each experiment have been detailed in the Figure legend and Method.

2. Data exclusions
   
   Describe any data exclusions.
   
   For IP-MS:A contamination protein database in Maxquant was used to exclude peptides that match this database. The parameters for database searching were set as: 1) Carbamidomethylation of cysteines was set as fixed modification and protein N-terminal acetylutions as well as oxidation of methionines were set as variable modification for the peptide search; 2) A maximum mass deviation of 7.5 ppm was allowed for precursor’s identification and 0.5 Da was set as match tolerance for fragment identification (acquisition in orbitrap); 3) Up to two missed cleavages was allowed for trypsin digestion; 4) Peptide length of seven amino acids was considered for protein identification and quantification; 5) The minimum member of total peptides a protein group was set to 1 and the minimum member of razor+ unique peptides was set to 1; 6) The minimum Andromeda score for accepting an MS/MS identification for modified peptides was set to 40; 7) Using unique and razor peptides for protein quantification; 8) The false discovery rates (FDR) for peptide and protein identifications were set to a maximum 1% and 5% respectively.

For ChIP-seq: Raw reads (56-72 Million reads per sample) were first cleaned for adapter sequences using Trim Galore(Version 0.4.4 , a wrapper tool of Cutadapt, www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with parameters--"--paired --stringency 1 --dont_gzip --length 20--q 20 --trim1", in which reads with length less than 20 (-length 20) or average quality score less than 20 (-q 20) were removed for downstream analysis. Cleaned reads were aligned to mm10 using Bowtie (version 1.2, Ben Langmead, Cole Trapnell, Mihai Pop andSteven L Salzberg Ultrafast and memory-efficient alignment of short DNA sequences to the human genome Genome Biology 2009 10:R25 with the parameters –"-p 10 -m 1 -v 2 -X 1500 -S". These parameters ensured that fragments up to 1500bp (-X1500) and mismatch up to 2 (-v2) were allowed to align, and that only unique aligning reads were collected (-m1).

For RNA-seq: Reads (32-45 Million reads per sample) were first cleaned for adapter sequences using Trim Galore(version 0.4.4) with parameters--"--paired --stringency 1 --dont_gzip --length 20 -q 25 --trim1". Cleaned reads were aligned to the UCSC mm10 reference genome using STAR(Dobin A et al. 2013 Jan 1;29(1):15-21) with default parameters.The reads count for each gene was calculated by featureCounts (Liao Y, Smyth GK and Shi W 2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics, 30(7):923-30 with parameter—"-T 6 -t exon -g gene_name -M".The quantification results were then analyzed with the Bioconductor package DESeq2(Love MI, Huber W and Anders S 2014)."Moderated estimation of
3. Replication
Describe whether the experimental findings were reliably reproduced.

The experimental findings were reliably reproduced.

4. Randomization
Describe how samples/organisms/participants were allocated into experimental groups.

Mice with needed genotypes and ages were chosen randomly. For example, different group of mice (e.g. WT and KO, littermates if possible) were mixed in one cage, and treated equally without knowing the identity of the mice. The mice were then identified upon the completion of the experiments.

5. Blinding
Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Samples were labeled with simple numbers. The person who performed the experiments did not know the exact samples until after data were analyzed.

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 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 summary of the descriptive 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, MAC version, 9.3.2, Graghpad Prism 6.0c, XCalibur software version 2.1, MaxQuant version 1.5.0.25, Trim Galore version 0.4.4, Bowtie version 1.2, STAR, featureCounts, Bioconductor package DESeq2, HOMER Version 4, GREAT Version 3

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The Nature Methods guidance for providing algorithms and software for publication may be useful for any submission.

‒ 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.

No restricted material was used.
9. Antibodies

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

Antibodies including anti-CD4 (GK1.5), anti-CD25 (PC61.5), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-IFN-γ 188 (XMG1.2), anti-Thy1.1 (OX-7) and anti-IL-17A (TC11-18H10.1) from Biolegend; anti-CD3 (145-2C11), anti-CD28 (37.51), anti-IFN-γ (XMG1.2) and anti-IL-4 (11B11) from BioXcell; anti-Ski (H-329), anti-217 RORγt, anti-β-actin (I-19) and anti-IgG (sc-2027) from Santa Cruz, anti-Smad4 (D3M6U) and anti-pSmad3(C25A9) from Cell Signaling Technology, anti-Smad4 (EP618Y) from Abcam were obtained from indicated commercial vendors with ensured quality. In addition, all the antibodies has been used in multiple experiments to detect intended markers and proteins in control samples with expected results to validate their effectiveness in our study. Moreover, Smad4, RORγt, and Ski deficient T cells have been used to further validate related antibodies in our study.

10. Eukaryotic cell lines

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

b. Describe the method of cell line authentication used.

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

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

- No eukaryotic cell lines were used in the presented data.
- No eukaryotic cell lines were used in the presented data.
- No eukaryotic cell lines were used in the presented data.
- No commonly misidentified cell lines were used in the presented data.

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.

Smad4fl/fl, Tgfb2fl/fl, Cd4cre, ERCre, Rag1-/-, Rorc-/-, Cre-dependent-Cas9 knockin (CdC) and CD45.1 congenic wild-type mice were on the C57BL/6 background. 6-18 weeks old same sex (both males and females) mice were used. Primary T cells or HSC were isolated from the mice and then used for the subsequent experiments.

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.

Study did not involve human research participants
**Data deposition**

1. For all ChIP-seq data:
   - Confirm that both raw and final processed data have been deposited in a public database such as GEO.
   - Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

2. Provide all necessary reviewer access links. (The entry may remain private before publication.)

   - track type=bigWig name="Smad4_WT1" db=mm10 visibility=full alwaysZero=on maxHeightPixels=40 color=0,0,153 bigDataUrl=http://snpinfo.niehs.nih.gov/ucscview/takaku/Smad4_MT-384.singleFrag.Normalized.bigWig
   - track type=bigWig name="Smad4_WT2" db=mm10 visibility=full alwaysZero=on maxHeightPixels=40 color=0,0,153 bigDataUrl=http://snpinfo.niehs.nih.gov/ucscview/takaku/Smad4_MT-385.singleFrag.Normalized.bigWig
   - track type=bigWig name="Smad4_KO1" db=mm10 visibility=full alwaysZero=on maxHeightPixels=40 color=0,0,153 bigDataUrl=http://snpinfo.niehs.nih.gov/ucscview/takaku/Smad4_MT-386.singleFrag.Normalized.bigWig
   - track type=bigWig name="Smad4_KO2" db=mm10 visibility=full alwaysZero=on maxHeightPixels=40 color=0,0,153 bigDataUrl=http://snpinfo.niehs.nih.gov/ucscview/takaku/Smad4_MT-387.singleFrag.Normalized.bigWig

3. Provide a list of all files available in the database submission.

4. If available, provide a link to an anonymized genome browser session (e.g. UCSC).

   - ChIP-seq data for WT#1, WT#2, KO#1, KO#2

**Methodological details**

5. Describe the experimental replicates.

   - We used two replicates for ChIP-seq. (Two independent experiments)
6. Describe the sequencing depth for each experiment. Total numbers of reads are as follows. Smad4 ChIP-seq WT1: 72563366, Smad4 ChIP-seq WT2: 62982483, Smad4 ChIP-seq KO1: 60135618, Smad4 ChIP-seq KO2: 61833161, input WT1: 56967653, input WT2: 64144763, input KO1: 63174257, input KO2: 66197177. For the analysis, we used uniquely-mapped and deduplicated reads. (Smad4 ChIP-seq WT1: 33955608, Smad4 ChIP-seq WT2: 39070319, Smad4 ChIP-seq KO1: 35688532, Smad4 ChIP-seq KO2: 33577809, input WT1: 32558640, input WT2: 36817054, input KO1: 32535178, input KO2: 35203178)

7. Describe the antibodies used for the ChIP-seq experiments. Anti-Smad4 (EP618Y, Abcam)

8. Describe the peak calling parameters. We used program called findPeaks from Homer (Version 4) to call all reported ChIP-seq peaks in this manuscript. The basic idea is to identify regions in the genome where we find more sequencing reads than we would expect to see by chance. Two input data was pooled together before to do peak calling. findPeaks was run default parameters. All significant peaks were associated with genes using GREAT Version 3.

9. Describe the methods used to ensure data quality. We observed significant enrichment at many loci including previously known Smad4 binding sites. We prepared ChIP-seq libraries from Smad4 KO cells using the same protocol, and the signals were significantly lower in the Smad4 KO ChIP-seq data. More than 20,000 peaks were identified in the Smad4 WT cells while the KO cells only showed ~1,200 peaks.

10. Describe the software used to collect and analyze the ChIP-seq data. HOMER Version 4
                 GREAT Version 3
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. Naive CD4+ T cells were harvested from mouse spleen and lymph nodes, then MACS purified with CD4-beads or with FACS sorter. After activation, cells were collected and Ficol was used to remove cell debris. Spinal cord lymphocytes: mice were euthanized, then perfused with 50-60ml PBS containing heparin. Spinal cord removed and digested with 2mg/ml collagenase D. for 45min at 37C with brief vortexing every 15min, then filtered with 40ml nylon mesh. Cells were washed with PBS and then re-suspended in 6ml of 38% Percoll solution. Cells were separated at 2000rpm for 20m. Cell pellets were washed with 1xPBS and then re-suspend in PBS or culture media for subsequent procedures.

6. Identify the instrument used for data collection. BD Biosciences, FACSCanto II.

7. Describe the software used to collect and analyze the flow cytometry data. Flowjo, MAC version,v9.3.2

8. Describe the abundance of the relevant cell populations within post-sort fractions. The purity of sorted cell purity was >95%, determined by flow-cytometry.

9. Describe the gating strategy used. Preliminary FSC/SSC gating was used to gate on lymphocytes population. Specific T cell populations were gated based on the specific antibody staining as described for each experiment. Positive and negative populations were defined and gated clearly by the negative control sample and samples with control IgG staining.

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