Homeostatic control of metabolic and functional fitness of T<sub>reg</sub> cells by LKB1 signalling

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Regulatory T cells (T<sub>reg</sub> cells) have a pivotal role in the establishment and maintenance of immunological self-tolerance and homeostasis<sup>1,2</sup>. Transcriptional programming of regulatory mechanisms facilitates the functional activation of T<sub>reg</sub> cells in the prevention of diverse types of inflammatory responses<sup>3,4</sup>. It remains unclear how T<sub>reg</sub> cells orchestrate their homeostasis and interplay with environmental signals. Here we show that liver kinase B1 (LKB1) programs the metabolic and functional fitness of T<sub>reg</sub> cells in the control of immune tolerance and homeostasis. Mice with a T<sub>reg</sub>-specific deletion of LKB1 developed a fatal inflammatory disease characterized by excessive TH2-type-dominant responses. LKB1 deficiency disrupted T<sub>reg</sub> cell survival and mitochondrial disease characterized by excessive TH2-type-dominant responses. LKB1 deficiency disrupted T<sub>reg</sub> cell survival and mitochondrial disease characterized by excessive TH2-type-dominant responses. LKB1 deficiency disrupted T<sub>reg</sub> cell survival and mitochondrial disease characterized by excessive TH2-type-dominant responses.

The tumour suppressor LKB1 (encoded by <i>Stk11</i>) is a bioenergetic sensor that controls cell metabolism and growth<sup>5</sup>. To define the role of LKB1 in T<sub>reg</sub> cells, we crossed mice carrying loxP-flanked <i>Stk11</i> alleles (<i>Stk11<sup>fl/fl</sup></i>) with Foxp3<sup>Cre</sup> mice<sup>6</sup> to specifically delete <i>Stk11</i> in T<sub>reg</sub> cells (denoted hereafter as Foxp3<sup>CreStk11fl/fl</sup>) mice (Extended Data Fig. 1a). Compared with Foxp3<sup>Cre</sup> controls, Foxp3<sup>CreStk11fl/fl</sup> mice had a significantly shorter lifespan (Fig. 1a) and lower body weight (Fig. 1b), and manifested a reduced body size, skin ulceration and crusting of ears (Fig. 1c). Moreover, the mutant mice had splenomegaly and lymphadenopathy (Fig. 1d) and infiltration of immune cells in multiple organs (Fig. 1e). Notably, despite largely normal morphology of the colon and caecum in Foxp3<sup>CreStk11fl/fl</sup> mice (Extended Data Fig. 1b), these organs had notable accumulation of interepithelial mucosal mast cells<sup>7</sup> (Extended Data Fig. 1c). The development of a fatal inflammatory disease in Foxp3<sup>CreStk11fl/fl</sup> mice suggests that LKB1 has a crucial role in the function of T<sub>reg</sub> cells.

Among serum cytokines, levels of IL-4 and IL-5 were markedly elevated in Foxp3<sup>CreStk11fl/fl</sup> mice (Fig. 1f, g), but normal populations of splenocytes and macrophages (Extended Data Fig. 1h, i). Histological analysis revealed markedly increased numbers of eosinophils and Ym1<sup>+</sup> M2 macrophages in the skin of Foxp3<sup>CreStk11fl/fl</sup> mice (Extended Data Fig. 1j, k). Compared to wild-type controls, T cells in Foxp3<sup>CreStk11fl/fl</sup> mice showed the memory/effector phenotype (CD4<sup>+</sup>CD62L<sup>lo</sup>) (Extended Data Fig. 2a), with a large number of CD4<sup>+</sup> T cells, including T<sub>reg</sub> cells, expressing the Th1 cytokines IL-4 and IL-5 (Extended Data Fig. 2b, c). IFN-γ and IL-17 expression was also elevated, but to a lesser extent (Extended Data Fig. 2d, e). Moreover, CD4<sup>+</sup> T cells from the lung and colonic lamina propria of Foxp3<sup>CreStk11fl/fl</sup> mice upregulated IL-4 (Extended Data Fig. 2f). These abnormal type 2 immune responses were observed even in CD4<sup>+</sup> T cells from young Foxp3<sup>CreStk11fl/fl</sup> mice (Fig. 1h), Extended Data Fig. 2g), whereas Foxp3<sup>CreStk11fl/fl</sup> mice showed normal immune homeostasis (data not shown). These results reveal an excessive Th2-dominant inflammatory disorder in Foxp3<sup>CreStk11fl/fl</sup> mice.

The T<sub>reg</sub> cell compartment was reduced in percentage and cellularity in Foxp3<sup>CreStk11fl/fl</sup> mice (Fig. 2a, Extended Data Fig. 3a). LKB1-deficient T<sub>reg</sub> cells showed elevated 5-bromodeoxyuridine (BrDU) incorporation (Extended Data Fig. 3b), but also higher levels of caspase-3 activity (Fig. 2b) and expression of Bim (Extended Data Fig. 2c). The survival defects were observed in LKB1-deficient T<sub>reg</sub> cells from the mixed bone marrow chimera that avoided spontaneous inflammation (Fig. 2d, Extended Data Fig. 3c, d), indicating cell-intrinsic effects. Interestingly, Foxp3<sup>CreStk11fl/fl</sup> mice at 2 weeks old had a largely normal proportion of T<sub>reg</sub> cells (Extended Data Fig. 3e) with normal expression of Bim (Extended Data Fig. 3f). However, activation of T cells, infiltrations of immune cells into the lung, and elevation of serum IgE were appreciable in these mice (Extended Data Fig. 3g–i). Depletion of Bim (encoded by Bcl2l11) in LKB1-deficient T<sub>reg</sub> cells substantially restored T<sub>reg</sub> cell cellularity (Extended Data Fig. 3j), but Foxp3<sup>CreStk11fl/flBcl2l11fl/fl</sup> mice still had elevated frequencies of CD4<sup>+</sup>CD62L<sup>lo</sup> cells (Extended Data Fig. 3k) and IL-4-producing CD4<sup>+</sup> T cells (Fig. 2e), compared with wild-type or Foxp3<sup>CreBcl2l11fl/fl</sup> mice (Extended Data Fig. 3l, m). These results identify important roles of LKB1 in mediating both T<sub>reg</sub> cell survival and function.

The regulatory function is closely associated with signature molecules expressed by T<sub>reg</sub> cells<sup>1,2</sup>. The negative co-receptor PD-1 and TNF receptor superfamily proteins GITR and OX40 were markedly elevated on LKB1-deficient T<sub>reg</sub> cells from Foxp3<sup>CreStk11fl/fl</sup> mice (Extended Data Fig. 4a) and mixed bone marrow chimaeras (Fig. 2f), indicating cell-autonomous effects. By contrast, wild-type and LKB1-deficient T<sub>reg</sub> cells had largely comparable or slightly altered expression of CD62L, CD44 (Extended Data Fig. 4b), GATA3, IRF4, T-bet, RORγt (Extended Data Fig. 4c), Foxp3, ICOS, CTLA-4 and CD25 (Extended Data Fig. 4d, e). The co-receptors PD-1, GITR and OX40 have been implicated in the control of T<sub>reg</sub> cell function and generation<sup>8–13</sup>. To examine

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whether LKB1 directly regulates these Treg signatures in mature Treg cells, we generated Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) Rosa26\(^{RFP}\) mice (designated Foxp3\(^{Cre-ERT2}\)Stk11\(^{fl/fl}\) mice) to delete Stk11 in Treg cells upon tamoxifen treatment\(^{14}\) (Extended Data Fig. 4f). Longer duration of LKB1 loss diminished Treg cells (Extended Data Fig. 4g), associated with increased cell death (Extended Data Fig. 4h), whereas Treg cells with acute deletion of LKB1 maintained intact homeostasis (Extended Data Fig. 4i) and Bim expression (Fig. 2g). In this setting of an inflammation-free environment (Extended Data Fig. 4j, k), loss of LKB1 led to upregulated PD-1, GITR and OX40 expression (Fig. 2g), without affecting Foxp3, ICOS and CD25 (Extended Data Fig. 4i) or the distribution of resting and activated Treg subsets (Extended Data Fig. 4m). Collectively, LKB1 acts in a cell-autonomous and direct manner to suppress a select group of Treg signature molecules.

Continuous T cell receptor (TCR) signalling drives Treg cell function in maintaining immune tolerance\(^{15,16}\). Stimulation of Treg cells with anti-CD3 and anti-CD28 (anti-CD3/CD28) antibodies resulted in LKB1 phosphorylation and modestly increased LKB1 expression (Fig. 3a).

Figure 2 | LKB1 controls Treg cell survival and expression of selective co-receptors PD-1, GITR and OX40. a, Treg cells from wild-type and Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) mice. Right, proportion and number of Treg cells (WT, n = 5; Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\), n = 4). b, C. Caspase-3 activity (b) and Bim expression (c) in Treg cells. d. Donor wild-type and Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) Treg cells from mixed bone marrow chimaeras. e, IL-4- and IL-17-producing CD4\(^{+}\) T cells from the indicated mice. f, PD-1, GITR and OX40 expression on Treg cells in d. g, PD-1, GITR, OX40, and Bim expression in wild-type Treg cells and Treg cells with acute deletion of LKB1. Data are representative of at least three (a–g) independent experiments. Data are mean ± s.e.m. *P values are determined by Mann–Whitney test (a, cell proportion) or two-tailed Student’s t-test (a, cell number). **P < 0.005, ***P < 0.0005. Numbers above graphs indicate the mean fluorescence intensity; numbers in quadrants indicate percentage of cells.
Figure 3 | LKB1 regulates Treg cell metabolism through connecting immune signals and mitochondrial function. a, Phosphorylation and expression of LKB1 in resting and activated Treg cells. b, Phosphorylation of S6 and 4E-BP1 in resting Treg cells. c, Treg cells from wild-type and Foxp3<sup>Cre</sup>Prkaa1<sup>fl/fl</sup>Prkaa2<sup>fl/fl</sup> mice. d, Heat maps of differentially expressed intracellular metabolites and unsupervised hierarchical clustering (Foxp3<sup>Cre</sup>Stk11<sup>fl/fl</sup>, n = 3; Foxp3<sup>Cre</sup>Stk11<sup>fl/fl</sup>, n = 4). FAO, fatty acid β-oxidation. e, Relative abundance of histamine in the cells and culture medium in d (Foxp3<sup>Cre</sup>Stk11<sup>fl/fl</sup>, n = 3; Foxp3<sup>Cre</sup>Stk11<sup>fl/fl</sup>, n = 4).

mTORC1 and HIF-1α are important effector pathways aberrantly upregulated in LKB1-deficient cancer cells and conventional T cells<sup>17,18</sup>. Unexpectedly, Foxp3<sup>Cre</sup>Stk11<sup>fl/fl</sup> Treg cells had slightly reduced mTORC1 activity under steady state (Fig. 3b) and largely normal phosphorylation of S6, AKT (S473) and FOXO1 upon anti-CD3/CD28 stimulation (Extended Data Fig. 5a, b). Additionally, depletion of HIF-1α in LKB1-deficient Treg cells failed to rescue the defective Treg cell homeostasis or inflammatory disorder (Extended Data Fig. 5c–e). 5′AMP-activated protein kinase (AMPK) is a well-documented substrate of LKB1 (ref. 5), the activation of which was lost in LKB1-deficient Treg cells (Extended Data Fig. 5f). Nonetheless, deletion of AMPKα1 and AMPKα2 in Treg cells did not affect Treg cell proportion or immune homeostasis (Fig. 3c, Extended Data Fig. 5g, h). Taken together, these results show that LKB1 is activated by TCR signalling in Treg cells but functions independently of mTORC1–HIF-1α axis or AMPK signalling.

In the transcriptome of wild-type and LKB1-deficient Treg cells from the mixed bone marrow chimaeras, gene set enrichment analysis (GSEA) showed that LKB1 deficiency impaired gene expression implicated in multiple metabolic pathways (Extended Data Fig. 6a), including tricarboxylic acid (TCA) cycle and mitochondrial protein import (Extended Data Fig. 6b, c). Unbiased metabolomic profiling in activated Foxp3<sup>Cre</sup>Stk11<sup>fl/fl</sup> Treg cells (Extended Data Fig. 6d) revealed a marked reduction of metabolic intermediates associated with mitochondrial function including TCA cycle and fatty acid β-oxidation, as well as those in purine and pyrimidine metabolism, whereas metabolites in glycolysis were not uniformly altered (Fig. 3d, Extended Data Fig. 6e, f). Notably, histamine was increased in LKB-deficient Treg cells (Fig. 3e). Histidine decarboxylase, an enzyme that converts histidine to histamine, was increased in LKB1-deficient Treg cells (Fig. 3f). As histamine has been implicated in suppressing Treg cell function<sup>19</sup>, an increased production of histamine in LKB-deficient Treg cells could contribute to their defects in an autocrine manner. Metabolite set enrichment analysis indicated that LKB1 deficiency in Treg cells downregulated multiple metabolic programs, but upregulated the pathway for biosynthesis of unsaturated fatty acids (Fig. 3g). LKB1-deficient Treg cells had defective mitochondrial fitness, indicated by reduced mitochondrial mass, mitochondrial membrane potential, and reactive oxygen species (Extended Data Fig. 6g, h), associated with diminished intracellular ATP (Extended Data Fig. 6i). LKB1 deletion reduced oxygen consumption rate without significantly affecting extracellular acidification rate (Extended Data Fig. 6j), further confirming impaired mitochondrial function. Furthermore, LKB1-deficient Treg cells showed a notable accumulation of lipids (Extended Data Fig. 6k), associated with defective fatty acid β-oxidation (Extended Data Fig. 6l) but normal lipogenesis (Extended Data Fig. 6m). Therefore, LKB1 orchestrates metabolic rewiring and mitochondrial function in Treg cells.

We next explored the cellular processes underlying excessive Th2 responses in Foxp3<sup>Cre</sup>Stk11<sup>fl/fl</sup> mice. Deletion of IL-4 partly rescued Th2 responses in Foxp3<sup>Cre</sup>Stk11<sup>fl/fl</sup> mice, as indicated by partially mitigated proportions of IL-13- and IL-5-producing CD4<sup>+</sup> T cells, accumulation of eosinophils and PD-L2<sup>+</sup> dendritic cells (DCs) that are associated with Th2 responses<sup>20</sup>, and production of serum IgE (Extended Data Fig. 7a). However, the Treg cell compartment remained defective in Foxp3<sup>Cre</sup>Stk11<sup>fl/fl</sup> mice, as indicated by a reduced proportion of Treg cells (Extended Data Fig. 7f) and aberrant PD-1, GITR and OX40 expression (Extended Data Fig. 7g). Moreover, these mice had increased proportions of CD44<sup>hi</sup>CD62L<sup>lo</sup> memory/effector cells and IFN-γ-producing CD4<sup>+</sup> T cells and extensive inflammation in a number of organs (Extended Data Fig. 7h–j). Collectively, these results indicate that IL-4 contributes to amplifying
Figure 4  | The LKB1–β-catenin signalling axis enforces Treg-mediated suppression of T\(_{h2}\) responses through the control of PD-1 expression. a. Production of TSLP in the lung (WT, n = 7; Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\), n = 5). b. Fold change of PD-1 expression on DCs with or without TSLP stimulation (n = 3 each group). c, CD11b+ PD-L2+ DCs from wild-type and Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) Treg cells (n = 4 each group). d. Mean fluorescence intensity (MFI) of PD-L2 expression on DCs (with TSLP) cultured alone, or together with wild-type or Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) Treg cells (n = 4 each group). e. Expression of IL-4 and IFN-γ in CD4+ T cells co-cultured with TSLP-DCs, alone, or together with Foxp3\(^{Cre}\)ERT2Stk11\(^{fl/fl}\) or Foxp3\(^{Cre}\)ERT2Stk11\(^{fl/+}\) Treg cells. f. Expression of IL-4 and IFN-γ in CD4+ T cells co-cultured with TSLP-DCs and bone marrow chimaeras using bone marrow cells from Treg-deficient Scurfy mice, together with those from wild-type or Pdcd1\(^{−/−}\) mice. Scurfy–Pdcd1\(^{−/−}\) chimaeras had a reduced proportion of lung and spleen eosinophils (Extended Data Fig. 9e) and concentration of serum IgG1 (Extended Data Fig. 9f), but normal concentration of IgG2a/c (Extended Data Fig. 9f). Finally, blocking PD-1 and its ligands largely reversed the defect of LKB1-deficient Treg cells in suppressing T\(_{h2}\) cell differentiation (Fig. 4f). Therefore, LKB1 restrains PD-1 expression as an important mechanism to ensure the functional integrity of Treg cells in repressing T\(_{h2}\) responses.

We next explored how LKB1 regulates PD-1 and other Treg signature molecules. Wnt signalling, which is implicated in T\(_{h2}\)-mediated inflammation\(^{23}\) and Treg cell survival and function\(^{24,25}\), was significantly underrepresented in gene sets from LKB1-deficient Treg cells (Extended Data Fig. 10a). Activated wild-type Treg cells upregulated the expression of β-catenin, a key mediator of Wnt signalling, while the induction was attenuated in LKB1-deficient Treg cells (Fig. 4g). As a result of the modestly reduced β-catenin (Ctnnb1) mRNA (Extended Data Fig. 10b), LKB1-deficient Treg cells had increased phosphorylation of β-catenin that mediates its degradation (Extended Data Fig. 10c). Moreover, β-catenin bound to a T-cell factor 3 (TCF3) motif in the Pdcd1 locus in wild-type Treg cells, but this binding was lost in LKB1-deficient Treg cells (Extended Data Fig. 10d). Expression of constitutively active β-catenin by retrovirus (β-cat–RV) reversed the aberrant expression of PD-1 and GITR on LKB1-deficient Treg cells (Fig. 4h, Extended Data Fig. 10e), but did not affect CD25 expression (Extended Data Fig. 10e) or survival of Treg cells (data not shown). Similar rescue effects were observed in β-catenin-expressing Treg cells after acute deletion of LKB1 (Extended Data Fig. 10f). Moreover, β-cat–RV substantially rectified the defects of LKB1-deficient Treg cells in suppressing DC expression of PD-L2, CD80 and CD86 (Extended Data Fig. 10g) and T\(_{h2}\) cell differentiation polarized by TSLP-DCs (Fig. 4i). Collectively, these results indicate an important role of the LKB1–β-catenin signalling axis in the control of T\(_{h2}\) responses through modulating PD-1 and Treg cell signature molecules.
Emerging studies highlight the importance of metabolic reprogramming in immune cell activation and differentiation\textsuperscript{20,26}, but how metabolism and immune function are integrated is not fully understood. Here, we identify LKB1 as a crucial checkpoint to maintain T\textsubscript{reg} cell survival and function through coordinating metabolic and functional fitness of T\textsubscript{reg} cells (Extended Data Fig. 10h). LKB1 connects immunological signals and cellular metabolism including mitochondrion-related functions. Furthermore, LKB1 curtails the aberrant induction of PD-1, GITR and OX40 in a β-catenin-dependent manner, and blockade of PD-1 signalling reinvigorates the ability of LKB1-deficient T\textsubscript{reg} cells in the repression of T\textsubscript{eff} responses. PD-1 upregulation and metabolic alteration have long been observed in exhausted CD8\textsuperscript{+} T cells\textsuperscript{27}, and increasing evidence reveals the interplay between immune checkpoints and cell metabolism\textsuperscript{28–30}. Our results suggest that T\textsubscript{reg} cells not only exist at a resting or activated state\textsuperscript{1}, but could also undergo functional exhaustion. Given the constitutive expression of the epithelial cytokine TSLP and the ensuing stimulation of DCs at the environmental interface, T\textsubscript{reg} cells require LKB1 to actively maintain their metabolic and immunological homoeostasis, the loss of which results in the apoptotic and functional exhaustion of T\textsubscript{reg} cells. Our findings point to a previously unrecognized mechanism enforcing homoeostatic control of T\textsubscript{reg} cells, and provide a framework to further understand integration of metabolic signalling and lymphocyte fate and state.

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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METHODS

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

Mice. C57BL/6, CD45.1−, Stk11fl/fl, Bcl2l11a−/−, Hif1afl/fl, Prkaad−/−, Prkaca−/−, Rag1−/−, Btμ−/−, Pdcd1−/−, and Rosa26reporter (a locus-site-flanked STOP cassette followed by the YFP-encoding sequence inserted into the Rosa26 locus) mice were purchased from the Jackson Laboratory. Foxp3−/− and Foxp3−/−Stk11−/− mice were used at 3–5 weeks old unless otherwise noted, with the age- and gender-matched wild-type mice containing the Foxp3−/− allele as controls. Other mice were used at 8–10 weeks old unless otherwise noted. Bone marrow chimerae were generated by transferring 1 × 10^7 T-cell-depleted bone marrow cells into sub-lethally irradiated (5.5 Gy) Rag1−/− mice, followed by reconstitution for at least 2 months. For tamoxifen administration, mice were injected intraperitoneally with tamoxifen (2 mg per mouse) in corn oil every other day for 6 times, and then analysed 6–7 days after the last injection (unless otherwise noted). All mice were kept in a specific pathogen-free facility in the Animal Resource Center at St Jude Children’s Research Hospital, and animal protocols were approved by the Institutional Animal Care and Use Committee.

Flow cytometry. For analysis of surface markers, cells were stained in PBS containing 2% (wt/vol) BSA, with anti-CD4 (RM4-5), anti-CD8α (53-6.7), -TcrC3 (H57-597), anti-CD25 (PC61.5), anti-CD44 (1M7), anti-CD62L (MEL-14), anti-CD45.1 (Ly5.1), anti-CD45.2 (Ly5.2), anti-CD11c (N418), anti-Ly6G (RB6-8C5; all from BD Biosciences). Intracellular levels of ATP in Treg cells were measured using luminescent ATP detection assay kit (Abcam). BFA and TCR stimulation (1 μg/ml of anti-CD3 and 2 μg/ml of anti-CD28 for an additional 5 days). To block PD-1 (20 μg/ml of 29F.1A3), CD11c, and CD25 (5 μg/ml each) from wild-type and Foxp3−/−Stk11−/−, Ctnnb1−/−, and Prkaa2−/− mice were cultured in Click’s medium (plus 5% fetal bovine serum, 100 μg/ml of gentamicin, and 0.05 ng/ml of 1,25-dihydroxyvitamin D₃ as internal standards (Cambridge Isotope Laboratories). The samples were centrifuged (10 min, 9,000g, 4°C) and the supernatants were collected. Three liquid chromatography tandem mass spectrometry (LC–MS) systems were used to profile metabolites. Negative-ion targeted profiling of polar metabolites was performed using an ACQUITY UPLC (Waters Corp) coupled to a 550 QTRAP triple quadrupole mass spectrometer (AB SCIEX). Extracts (10 μl) were injected directly onto a 150 × 2.0 mm Atlantis HILIC column (Waters). The column was eluted at a flow rate of 400 μl min⁻¹ with initial conditions of 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide (Sigma–Aldrich) in water (YVVR)) and 90% mobile phase B (10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol (VWR)) followed by a 10 min linear gradient to 100% mobile phase A. The ion spray voltage was −4.5 kV and the source temperature was 50°C. Positive ionization mode profiling of polar metabolites was performed using a Nexera X2 U-HPLC (Shimadzu)–Q Exactive Orbitrap (Thermo Fisher Scientific) LC–MS system. The 80% methanol extracts (100 μl) were dried using a nitrogen evaporator (TurboVap LV; Biotage) and then resuspended in 10 μl of water and 90 μl of 74:24:9.2:0.2 vol/vol/vol acetonitrile/methanol/formic acid containing stable isotope-labelled internal standards (valine-d8, isotic, and phenylalanine-d8, Cambridge Isotope Laboratories). The samples were centrifuged (10 min, 9,000g, 4°C) and the supernatants were injected directly onto a 150 × 2.0 mm Atlantis HILIC column (Waters). The column was eluted isocratically at a flow rate of 250 μl min⁻¹ with 5% mobile phase A (10 mM ammonium formate and 0.1% formic acid in water) for 1 min followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 min. The electrospray ionization voltage was 3.5 kV and data were acquired using full scan analysis over m/z 70–800 at 70,000 resolution. Reversed-phase C18 chromatography/negative ion mode mass spectrometry analyses of free fatty acids and bile acids were conducted using an LC–MS system comprised of a Shimadzu Nexera X2 U-HPLC (Shimadzu Corp.) coupled to a Q Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific). Medium (30 μl) was extracted using 90 μl of methanol containing PGE2-d4 (Cayman Chemical Co.) and centrifuged (10 min, 9,000g, 4°C) and the cell extracts were analysed directly. The samples were injected onto a 150 × 2.1 mm ACQUITY BEH C18 column (Waters). The column was eluted isocratically at a flow rate of 450 μl min⁻¹ with 80% mobile phase A (0.01% formic acid in water) for 3 min followed by a linear gradient to 100% mobile phase B (acetonitrile with 0.01% acetic acid) over 12 min. MS analyses were carried out in the negative ion mode using electrospray ionization, full-scan MS acquisition over 70–850 m/z, and a resolution setting of 70,000. Metabolite identities were confirmed using authentic reference standards. Other mass spectrometry settings were: spray voltage −3.5 kV, capillary temperature 320 °C, and heater temperature 300 °C. LC–MS data were processed and visually inspected using MultiQuant 2.1 (SCIEX) and TraceFinder 3.1 software (Thermo Fisher Scientific). Metabolite set enrichment analysis was performed as described previously 35.

Metabolic assays. Treg cells were stimulated with plate-bound anti-CD3/CD28 for 48 h. To measure de novo lipid synthesis, d-[1-14C]glucose (Perkin Elmer) was added to the cells for the final 8 h of culture. Cells were lysed in 0.5% Triton X-100, and the lipid fraction was extracted by addition of chloroform and methanol (2:1 v/v) with vortexing, followed by addition of water with vortexing. After centrifugation, the lower chloroform phase obtained was evaporated to dryness and the residue was measured with a Beckman LS6500 scintillation counter. Oxygen consumption rate and extracellular acidification rate were measured in XF media (non-buffered DMEM containing 5 mM glucose, 2 mM l-glutamine and 1 mM sodium pyruvate), under basal conditions and in response to 1 μM oligomycin, 2 μM furosemide and 10 μM sodium cyanide (FCCP), 200 μM Etomoxir (Eto) and 1 μM rotenone/antimycin A (Rot/AA) using the XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). Intracellular levels of ATP in Treg cells were measured using luminescent ATP detection assay kit (Abcam).

Gene expression profiling. RNA samples from donor-derived Treg cells from C57BL/6, CD45.1−, wild-type and CD45.2−/− mixed bone marrow chimaeas (n = 5 each group) were analysed using the Mouse Gene 2.0 ST Signals array. Differentially expressed transcripts were identified by ANOVA (Partek Genomics Suit 6.5), and the Benjamini–Hochberg method was used to estimate the false discovery rate (FDR). Gene set enrichment analysis (GSEA) was performed as using the following antibodies: phospho-LKB1 (Ser428; C67A3), LKB1 (D605C5), β-catenin (D10A8), phospho-β-catenin (Ser33/37/Thr41), phospho-STAT5 (Ser253/256; D57.2.2E), phospho-AKT (Ser473; D9E), phospho-FOXO1 (Ser256), phospho-AMPK (Thr172) (all from Cell Signaling Technology), and β-actin (Sigma).

Differentially expressed transcripts were identified by ANOVA (Partek Genomics Suit 6.5), and the Benjamini–Hochberg method was used to estimate the false discovery rate (FDR). Gene set enrichment analysis (GSEA) was performed as using the following antibodies: phospho-LKB1 (Ser428; C67A3), LKB1 (D605C5), β-catenin (D10A8), phospho-β-catenin (Ser33/37/Thr41), phospho-STAT5 (Ser253/256; D57.2.2E), phospho-AKT (Ser473; D9E), phospho-FOXO1 (Ser256), phospho-AMPK (Thr172) (all from Cell Signaling Technology), and β-actin (Sigma).

RNA and immunoblot analysis. Real-time PCR analysis was performed with probe sets Stk11 (Mm00488473_g1), Hdc (Mm00456104_m1), Ctnnb1 (Mm00483039_m1), and Actb (Mm00607939_s1) (all from Thermofisher Scientific). Immunoblots were performed and quantified as described previously 34.
described previously. The microarray data have been deposited in the Gene Expression Omnibus under accession number GSE83088.

**Serum antibodies.** Immunoglobulin subclasses were measured with kits from Alpha Diagnostic International (5110) and Millipore (MGAMMAG-300K), respectively.

**OVA-induced allergic airway inflammation.** Mice were sensitized with mixture of OVA (50 μg ml⁻¹ per mouse) and alum adjuvant (1:2 dilution) injected intraperitoneally (100 μl per mouse), followed by the second sensitization 12 days later. Eight days after the second sensitization, mice were intranasally challenged with OVA for 4 consecutive days. At 18 h after the last challenge, lymphocytes were isolated from the spleen and lung and analysed by flow cytometry.

**Chromatin immunoprecipitation (ChIP).** ChIP methodology was performed as described. Briefly, cells were cross-linked for 40 min using 12.5 μM ethylene glycol-bis(succinimidyl succinate) (ThermoFisher Scientific), followed by the addition of formaldehyde (1% final concentration) and incubation for additional 20 min. After the reactions were quenched with glycine, cell pellets were lysed in cell lysis buffer (25 mM HEPES pH 7.8, 1.5 mM MgCl₂, 10 mM KCl, 0.3% NP-40 and 1 mM DTT) containing a protease inhibitor tablet (Roche) for 10 min on ice. Nuclei were pelleted and lysed in nuclear lysis buffer (50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.2% SDS) containing a protease inhibitor tablet for 10 min on ice, before sonication into 500-bp pieces using a Diagenode Bioruptor. Sheared chromatin was cleared of debris and incubated with IgG (Santa Cruz) or anti-β-catenin antibody (Cell Signaling; 1:50) and blocker (Active Motif) rotating overnight at 4°C. Chromatin immunoprecipitation and subsequent DNA purification were performed using the ChIP-IT High Sensitivity Kit (Active Motif) per manufacturer's instructions. Real-time PCR was performed using the primers spanning the TCF3 motif in the Pdcd1 locus (http://rstats.immgen.org/Chromatin/): forward, 5′-GGAAACAGGGCACCAAGACAT-3′, reverse, 5′-CTACTCAATCCGTGGGAGGA-3′, and negative control primer sets (Active Motif 71011). Data analysis was performed using the ‘Percent Input’ normalization method.

**Statistical analysis.** P values were calculated by Mann–Whitney test, two-tailed unpaired Student’s t-test, one-way ANOVA or two-way ANOVA as indicated using GraphPad Prism, unless otherwise noted. Statistical analysis of mouse survival and respective P values were determined using the log-rank test. P < 0.05 was considered as significant. All error bars represent the s.e.m.

**Data availability.** The microarray data that support the findings of this study have been deposited in the Gene Expression Omnibus under accession number GSE83088.

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Extended Data Figure 1  |  Disrupted immune homeostasis in Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus} mice. a, Expression of Stk11 mRNA (WT, n = 3; Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus}, n = 4) and LKB1 protein in CD4\textsuperscript{+}YFP\textsuperscript{+} T cells (Treg cells) from wild-type and Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus} mice. b, Representative images of haematoxylin and eosin staining of colon (original magnification, \(\times 20\)) and caecum (\(\times 10\)) from wild-type and Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus} mice. c, Representative images of MCPT1 (mast cell protease 1) staining of colon (original magnification, \(\times 20\)) and caecum (\(\times 20\)) from wild-type and Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus} mice. MCPT1 (brown) labels the interepithelial mucosal mast cells (ieMMCs). d, Quantification of serum MCP-1 (WT, n = 10; Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus}, n = 12), TNF-\(\alpha\) (WT, n = 11; Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus}, n = 12) and IFN-\(\gamma\) (WT, n = 9; Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus}, n = 8). e, Quantification of IgG2a/c, IgG2b, IgA and IgM in the serum from wild-type and Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus} mice (n = 6 each group). f, g, Flow cytometry of eosinophils (CD11b\textsuperscript{+}Siglec-F\textsuperscript{+}) in the spleen (f) and lung (g), and alveolar macrophages (CD11b\textsuperscript{+}Siglec-F\textsuperscript{+}) in the lung (g) from wild-type and Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus} mice. h, i, Flow cytometry of neutrophils (CD11b\textsuperscript{+}Ly6G\textsuperscript{+}), h and macrophages (CD11b\textsuperscript{+}F4/80\textsuperscript{+}) in the spleen from wild-type and Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus} mice. j, k, Representative images of MBP (major basic protein, pink, which labels eosinophils) (j) and YM1 (brown, which labels M2 macrophages) (k) staining of skin (\(\times 20\)) from wild-type and Foxp3\textsuperscript{cre}\textsuperscript{Stk11\textminus/\textminus} mice. Data are representative of two (a–c, j, k), one (d, e) or at least three (f–i) independent experiments. Data are mean \(\pm\) s.e.m. \(P\) values are determined by two-tailed Student’s \(t\)-test (a, d, e). NS, not significant; *\(P < 0.05\), **\(P < 0.005\), ***\(P < 0.0005\). Numbers in gates indicate percentage of cells.
Extended Data Figure 2 | Excessive T cell activation and T\(_H\)2-dominant inflammatory responses in Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) mice. a, Expression of CD62L and CD44 on CD4\(^{+}\) and CD8\(^{+}\) T cells in the spleen from wild-type and Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) mice. b, Flow cytometry and quantification of IL-4- (WT, \(n = 4\); Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\), \(n = 5\)) and IL-5- (\(n = 4\) each group) producing CD4\(^{+}\) T cells in the spleen from wild-type and Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) mice (approximately 4–5 weeks old) after in vitro stimulation for 4 h. c, Flow cytometry of IL-4- and IL-5-producing T\(_{reg}\) cells in the spleen from wild-type and Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) mice. d, e, Flow cytometry and quantification of IFN-\(\gamma\)- (\(n = 4\) each group; d) and IL-17-producing (WT, \(n = 4\); Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\), \(n = 5\); e) CD4\(^{+}\) T cells in the spleen from the mice in b, f. Flow cytometry of IL-4-producing CD4\(^{+}\) T cells in the lung (left panel) and colon (right panel) from wild-type and Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) mice. g, Expression of IL-5 and IL-17 in CD4\(^{+}\) T cells in the spleen from young wild-type and Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) mice (approximately 16 days old). Right, fold changes of IL-5- or IL-17-producing CD4\(^{+}\) T cells from Foxp3\(^{Cre}\)Stk11\(^{fl/fl}\) versus wild-type counterparts (\(n = 5\) each group). Data are representative of at least three (a–g) independent experiments. Data are mean ± s.e.m. \(P\) values are determined by Mann–Whitney test (b, d, e) or two-tailed Student's \(t\)-test (g). *\(P < 0.05\), **\(P < 0.005\), ***\(P < 0.0005\). Numbers in quadrants or gates indicate percentage of cells.
Extended Data Figure 3 | LKB1 regulates T<sub>reg</sub> cell homeostasis and function. a, Flow cytometry of T<sub>reg</sub> cells in the lung (left panel) and colon (right panel) from wild-type and Foxp3<sup>creStk11<sup>fl/fl</sup></sup> mice. b, BrdU incorporation in splenic T<sub>reg</sub> cells from wild-type and Foxp3<sup>creStk11<sup>fl/fl</sup></sup> mice at 16 h after injection of BrdU. c, d, Expression of active caspase-3 (c) and Bim (d) in CD45.2<sup>+</sup> T<sub>reg</sub> cells from CD45.1<sup>+</sup> mixed bone marrow chimaeras. e, Flow cytometry of splenic T<sub>reg</sub> cells from wild-type and Foxp3<sup>creStk11<sup>fl/fl</sup></sup> mixed bone marrow chimaeras. f, g, Expression of Bim (f), and CD62L and CD44 (g) in splenic CD4<sup>+</sup> T cells from young wild-type and Foxp3<sup>creStk11<sup>fl/fl</sup></sup> mice (approximately 16 days old). h, i, Representative images of haematoxylin and eosin staining of the lung (h) and measurement of IgE in the serum (i) from young (approximately 16 days old) wild-type (n = 7) and Foxp3<sup>creStk11<sup>fl/fl</sup></sup> mice (n = 4). j, Flow cytometry of splenic T<sub>reg</sub> cells from wild-type, Foxp3<sup>creStk11<sup>fl/fl</sup></sup> and Foxp3<sup>creBcl2l11<sup>fl/fl</sup></sup> mice. k, Expression of CD62L and CD44 on CD4<sup>+</sup> T cells from wild-type, Foxp3<sup>creStk11<sup>fl/fl</sup></sup> and Foxp3<sup>creBcl2l11<sup>fl/fl</sup></sup> mice. l, Flow cytometry of splenic T<sub>reg</sub> cells from wild-type and Foxp3<sup>creBcl2l11<sup>fl/fl</sup></sup> mice. m, Expression of IL-4 and IFN-γ in CD4<sup>+</sup> T cells from wild-type and Foxp3<sup>creBcl2l11<sup>fl/fl</sup></sup> mice after in vitro stimulation for 4 h. Data are representative of at least three (a–d) or two (e–m) independent experiments. Data are mean ± s.e.m. P values are determined by Mann–Whitney test (e) or two-tailed Student’s t-test (i). NS, not significant; *P < 0.01. Numbers above graphs indicate the mean fluorescence intensity; numbers in quadrants or gates indicate percentage of cells.
Extended Data Figure 4 | LKB1 regulates the expression of distinct T<sub>reg</sub> signature molecules. a, Comparison of PD-1, GITR and OX40 expression on T<sub>reg</sub> cells from wild-type and Foxp3<sup>creStk11<sup>fl/fl</sup></sub> mice. b, Flow cytometry of resting (CD62L<sup>hi</sup>CD44<sup>lo</sup>) and activated (CD62L<sup>lo</sup>CD44<sup>hi</sup>) T<sub>reg</sub> cells in the spleen from wild-type and Foxp3<sup>creStk11<sup>fl/fl</sup></sub> mice. c, Comparison of GATA3, IRF4, T-bet and ROR<sup>γ</sup> in T<sub>reg</sub> cells from wild-type and Foxp3<sup>creStk11<sup>fl/fl</sup></sub> mice. d, Comparison of Foxp3, ICOS, CTLA-4 and CD25 expression in T<sub>reg</sub> cells from wild-type and Foxp3<sup>creStk11<sup>fl/fl</sup></sub> mice. e, Comparison of Foxp3, ICOS, CTLA-4 and CD25 expression in CD45.2<sup>+</sup> wild-type and Foxp3<sup>creStk11<sup>fl/fl</sup></sub> T<sub>reg</sub> cells from mixed bone marrow chimaeras. f, Expression of Stk11 mRNA in CD4<sup>+</sup> Foxp3–GFP<sup>+</sup> T<sub>reg</sub> cells from Foxp3<sup>cre-ERT2Stk11<sup>fl/fl</sup></sub> and Foxp3<sup>cre-ERT2Stk11<sup>fl/fl</sup></sub> mice (n = 7 each group). g, Flow cytometry of Foxp3–GFP<sup>+</sup> YFP<sup>+</sup> T<sub>reg</sub> cells in g, l, Flow cytometry of Foxp3–GFP<sup>+</sup> YFP<sup>+</sup> T<sub>reg</sub> cells from Foxp3<sup>cre-ERT2Stk11<sup>fl/fl</sup></sub> and Foxp3<sup>cre-ERT2Stk11<sup>fl/fl</sup></sub> mice at day 6 after the last tamoxifen administration. j, k, Expression of IL-4 versus IL-13 (j) and IFN-γ versus IL-17 (k) in CD4<sup>+</sup> T cells from Foxp3<sup>cre-ERT2Stk11<sup>fl/fl</sup></sub> and Foxp3<sup>cre-ERT2Stk11<sup>fl/fl</sup></sub> mice after in vitro stimulation for 4 h. l, Comparison of Foxp3–GFP, ICOS and CD25 expression on Foxp3–GFP<sup>+</sup> YFP<sup>+</sup> T<sub>reg</sub> cells from Foxp3<sup>cre-ERT2Stk11<sup>fl/fl</sup></sub> and Foxp3<sup>cre-ERT2Stk11<sup>fl/fl</sup></sub> mice. m, Flow cytometry of resting (CD62L<sup>hi</sup>CD44<sup>lo</sup>) and activated (CD62L<sup>lo</sup>CD44<sup>hi</sup>) T<sub>reg</sub> cells in the spleen from Foxp3<sup>cre-ERT2Stk11<sup>fl/fl</sup></sub> and Foxp3<sup>cre-ERT2Stk11<sup>fl/fl</sup></sub> mice. Data are representative of at least three (a–i, l, m) or two (j, k) independent experiments. Data are mean ± s.e.m. P values are determined by two-tailed Student’s t-test (f). ***P < 0.0001. Numbers above graphs indicate the mean fluorescence intensity; numbers in quadrants or gates indicate percentage of cells.
Extended Data Figure 5 | LKB1 regulates T<sub>reg</sub> cell function independently of mTOR–HIF-1α axis or AMPK signalling. a, b, Phosphorylation of S6 (a), and phosphorylation of AKT S473 and FOXO1 (b) in wild-type and Foxp<sup>3</sup>WT/Stk11<sup>fl/fl</sup> T<sub>reg</sub> cells stimulated with or without anti-CD3/CD28 antibodies for 48 h. c, Flow cytometry of splenic T<sub>reg</sub> cells from wild-type, Foxp<sup>3</sup>WT/Stk11<sup>fl/fl</sup>, Foxp<sup>3</sup>WT/Hif1α<sup>fl/fl</sup> and Foxp<sup>3</sup>WT/Stk11<sup>fl/fl</sup>Hif1α<sup>fl/fl</sup> mice. d, Expression of CD62L and CD44 on CD4<sup>+</sup> T cells from the mice in c. e, Expression of IL-4 and IL-17 in CD4<sup>+</sup> T cells from the mice in c after in vitro stimulation for 4 h. f, Phosphorylation of AMPK in wild-type and LKB1-deficient T<sub>reg</sub> cells stimulated with anti-CD3/CD28 antibodies for 48 h. g, Expression of CD62L and CD44 on CD4<sup>+</sup> (upper) and CD8<sup>+</sup> T cells (lower) from wild-type and Foxp<sup>3</sup>WT/Prkaa1<sup>fl/fl</sup>/Prkaa2<sup>fl/fl</sup> mice. h, Expression of IFN-γ, IL-4 and IL-17 in CD4<sup>+</sup> (upper) and CD8<sup>+</sup> T cells (lower) from wild-type and Foxp<sup>3</sup>WT/Prkaa1<sup>fl/fl</sup>/Prkaa2<sup>fl/fl</sup> mice after in vitro stimulation for 4 h. Data are representative of two (a–f) or three (g, h) independent experiments. Numbers in quadrants or gates indicate percentage of cells.
### Extended Data Figure 6

| Name                                      | NES  |
|-------------------------------------------|------|
| Reactome_cholesterol_biosynthesis         | -2.50|
| Reactome_trna_aminoacylation              | -2.48|
| Reactome_mitochondrial_protein_import     | -2.44|
| Kegg_aminoacyl_trna_biosynthesis          | -2.43|
| Reactome_translation                      | -2.42|
| Reactome_tca_cycle_and_respiratory_electron_transport | -2.42|
| Reactome_metabolism_of_rna                | -2.41|
| Reactome_mrna_processing                   | -2.39|
| Reactome_respiratory_electron_transport_atp_synthesis | -2.39|
| GeneGo_translation_regulation_of_translation_initiation | -2.37|

**Additional Notes:**

- **Figure d:** Heatmap showing the expression levels of various genes in different conditions.
- **Figure b:** Enrichment plot illustrating the reaction TCA cycle and respiratory electron transport.
- **Figure c:** Enrichment plot for the reaction mitochondrial protein import.
- **Figure e:** Bar charts showing fold change in metabolites such as Citrate, Fumarate, Isocitrate, Malate, and Succinate.
- **Figure f:** Comparison of glucose, Hexose diphosphate, DHAP, PEP, Lactate, and Hexose monophosphate levels between Foxp3<sup>Cre<sup>-</sup></sup>Stk11<sup>11<sup>-</sup></sup> and Foxp3<sup>Cre<sup>-</sup></sup>Stk11<sup>11<sup>+/</sup></sup>.
- **Figure g:** Graph showing MitoTracker-APC, TMRM-PE, and ROS-PE signals.
- **Figure h:** Comparison of ATP (relative amount) between WT and Foxp3<sup>Cre<sup>-</sup></sup>Stk11<sup>11<sup>-</sup></sup>.
- **Figure i,j,k,l,m:** Various measurements and graphs showing OCR, ECAR, and lipid metabolism.

**Caption:** See next page for caption.
Extended Data Figure 6 | LKB1-dependent gene expression and metabolic programs in T<sub>reg</sub> cells. a, Gene set enrichment analysis (GSEA) of transcriptional profiles in wild-type and Foxp3<sup>Cre</sup>/Stk11<sup>fl/fl</sup> T<sub>reg</sub> cells from mixed bone marrow chimaeras (n = 5 each group). The list of top 10 gene sets downregulated in Foxp3<sup>Cre</sup>/Stk11<sup>fl/fl</sup> T<sub>reg</sub> cells were shown. NES, normalized enrichment score. b, c, The gene sets of TCA cycle and respiratory electron transport (b) and mitochondrial protein import (c) were enriched among the top 10 downregulated pathways in Foxp3<sup>Cre</sup>/Stk11<sup>fl/fl</sup> T<sub>reg</sub> cells. d, Heat maps showing relative abundance of metabolites differentially expressed and unsupervised hierarchical clustering. e, f, Relative abundance of metabolites implicated in TCA cycle or glycolytic pathways (f) in Foxp3<sup>Cre</sup>/Stk11<sup>fl/fl</sup> and Foxp3<sup>Cre</sup>/Stk11<sup>fl/fl</sup> T<sub>reg</sub> cells (Foxp3<sup>Cre</sup>/Stk11<sup>fl/+</sup>, n = 3; Foxp3<sup>Cre</sup>/Stk11<sup>fl/fl</sup>, n = 4). DHAP, gliceraldehyde 3P; PEP, phosphoenolpyruvic acid. g, Comparison of mitochondrial mass, TMRM (indicative of mitochondrial membrane potential) and ROS production in wild-type and LKB1-deficient T<sub>reg</sub> cells. h, Relative amount of intracellular ATP in wild-type and LKB1-deficient T<sub>reg</sub> cells (n = 4 each group). i, j, Oxygen consumption rate (OCR) (n = 8 each group, i) and extracellular acidification rate (ECAR) (n = 4 each group, j) of activated Foxp3–GFP<sup>+</sup>YFP<sup>+</sup> T<sub>reg</sub> cells from Foxp3<sup>Cre</sup>/ERT2/Stk11<sup>fl/fl</sup> and Foxp3<sup>Cre</sup>/ERT2/Stk11<sup>fl/fl</sup> mice. k, Comparison of lipid droplets in wild-type and LKB1-deficient T<sub>reg</sub> cells. l, Oxygen consumption rate of activated Foxp3–GFP<sup>+</sup>YFP<sup>+</sup> T<sub>reg</sub> cells responding to the treatment of inhibitors oligomycin (Oligo), FCCP, etomoxir (Eto) and Rotenone/antimycin A (Rot/AA). m, De novo lipid biosynthesis of Foxp3–GFP<sup>+</sup>YFP<sup>+</sup> T<sub>reg</sub> cells stimulated with anti-CD3/CD28 antibodies for 48 h (WT, n = 4; Foxp3<sup>Cre</sup>/Stk11<sup>fl/fl</sup>, n = 3). Data are representative of one (a–c; n = 5 mice each group; d–f; Foxp3<sup>Cre</sup>/Stk11<sup>fl/+</sup>, n = 3; Foxp3<sup>Cre</sup>/Stk11<sup>fl/fl</sup>, n = 4) or two (g–m) independent experiments. Data are mean ± s.e.m. P values are determined by two-tailed Student's t-test (e, f, h–j, m) or two-way ANOVA (l). NS, not significant; *P < 0.05, **P < 0.005. Numbers above graphs indicate the mean fluorescence intensity.
Extended Data Figure 7 | See next page for caption.
Extended Data Figure 7 | Deletion of IL-4 partially rescues dysregulated type 2 immune responses, but not T<sub>reg</sub> cell function or overall immune homeostasis. a, b, Expression of IL-4 and IL-13 (a) and IL-5 (b) in splenic CD4<sup>+</sup> T cells from wild-type, Foxp3<sup>Cre/Stk11<sup>fl/fl</sup></sup>, Il4<sup>−/−</sup> and Foxp3<sup>Cre/Il4<sup>−/−</sup></sup> mice after in vitro stimulation for 4 h. c, d, Flow cytometry of eosinophils (CD11b<sup>+</sup> Siglec-F<sup>+</sup>) (c) and CD11b<sup>+</sup>PD-L2<sup>+</sup>DCs (gated on CD11c<sup>+</sup>MHC-II<sup>+</sup>) (d) in the spleen from the mice in (a). e, Quantification of IgE and IgG1 in the serum from the mice in a (n = 3 each group). f, Flow cytometry of splenic T<sub>reg</sub> cells from the mice in a. g, Mean fluorescence intensity (MFI) of PD-1, GITR and OX40 expression on T<sub>reg</sub> cells from the mice in a (WT, n = 4; Foxp3<sup>Cre/Il4<sup>−/−</sup></sup>, n = 3; Il4<sup>−/−</sup>, n = 3; Foxp3<sup>Cre/Il4<sup>−/−</sup></sup>, n = 3). h, Expression of CD62L and CD44 on splenic CD4<sup>+</sup> T cells from the mice in a. i, Expression of IFN-γ and IL-4 in splenic CD4<sup>+</sup> T cells from the mice in a after in vitro stimulation for 4 h. j, Representative images of haematoxylin and eosin staining of the lung (original magnification, ×10), liver (×10) and skin (×20) from the mice in a. Data are representative of at least three (a–d, f, h, i) or two (e, g, j) independent experiments. Data are mean ± s.e.m. P values are determined by one-way ANOVA (e, g). NS, not significant; *P < 0.05, **P < 0.005. Numbers in quadrants or gates indicate percentage of cells.
Extended Data Figure 8 | Defects of LKB1-deficient T<sub>reg</sub> cells in suppressing DC maturation and Th2 cell differentiation. 

**a** Production of IL-25 and IL-33 in the homogenate of the lung from wild-type and Foxp3<sup>cre<sub>Stk11<sup>fl/fl</sup></sub></sup> mice, normalized by the weight of the lung (WT, <i>n</i> = 7; Foxp3<sup>cre<sub>Stk11<sup>fl/fl</sup></sub></sup>, <i>n</i> = 5). 

**b** Fold change of CD80 and CD86 expression on splenic DCs from C57BL/6 mice stimulated with TSLP for 18 h (<i>n</i> = 3 each group). 

**c** Mean fluorescence intensity (MFI) of CD80 and CD86 expression on DCs (with or without TSLP) cultured alone, or together with wild-type or Foxp3<sup>cre<sub>Stk11<sup>fl/fl</sup></sub></sup> T<sub>reg</sub> cells for 48 h (<i>n</i> = 4 each group). 

**d** Naive CD4<sup>+</sup> T cells were co-cultured with TSLP-DCs alone, or together with T<sub>reg</sub> cells from Foxp3<sup>cre-ERT2<sub>Stk11<sup>fl</sup></sub></sup> or Foxp3<sup>cre-ERT2<sub>Stk11<sup>fl</sup></sub></sup> mice (after tamoxifen treatment) for 5 days, and expression of Il4, Il5 and Il13 mRNA in co-cultured CD4<sup>+</sup> T cells was measured after restimulation with anti-CD3 for 5 h. 

**e**, f Naive CD4<sup>+</sup> T cells were co-cultured with LPS-primed DCs alone, or together with T<sub>reg</sub> cells from Foxp3<sup>cre-ERT2<sub>Stk11<sup>fl</sup></sub></sup> or Foxp3<sup>cre-ERT2<sub>Stk11<sup>fl</sup></sub></sup> mice (after tamoxifen treatment) for 5 days, followed by analyses of IFN-γ expression after in vitro stimulation for 4 h (e), and Ifng mRNA expression after restimulation with anti-CD3 for 5 h (f). Data are representative of two (a, d–f) or three (b, c) independent experiments. 

Data are mean ± s.e.m. <i>P</i> values are determined by two-tailed Student’s <i>t</i>-test (a, b) or one-way ANOVA (c). NS, not significant; *<i>P</i> < 0.05, **<i>P</i> < 0.005. Numbers in gates indicate percentage of cells.
Extended Data Figure 9 | Appropriate control of PD-1 expression is important for T_{reg} cells in suppressing T\_H2 immune responses.

a, b, Fold change of PD-1, GITR and OX40 expression on lung T_{reg} cells (a), or splenic T_{reg} cells (b) from OVA-sensitized C57BL/6 mice with or without OVA inhalation challenge (n = 3 each group). c, Expression of IL-4 in CD4^{+} T cells co-cultured with TSLP-DCs, or together with wild-type or PD-1-deficient T_{reg} cells. d, Expression of IL-4 in CD4^{+} T cells co-cultured with TSLP-DCs, or together with wild-type or PD-1-deficient T_{reg} cells. e, Flow cytometry of eosinophils (CD11b^{+} Siglec-F^{+}) in the lung and spleen from Rag1^{-/-} mice reconstituted with Scurfy: wild-type or Scurfy: Pdcd1^{-/-} bone marrow cells. f, Concentration of IgG1 and IgG2a/c in the serum from the mice in (e) (n = 4 each group). Data are representative of two (a–f) independent experiments. Data are mean ± s.e.m. P values are determined by two-tailed Student's t-test (a, b, f). NS, not significant; *P < 0.05, **P < 0.005. Numbers in gates indicate percentage of cells.
Extended Data Figure 10 | Treg cells require the LKB1–β-catenin signalling axis to enforce their functional fitness in maintaining immune homeostasis. a, GSEA reveals the significant enrichment of the Wnt signalling gene set among the downregulated pathways in LKB1-deficient Treg cells. b, Relative expression of Ctnnb1 mRNA in activated wild-type and LKB1-deficient Treg cells. c, Expression of total and phosphorylated β-catenin in activated wild-type and LKB1-deficient Treg cells. Right, relative phosphorylation of β-catenin normalized by total β-catenin. d, ChIP and real-time PCR analysis of β-catenin-bound DNA of the Pdcd1 locus from activated Foxp3<sup>CreERT2</sup>Stk11<sup>fl/fl</sup> and Foxp3<sup>CreERT2</sup>Stk11<sup>−/−</sup> Treg cells (following in vivo tamoxifen treatment) (n = 4 each group). e, Mean fluorescence intensity (MFI) of GITR and CD25 expression on wild-type and LKB1-deficient Treg cells transduced with control retrovirus (RV) or mutant β-catenin-expressing retrovirus (β-cat-RV) (n = 3 each group). f, MFI of PD-1, GITR and CD25 expression on Foxp3<sup>CreERT2</sup>Stk11<sup>−/−</sup> (n = 5) and Foxp3<sup>CreERT2</sup>Stk11<sup>fl/fl</sup> (n = 4) Treg cells (following in vivo tamoxifen treatment) transduced with control RV or β-cat-RV. g, Expression of PD-L2, CD80 and CD86 on DCs co-cultured with wild-type or LKB1-deficient Treg cells transduced with RV or β-cat-RV. Numbers above graphs indicate the MFI. Data are representative of one (a) or two (b–g) independent experiments. Data are mean ± s.e.m. P values are determined by two-tailed Student’s t-test (d) or two-way ANOVA (e, f). NS, not significant; **P < 0.005, ***P < 0.0005.

h, Schematics of LKB1 signalling in the regulation of Treg cell function and immune homeostasis. LKB1 signalling in Treg cells establishes metabolic and homeostatic fitness required for preventing undesired immune responses through selectively controlling the expression of inhibitory regulators, including PD-1, GITR and OX40. Consequently, uncontrolled expression of PD-1 and possible other receptors impairs the capability of Treg cells in suppressing T<sub>H2</sub> immune responses triggered by TSLP-induced PD-L2<sup>+</sup> DCs. Although not depicted here, IL-4 contributes to the induction of PD-L2 on DCs and the amplification of T<sub>H2</sub>-mediated immunopathology.
Experimental design

1. Sample size
Describe how sample size was determined. Sample size was selected to maximize the chance of uncovering mean difference which is also statistically significant.

2. Data exclusions
Describe any data exclusions. No data were excluded.

3. Replication
Describe whether the experimental findings were reliably reproduced. All the experimental findings were reliably reproduced.

4. Randomization
Describe how samples/organisms/participants were allocated into experimental groups. Randomization was not used.

5. Blinding
Describe whether the investigators were blinded to group allocation during data collection and/or analysis. Blinding was not used.

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.
7. Software

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

Flowjo 9.7.7 (Tree Star) for FACS results; GraphPad Prism 5 for statistics.

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.

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 materials were used.

9. Antibodies

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

Antibody information has been provided in the Methods of the manuscript: ‘Flow cytometry’ (page 18), ‘Cell purification and culture’ (page 19) and ‘RNA and immunoblot analysis’ (page 20) subsections.

10. Eukaryotic cell lines

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

No eukaryotic cell lines were used.

b. Describe the method of cell line authentication used.

No eukaryotic cell lines were used.

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

No eukaryotic cell lines were used.

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.

No commonly misidentified cell lines were used.

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.

  The spleens, peripheral lymph nodes (plns) and mesenteric lymph nodes (mlns) were gently grinded under nylon mesh using the flat end of a 3-mL syringes. Red blood cells were removed by ACK lysing buffer, followed by washing cells with isolation buffer. After spin down, the cell pellets were resuspended and filtered with nylon mesh before staining. For the examination of lymphocytes, the lung from the perfused mice were cut into small pieces, followed by digestion with RPMI 1640 medium containing 2% FBS and 1 mg/ml collagenase IV at 37 degree for 45 min. The red blood cells were removed by ACK lysing buffer, followed by washing cells and filtered with nylon mesh before staining. To isolate lymphocytes in the colon lamina propria, we first cleaned the colons and cut them into small pieces, followed by removing intraepithelial lymphocytes (IELs) with HBSS containing 5 mM EDTA, 1 mM DTT and 2% FBS. After washing the colons, we digested them with RPMI 1640 containing 0.1 mg/ml liberase at 37 degree for 1 h. The lamina propria lymphocytes were isolated by Percoll.

  6. Identify the instrument used for data collection.

  LSRII or LSR Fortessa (BD Biosciences)

  7. Describe the software used to collect and analyze the flow cytometry data.

  Flowjo 9.7.7 (Tree Star)

  8. Describe the abundance of the relevant cell populations within post-sort fractions.

  The purities of the sorted Treg cells, naive CD4 T cells and DCs were more than 99%.

  9. Describe the gating strategy used.

  Based on the pattern of FSC-A/SSC-A, cells in the lymphocyte gate were used for analysis of T cell subsets; cells in the granulocyte gate were used for analysis of myeloid cells. Singlets were gated according to the pattern of FSC-H vs. FSC-A. Positive populations were determined by the specific antibodies, which were distinct from negative populations.

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