mTORC1 couples immune signals and metabolic programming to establish T\textsubscript{reg}−cell function

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The mechanistic target of rapamycin (mTOR) pathway integrates diverse environmental inputs, including immune signals and metabolic cues, to direct T-cell fate decisions\textsuperscript{4}. The activation of mTOR, which is the catalytic subunit of the mTORC1 and mTORC2 complexes, delivers an obligatory signal for the proper activation and differentiation of effector CD4\textsuperscript{+} T cells\textsuperscript{2,3}, whereas in the regulatory T-cell (T\textsubscript{reg}) compartment, the Akt–mTOR axis is widely acknowledged as a crucial negative regulator of T\textsubscript{reg}−cell de novo differentiation\textsuperscript{4–8} and population expansion\textsuperscript{6}. However, whether mTOR signalling affects the homeostasis and function of T\textsubscript{reg} cells remains largely unexplored. Here we show that mTORC1 signalling is a pivotal positive determinant of T\textsubscript{reg}−cell function in mice. T\textsubscript{reg} cells have elevated steady-state mTORC1 activity compared to naive T cells. Signals through the T-cell antigen receptor (TCR) and interleukin-2 (IL-2) provide major inputs for mTORC1 activation, which in turn programs the suppressive function of T\textsubscript{reg} cells. Disruption of mTORC1 through T\textsubscript{reg}−specific deletion of the essential component raptor leads to a profound loss of T\textsubscript{reg}−cell suppressive activity \textit{in vivo} and the development of a fatal early onset inflammatory disorder. Mechanistically, raptor/mTORC1 signalling in T\textsubscript{reg} cells promotes cholesterol and lipid metabolism, with the mevalonate pathway particularly important for coordinating T\textsubscript{reg}−cell proliferation and upregulation of the suppressive molecules CTLA4 and ICOS to establish T\textsubscript{reg}−cell functional competency.

The evolutionarily conserved mTORC1 signalling pathway couples cell growth and proliferation to nutrient availability and metabolic cues\textsuperscript{9}. To investigate the function of mTORC1 in naturally occurring T\textsubscript{reg} cells, we compared mTORC1 activity between T\textsubscript{reg} cells and naive T cells at steady state. T\textsubscript{reg} cells had relatively increased phosphorylation of S6 and 4E-BP1, two major mTORC1 downstream targets (Fig. 1a and Supplementary Fig. 1a–c), whereas STAT5 phosphorylation was similar in both sets of cells (Supplementary Fig. 1d). This finding is consistent with a recent study describing elevated S6 phosphorylation in T\textsubscript{reg} cells compared with non-T\textsubscript{reg} cells\textsuperscript{11}. T\textsubscript{reg} cells also contained a higher abundance of CD71 (the transferrin receptor) and, to a lesser extent, CD98 (a subunit of the L-amino acid transporter), key nutrient receptors that depend on mTORC1 activity for expression\textsuperscript{12} (Fig. 1b).

![Figure 1](image-url)

Figure 1 | mTORC1 signalling is constitutively active in T\textsubscript{reg} cells and its resultation occurs in a fatal early onset inflammatory disorder.

\textbf{a}, Comparison of phosphorylation (p) of S6 and 4E-BP1 between naive T cells (CD4\textsuperscript{+}CD44\textsuperscript{lo}Foxp3\textsuperscript{+}) and T\textsubscript{reg} cells (CD4\textsuperscript{+}Foxp3\textsuperscript{+}) from Foxp3\textsuperscript{−/−}YFP\textsuperscript{−}Cre mice. \textbf{b}, Comparison of CD71 and CD98 expression between naive T cells and T\textsubscript{reg} cells. Mean fluorescent intensity (MFI) is presented above the plots. \textbf{c}, T\textsubscript{reg} cells from C57BL/6J mice were activated with anti-CD3 or anti-CD3 and IL-2 for 3 days, washed and then used for \textit{in vitro} suppression assays at multiple T\textsubscript{reg} versus T\textsubscript{eff} ratios; freshly isolated T\textsubscript{reg} cells were included for comparison (shown only for 1:4 and 1:8 ratios). Error bars represent standard deviation (s.d.) (\(n = 3\)). \textbf{d}, Comparison of phosphorylation of S6 and 4E-BP1 and expression of CTLA4 and ICOS between freshly isolated and pre-activated T\textsubscript{reg} cells. MFI is presented above the plots. \textbf{e}, Images of 42-day-old wild-type (WT) and Foxp3\textsuperscript{−/−}Raptor\textsuperscript{fl/fl} mice. Arrows indicate the scaly tail and ulceration of the body. \textbf{f}, Haematoxylin and eosin staining of colon (original magnification, 20×) and skin (×20). Images of 42-day-old wild-type and Foxp3\textsuperscript{−/−}Raptor\textsuperscript{fl/fl} mice. g, IFN-γ, IL-17 and IL-4 production in CD4\textsuperscript{+} cells from wild-type and Foxp3\textsuperscript{−/−}Raptor\textsuperscript{fl/fl} mice. P values are determined by Mann–Whitney test. **P < 0.01. Results represent five (a, g), three (b) and two (c–f) independent experiments.

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Figure 2 | Raptor deletion abrogates Treg-cell suppressive activity. a, Foxp3-YFP and green fluorescent protein (GFP) expression in CD4+ T cells from 25-day-old Foxp3–/–Rosa26GFP and Foxp3+/–Rosa26GFP mice. b, Representative colon histology in Rag1–/– mice given Teff cells alone or in combination with wild-type (WT) or Foxp3–/–Rosa26GFP Treg cells (original magnification, ×20). c, Percentages of Treg cells in mixed chimaeras reconstituted with bone marrow cells from CD45.1+ mice and wild-type or Foxp3–/–Rosa26GFP mice (n = 3), MLN, mesenteric lymph nodes; PLN, peripheral lymph nodes. d, Expression of Treg signature molecules and mitochondrial membrane potential (tetramethylrhodamine methyl ester (TMRM)) and mass (MitoTracker) in the reconstituted chimaeras. e, Expression of Treg signature molecules in 6-week-old wild-type and Cd44–/–Rosa26GFP mice. f, In vitro suppression assays mediated by Treg cells from wild-type and Cd44–/–Rosa26GFP mice. Error bars represent s.d. (n = 3). g, Representative colon histology in Rag1–/– mice given Treg cells alone or in combination with wild-type or Cd44–/–Rosa26GFP Treg cells (original magnification, ×20). P values are determined by Mann–Whitney test (c) or t-test (f). *P < 0.05; **P < 0.01; ***P < 0.001. Results represent three (a, d, f), two (b, c, g) and five (e) independent experiments.

Surprisingly, Foxp3–/–Rosa26GFP mice developed profound inflammatory diseases, indicated by a reduced body size and hunched posture, crusting of ears, eyelids and tail, and skin ulceration particularly on the head and upper back (Fig. 1e). Foxp3–/–Rosa26GFP mice showed extensive lymphadenopathy (Supplementary Fig. 3a) and infiltrations of lymphocytes and myeloid cells in colon mucosa, lung, liver sinusoids and skin (Fig. 1f), among other organs, before they died at a young age (Supplementary Fig. 3b). Foxp3–/–Rosa26GFP mice had an increased CD8+–cell percentage and altered CD4+/CD8+ ratio, highly elevated memory/effecter phenotype (CD44hiCD62Llo) T cells, and expansion of CD11b+ granulocytes and macrophages (Supplementary Fig. 3c–e). Furthermore, T cells from Foxp3–/–Rosa26GFP mice showed increased interferon (IFN)-γ, IL-4 and IL-17-producing CD4+ T cells and IFN-γ-producing CD8+ T cells (Fig. 1g). These phenotypes are reminiscent of those observed in mice carrying the scurfy mutation23, indicating a loss of Treg function.

Despite developing severe autoimmune diseases, Foxp3–/–Rosa26GFP mice had increased numbers of Foxp3+ Treg cells (Supplementary Fig. 4a). Lineage-tracing experiments indicated that the increase of Treg cells was mainly ascribed to a more abundant population of canonical Treg cells, as indicated by concomitant expression of yellow fluorescent protein (YFP)-tagged Foxp3 and specific Cre activity (Fig. 2a). Raptor-deficient Treg cells had normal Foxp3 expression and either increased or normal expression of many Treg signature molecules examined, whereas CD62L expression was reduced (Supplementary Fig. 4b). Thus, in settings of immune activation, raptor-deficient Treg cells acquired activated phenotypes.

Figure 3 | Raptor deletion abrogates Treg-cell suppressive function and causes colitis. a, Quantification of Foxp3+ Treg cells in splenic and LN of wild-type or Rag1–/– mice given Treg cells alone or in combination with wild-type or Foxp3–/–Rosa26GFP Treg cells (original magnification, ×20). b, The number of Teff and Treg cells was quantified in mixed chimaeras. c, Typical colon histology in Rag1–/– mice given Treg cells alone or in combination with wild-type or Foxp3–/–Rosa26GFP Treg cells (original magnification, ×20). P values are determined by Mann–Whitney test (c) or t-test (f). *P < 0.05; **P < 0.01; ***P < 0.001. Results represent three (d-f), two (a, b) and five (e) independent experiments.

Previous studies have demonstrated a requirement of mTORC1 for mitochondrial metabolism11, the dysregulation of which could affect the homeostasis of memory T cells14 and haematopoietic stem cells15. Treg cells had reduced mitochondrial membrane potential, whereas their mitochondrial mass and reactive oxygen species (ROS) production were largely comparable to naive cells (Supplementary Fig. 1e). Thus, Treg cells exhibit distinct regulation of mTORC1 activity and metabolism under steady-state conditions.

One of the hallmarks of Treg cells is that they are anergic in vitro on TCR stimulation alone16, but are highly proliferative in vivo17,18. Whereas reversal of anergy has been suggested to impair Treg function16, Treg cells stimulated by TCR together with IL-2, or under lymphopenic conditions, possess enhanced suppressive activity8,19. Indeed, prior stimulation of Treg cells with anti-CD3 upregulated their suppressive activity, and this was further enhanced by concomitant treatment with IL-2 (Fig. 1c). Such functional enhancements were associated with increased mTORC1 activity and expression of CTLA4 and ICOS, important effector molecules mediating Treg function20,21 (Fig. 1d). Compared with IL-2, CD28-mediated co-stimulation had more modest enhancing effects on these events (Supplementary Fig. 1f, g). Thus, TCR and IL-2 are the predominant signals that promote mTORC1 activity and the suppressive function of Treg cells.

Although these results indicate that mTORC1 is positively correlated with Treg suppressive activity, a number of recent studies have revealed a negative role of mTORC1 in Treg cells, including in the suppression of Treg differentiation2,4–8 and population expansion2. To investigate the physiological relevance of mTORC1 signalling in Treg cells, we deleted raptor, an obligatory component of the mTORC1 complex3, in Treg cells by crossing Raptor–/– mice with Foxp3–/–Rosa26GFP mice2 (designated Foxp3–/–Rosa26GFP mice). Raptor expression and S6 and 4E-BP1 phosphorylation were abolished in raptor-deficient Treg cells, but were normal in Foxp3–/– populations (Supplementary Fig. 2a–c). Additionally, a slight increase of phosphorylation of Akt Ser 473 was observed in mutant Treg cells, whereas phosphorylation of Akt Thr 380 and Erk in Treg cells was not affected (Supplementary Fig. 2d). Thus, deletion of raptor specifically abrogated mTORC1 activity.
We speculated that the increased number and heightened activation phenotypes of Treg cells in Foxp3<sup>CreRptor<sub>fl/fl</sub></sup> mice could be due to a compensatory response to the continuing inflammation<sup>24</sup>. To test this, we generated mixed bone marrow chimaeras by reconstituting sublethally irradiated Rag1<sup>−/−</sup> mice with bone marrow cells from CD45.1<sup>+</sup> mice mixed with those from either wild-type or Foxp3<sup>CreRptor<sub>fl/fl</sub></sup> mice. Raptor-deficient Treg cells were underrepresented in the chimaeras (Fig. 2c), with impaired expression of CTLA4 and ICOS but largely normal CD25 levels and mitochondrial parameters (Fig. 2d). These findings were recapitulated in 10-day-old Foxp3<sup>CreRptor<sub>fl/fl</sub></sup> mice, which exhibited minimal immune activation (Supplementary Fig. 4e). Thus, in a disease-free environment, loss of raptor in Treg cells renders them at a competitive disadvantage and compromises the expression of CTLA4 and ICOS. By contrast, the pronounced mitochondrial defects in Treg cells from Foxp3<sup>CreRptor<sub>fl/fl</sub></sup> mice (Supplementary Fig. 4b) were not intrinsically due to raptor deficiency, but were probably a compensatory response, as observed in haematopoietic stem cells lacking the metabolic sensor LKB1 (ref. 15).

To facilitate mechanistic studies of raptor-deficient Treg cells without the influence of continuing inflammation, we generated Cd4<sup>CreRptor<sub>fl/fl</sub></sup> (Cd4<sup>creRptor<sub>fl/fl</sub></sup>) mice, in which raptor was deleted in all αβ T cells. Cd4<sup>creRptor<sub>fl/fl</sub></sup> mice showed a small reduction of Treg numbers in peripheral lymph nodes but not the spleen (Supplementary Fig. 5a), and immunoblots confirmed the loss of S6 and 4E-BP1 phosphorlyation in these Treg cells (Supplementary Fig. 5b). Expression of CTLA4, ICOS and CD71 was impaired in Treg cells from Cd4<sup>creRptor<sub>fl/fl</sub></sup> mice, whereas the levels of Foxp3, CD25 and other markers were normal (Fig. 2e and Supplementary Fig. 5c). In vitro assays revealed a loss of the suppressive activity of Treg cells from Cd4<sup>creRptor<sub>fl/fl</sub></sup> mice (Fig. 2f). These Treg cells also exhibited severely impaired <i>in vivo</i> suppressive activity, because they failed to suppress colitis or IFN-γ production mediated by T<sub>eff</sub> cells (Fig. 2g and Supplementary Fig. 5d). Analysis of mixed chimaeras composed of CD45.1<sup>+</sup> and Cd4<sup>creRptor<sub>fl/fl</sub></sup> cells confirmed that raptor-deficient Treg cells were reduced as a percentage of total CD4<sup>+</sup> T cells with impaired CTLA4 and ICOS expression (Supplementary Fig. 5e). Thus, Treg cells deficient in mTORC1 have an intrinsic defect in suppressive activity. Nonetheless, Cd4<sup>creRptor<sub>fl/fl</sub></sup> mice exhibited normal health status, which was probably due to impaired activation of conventional T cells (K.Y. and H.C., unpublished observations). We found a similar requirement of raptor in Treg suppressive activity after Cre-ERT2-mediated acute deletion of raptor in adult mice (Supplementary Fig. 6).

Cytokines produced by Treg cells represent an important immunomodulatory effect of Treg cells. We thus examined the cytokine signature of Treg cells from Cd4<sup>creRptor<sub>fl/fl</sub></sup> mice (Supplementary Fig. 7a, b). Although Treg cells from Foxp3<sup>creRptor<sub>fl/fl</sub></sup> mice expressed increased IFN-γ, which may compromise Treg function<sup>25</sup>, Treg cells from Cd4<sup>creRptor<sub>fl/fl</sub></sup> did not (data not shown), thereby excluding such phenotypes as cell intrinsic. Moreover, ablation of IFN-γ did not ameliorate the immune-activation phenotypes of Foxp3<sup>creRptor<sub>fl/fl</sub></sup> mice (Supplementary Fig. 7c). Thus, the severe autoimmune phenotypes of Foxp3<sup>creRptor<sub>fl/fl</sub></sup> mice probably occur independently of cytokine dysregulation.

We therefore focused on the effects of raptor on Treg surface effector molecules, especially CTLA4 and ICOS, which are greatly reduced in Treg cells from multiple raptor-deficient genetic models in an intrinsic manner (Fig. 2d, e and Supplementary Figs 4e, 5e). Moreover, CTLA4 and, to a lesser extent, ICOS were further increased upon stimulation (Supplementary Fig. 8a). Although wild-type Treg cells were transferred into unmanipulated CD45.1<sup>+</sup> mice, which was associated with impaired proliferation (Fig. 3a and Supplementary Fig. 8a). Moreover, when wild-type Treg cells were transferred into unmanipulated CD45.1<sup>−/−</sup> mice, a proportion of them divided and upregulated CTLA4 (Fig. 3b). However, Treg cells from Cd4<sup>creRptor<sub>fl/fl</sub></sup> or Foxp3<sup>creRptor<sub>fl/fl</sub></sup> mice failed to proliferate or upregulate CTLA4 on transfer into CD45.1<sup>−/−</sup> mice (Fig. 3b and Supplementary Fig. 8b). Markedly diminished homeostatic proliferation and CTLA4 upregulation were also observed when raptor-deficient Treg cells were transferred into Rag1<sup>−/−</sup> mice (Supplementary Fig. 8c). Altogether, loss of raptor inhibits Treg-cell proliferation <i>in vitro</i> and <i>in vivo</i>. Furthermore,
Furthermore, on TCR stimulation, raptor-deficient Treg cells failed to and isopentenyl-diphosphate delta isomerase 1 (IDI1), which was veri-
ified by immunobots. Figure 4 | Deletion of Rictor does not alter Treg-cell function but partially rescues inflammation in Foxp3<sup>−/−</sup>Rptor<sup>fl/fl</sup> mice. a, T<sub>reg</sub> percentage in 2–3-month-old wild-type (WT) and Foxp3<sup>−/−</sup>Rptor<sup>fl/fl</sup> mice. NS, not significant. b, Expression of CD62L and CD44 on splenic T cells from wild-type and Foxp3<sup>−/−</sup>Rptor<sup>fl/fl</sup> mice. c, T<sub>reg</sub> cells were anti-CD3 and IL-2 for 4 h followed by immunobots. d, Expression of CD62L and CD44 on splenic CD4<sup>+</sup> T cells from 3–4-week-old wild-type, Foxp3<sup>−/−</sup>Rptor<sup>fl/fl</sup> and Foxp3<sup>−/−</sup>Rictor<sup>fl/fl</sup> mice. Right, percentage of CD62L<sup>hi</sup>CD44<sup>lo</sup> naive CD4<sup>+</sup> cells in the spleen. e, IFN-γ production in splenic CD4<sup>+</sup> T cells from the spleen of wild-type, Foxp3<sup>−/−</sup>Rptor<sup>fl/fl</sup> and Foxp3<sup>−/−</sup>Rictor<sup>fl/fl</sup> mice. f, Numbers of total TCR-β<sup>+</sup> cells in peripheral lymph nodes from wild-type, Foxp3<sup>−/−</sup>Rptor<sup>fl/fl</sup> and Foxp3<sup>−/−</sup>Rictor<sup>fl/fl</sup> mice. g, In vitro suppression assays mediated by T<sub>reg</sub> cells from 3–4-week-old wild-type, C4d<sup>−/−</sup>Rptor<sup>fl/fl</sup> and C4d<sup>−/−</sup>Rictor<sup>fl/fl</sup> mice. Error bars represent s.d. (n = 3). h, CTLA4 expression in T<sub>reg</sub> cells from the spleen of wild-type, C4d<sup>−/−</sup>Rptor<sup>fl/fl</sup> and C4d<sup>−/−</sup>Rictor<sup>fl/fl</sup> mice. P values are determined by Mann–Whitney test (a) and analysis of variance (d, f, g). *P < 0.05; **P < 0.01; ***P < 0.001. Results represent three (a–f) and two (g, h) independent experiments.

Figure 4 | Deletion of Rictor does not alter T<sub>reg</sub> cell function but partially rescues inflammation in Foxp3<sup>−/−</sup>Rptor<sup>fl/fl</sup> mice.

To test the functional significance of lipid metabolism, we activated T<sub>reg</sub> cells in the presence of 25-hydroxycholesterol, and this general lipid synthesis inhibitor potently blocked T<sub>reg</sub> suppressive activity (Supplementary Fig. 10a). Direct inhibition of HMGCR, the rate-limiting enzyme in the synthesis of cholesterol and isoprenoid lipids, by simvastatin also impaired T<sub>reg</sub> suppressive activity. Importantly, simvastatin-induced inhibition was completely reversed by the simultaneous addition of mevalonate, the metabolite downstream of HMGCR (Fig. 3g). Similar effects were observed after treatments with atorvastatin and lovastatin (Supplementary Fig. 10b). The inhibitory effects of these agents on T<sub>reg</sub>-cell function were associated with impaired T<sub>reg</sub>-cell proliferation and effector molecule upregulation (Supplementary Fig. 10c) in a mevalonate-dependent manner (Supplementary Fig. 10d). Furthermore, proliferation and CTLA4 and ICOS upregulation in T<sub>reg</sub> cells transferred into conegenic CD45.1<sup>+</sup> mice were diminished by statin treatment in vivo (Supplementary Fig. 11). Altogether, our results show that raptor/mTORC1 signalling promotes the lipogenic program, with the mevalonate pathway particularly important for mediating T<sub>reg</sub>-cell proliferation, CTLA4 and ICOS upregulation, and functional fitness.

Moreover, lipid-metabolism-dependent regulation of CTLA4 and ICOS is also operative in conventional T cells, because naive T cells treated with inhibitors of this pathway or deficient in raptor failed to upregulate these molecules effectively after TCR stimulation (Supplementary Fig. 12). We speculate that this constitutes a feedback mechanism, which would account for the immunosuppressive activity of activated non-T<sub>reg</sub> cells previously described<sup>18,25</sup>, a notion that awaits further investigation.

Crosstalk between the mTORC1 and mTORC2 signalling pathways has been reported<sup>19</sup>, although the molecular details and functional...
We propose that Treg cells adopt the evolutionarily ancient mTORC1 and Treg suppressive molecules. The predominant effect of mTORC1 is to coordinate metabolic programs in vitro and suppressive activity (Supplementary Fig. 13c, d). Thus, in contrast to a crucial requirement of mTORC1 in programming Treg activity, mTORC2 probably contributes to Treg maintenance but is dispensable for Treg function.

To examine the crosstalk between the two mTOR complexes, we analysed mTORC2 activity in raptor-deficient Treg cells. Phosphorylation of well-established mTORC2 targets, Akt Ser473 and Foxo1/3, was elevated in raptor-deficient Treg cells after anti-CD3/IL-2 (Fig. 4c) or anti-CD3/CD28 stimulation (Supplementary Fig. 14), consistent with the observation in freshly isolated raptor-deficient Treg cells (Supplementary Fig. 2d). By contrast, Akt Ser473 phosphorylation was nearly abolished after Rictor deletion (Fig. 4c and Supplementary Fig. 14a). Thus, mTORC1 negatively feeds back on mTORC2 in Treg cells. To determine the contribution of mTORC2 activity to Foxp3 expression, we generated Foxp3creRptorfl/fl mice. Compared with T cells in Foxp3creRptorfl/fl mice, those in Foxp3creRptorfl/fl Rictorfl/fl mice contained an increased percentage of naive phenotypes (Fig. 4d) and showed modestly reduced IFN-γ production (Fig. 4e). Also, the lymphadenopathy phenotype observed in Foxp3creRptorfl/fl mice was ameliorated in Foxp3creRptorfl/fl Rictorfl/fl mice (Fig. 4f). To test the intrinsic suppressive activity of raptor- and Rictor-deficient Treg cells, we generated Cdx2creRptorflofl Rictorflofl mice that eliminated both mTORC1 and mTORC2 activities (Supplementary Fig. 15a, b). An in vitro suppression assay revealed an intermediate phenotype of raptor/Rictor-deficient Treg cells compared with raptor-deficient and wild-type cells (Fig. 4g). However, impaired CTLA4 expression and TCR-induced proliferation after raptor deletion were not restored by the concomitant loss of Rictor (Fig. 4h and Supplementary Fig. 15c). Furthermore, Foxp3creRptorflofl Rictorflofl mice still succumbed to an inflammatory disorder, albeit with a small extension of lifespan and less inflammation as compared with Foxp3creRptorfl/fl mice (Supplementary Fig. 16). Therefore, Raptor/mTORC1 signalling promotes Treg function in part through inhibiting mTORC2, although the predominant effect of mTORC1 is to coordinate metabolic programs and Treg suppressive molecules.

Much emphasis has been placed on the transcriptional mechanisms that orchestrate Treg suppressive activity, but how immunological signals are sensed and integrated by Treg cells for their functional activation remains obscure. We show that mTORC1-dependent metabolic programming is a central mechanism to couple immune signals, including TCR and IL-2, and Treg suppressive function. Raptor/mTORC1 promotes the lipogenic program, with the mevalonate pathway particularly important for coordinating Treg proliferation and the optimal induction of effector molecules CTLA4 and ICOS (Supplementary Fig. 17). Thus, mTORC1-dependent lipid metabolism provides a novel link between two crucial Treg-cell regulators, IL-2 and CTLA4, the loss of which causes fulminating autoimmune diseases. An additional mechanism is through the mTORC1-dependent inhibition of mTORC2, although the modest rescue effect of Rictor deletion in Foxp3creRptorflofl mice suggests a relatively minor contribution of this feedback pathway. Notably, mTORC1-dependent metabolic programming operates under both steady state and immune stimulation, and this provides important mechanistic insights into the apparently conflicting observations of the in vitro energy of Treg cells and their in vivo antigen–primed state. We propose that Treg cells adopt the evolutionarily ancient mTORC1 signalling pathway to link immunological inputs to metabolic activity and functional fitness, therefore implicating mTORC1 as a fundamental rheostat to program Treg-cell suppressive activity through a non-conventional mechanism.

METHODS SUMMARY

The mice used in this study were backcrossed onto the C57BL/6j background. Bone marrow chimaeras were generated by transferring 1 × 107 T-cell-depleted bone marrow cells into sub-lethally irradiated (5 Gy) Rag1−/− mice, followed by reconstitution for at least 2 months. T-cell-mediated colitis was induced by intraperitoneal transfer of 4 × 105 Tc eff cells (CD4+ CD45RB+CD25−) from CD45.1+ mice in the absence or presence of 2 × 105 Treg cells into Rag1−/− mice. Lymphocytes were stained with surface antibodies or intracellularly for Foxp3, CTLA4 and caspase 3. Staining for the phosphorylated signalling proteins was carried out with Phosflow (Becton–Dickinson). Treg cells were activated with anti-CD3, anti-CD28 and IL-2, in the presence of simvastatin (2 μM; EMD Millipore), 25-hydroxycholesterol (0.5 μg μl−1), Sigma, or vehicle. The de novo lipogenesis assay was performed by incubating activated Treg cells with [1-14C]glucose or [1-14C]acetate for 4 h, followed by cell lysis and lipid extraction.

Full Methods and any associated references are available in the online version of the paper.

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1. Chi, H. Regulation and function of mTOR signalling in T cell fate decisions. Nature Rev. Immunol. 12, 325–338 (2012).
2. Delgoffe, G. M. et al. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. Nature Immunol. 12, 295–303 (2011).
3. Lee, K. et al. Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. Immunity 32, 743–753 (2010).
4. Delgoffe, G. M. et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity 30, 832–844 (2009).
5. Hashimoto, S., Mathis, D. & Benoist, C. The AKT–mTOR axis regulates de novo differentiation of CD4 ‘Foxp3+’ cells. J. Exp. Med. 205, 565–574 (2008).
6. Sauer, S. et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. Proc. Natl Acad. Sci. USA 105, 7779–7802 (2008).
7. Liu, G. et al. The receptor S1P1 overrides regulatory T cell-mediated immune suppression through Akt–mTOR. Nature Immunol. 10, 769–777 (2009).
8. Liu, G., Yang, K., Burns, S., Shrestha, S. & Chi, H. The S1P1 receptor axis directs the reciprocal differentiation of Th1 and Treg cells. Nature Immunol. 11, 1047–1056 (2010).
9. Battaglia, M., Stabili, A. & Roncarolo, M. G. Rapamycin selectively expands regulatory T cell lineage commitment. J. Exp. Med. 210, 7797–7802 (2009).
10. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. Cell 140, 274–293 (2012).
11. Procaccini, C. et al. An oscillatory switch in mTOR kinase activity sets regulatory T cell responsiveness. Immunity 33, 929–941 (2010).
12. Kelly, A. P. et al. Notch-induced T cell development requires phosphoinositide-dependent kinase 1. EMBO J. 26, 3441–3450 (2007).
13. Cunningham, J. T. et al. mTOR controls mitochondrial oxidative function through a YY1–PGC-1α transcriptional complex. Nature 450, 736–740 (2007).
14. van der Windt, G. J. et al. Mitochondrial respiratory capacity is a critical regulator of CD8+ T cell memory development. Immunity 36, 68–78 (2012).
15. Gurumurthy, S. et al. The Lkb1 metabolic sensor maintains haematopoietic stem cell survival. Nature 468, 659–663 (2010).
16. Takahashi, T. et al. Immunolocal self-tolerance maintained by CD25+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int. Immunol. 14, 68–78 (2002).
17. Fisson, S. et al. Continuous activation of autoreactive CD4+CD25+ regulatory T cells in the steady state. J. Exp. Med. 198, 737–746 (2003).
18. Gavin, M. A. et al. The Lkb1 metabolic sensor maintains haematopoietic stem cell survival. Nature 468, 659–663 (2010).
19. Thornton, A. M., Donovan, E. E., Piccirillo, C. A. & Shevach, E. M. Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. J. Immunol. 172, 6519–6523 (2004).
20. Herman, A. E., Freeman, G. J., Mathis, D. & Benoist, C. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. J. Exp. Med. 199, 1479–1489 (2004).
21. Wing, K. et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science 322, 271–275 (2008).
22. Rubtsov, Y. P. et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 28, 546–558 (2008).
23. Kanagat, S. et al. Disease in the scurfy (sf) mouse is associated with overexpression of cytokine genes. Eur. J. Immunol. 26, 161–165 (1996).
24. Chaudhry, A. et al. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity 34, 566–578 (2011).
Author Contributions H.Z. designed and performed experiments, and wrote the manuscript; K.Y. contributed to cellular experiments; C.C. contributed to survival curves and technical support; G.N. performed bioinformatic analyses; P.V. performed histological analysis; H.C. designed experiments, contributed to writing the manuscript, and provided overall direction.

Author Information The microarray data have been deposited in the Gene Expression Omnibus under accession GSE46693. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to H.C. (hongbo.chi@stjude.org).
**METHODS**

Mice. CS7BL/6, CD45.1, Riptorfl/fl, Rag1l−/−, Ihh−/− and ROSA26GFP (a lox-site-flanked STOP cassette followed by the GFP-encoding sequence was inserted into the ROSA26 locus) mice were purchased from the Jackson Laboratory. Rictorfl/fl, Cd4Cre and ROSA26Cre-ER T2 mice have been described previously. Foxp3Ftp-Cre mice were a gift from A. Rudensky. Foxp3Riptorfl/fl mice were used at 3–4 weeks old unless otherwise noted, with the age and gender-matched wild-type mice containing the Foxp3fl allele as controls. Other mice were used at 8–10 weeks old unless otherwise noted. Bone marrow chimerae were generated by transferring 1 × 106 T-cell-depleted bone marrow cells into sub-lethally irradiated (5 Gy) Rag1l−/− mice, followed by reconstitution for at least 2 months. For treatment with tamoxifen, mice were injected intraperitoneally with tamoxifen (1 mg per mouse) in corn oil daily for 4 consecutive days and then analysed 7 days after the last injection. All mice were kept in a specific pathogen-free facility in the Animal Resource Center at St Jude Children’s Research Hospital. Animal protocols were approved by the Institutional Animal Care and Use Committee of St Jude Children’s Research Hospital. Animal protocols were approved by the Institutional

**Flow cytometry.** For analysis of surface markers, cells were stained in PBS containing 2% (wt/vol) fetal bovine serum (FBS) with antibodies from eBioscience, unless otherwise noted. Foxp3 staining was performed as per the manufacturer’s instruction (E bioscience). Intracellular staining of CTLA4 was performed together with Foxp3 using anti-CTLA4 antibody (UC10-4F10-11; BD Biosciences). Bcl2 and caspase-3 staining was performed as per the manufacturer’s instruction (BD Biosciences). For detection of phosphorylated signalling proteins, lymphocytes were rested in complete medium for 1 h. They were fixed with Phosflow Lyse/Fix buffer, followed by permeabilization with Phosflow Perm buffer III (BD Biosciences) and staining with antibodies to S6 phosphorylated at Ser235 and Ser236 (D57.2.2E; Cell Signaling Technology), E4-BP1 phosphorylated at Thr37 and Thr46 (236B4; Cell Signaling Technology), Akt phosphorylated at Ser473 (M689-61; BD Biosciences) and Thr308 (J1-223.371; BD Biosciences), and Erk phosphorylated at Thr202 and Tyr204 (2A; BD Biosciences). For detection of phosphorylated ERK1/2, lymphocytes were incubated for 30 min at 37°C with 10 nM MitoTracker Deep Red (Life Technologies) or 20 nM MTRMR (ImmunoChemistry Technologies) after staining surface markers. ROS were measured by incubation with 10 μM 5-((and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA; Life Technologies) or 2.5 μM CellROX Deep Red (Life Technologies) after staining surface markers. Flow cytometry data were acquired on LSRII or LSR Fortessa (BD Biosciences) and analysed using FlowJo software (Tree Star).

**Cell purification and culture.** Lymphocytes were isolated from lymphoid organs (spleen and peripheral lymph nodes that included inguinal, auxiliary and cervical lymph nodes) and naïve and Treg cells were sorted on a MoFlow (Beckman-Coulter) or Reflection (i-Cyt). Sorted naïve (CD4CD62L−CD44CD25+) and Treg cells were used for in vitro culture in Click’s medium (plus γ-mercaptoethanol) supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin. Treg cells were activated with plate-coated anti-CD3 (5 μg ml−1; 2C11; Bio X Cell), anti-CD28 (5 μg ml−1; 37.51; Bio X Cell) and human IL-2 (200 U ml−1), in the presence or absence of simvastatin (2μM; EMD Millipore), or 25-hydroxycholesterol (0.5 μM; Sigma). Thymidine incorporation assays were performed by adding 0.2 × 106 T cells and 1 × 105 irradiated splenocytes together with soluble anti-CD3 (0.2 μg ml−1) in the presence or absence of human IL-2 (100 U ml−1) for 72 h, followed by pulsing with [3H]-thymidine at 1 μCi per well for the last 12–16 h of culture. For labelling with CFSE and Celltrace violet (both from Life Technologies), 1 × 105 irradiated splenocytes together with soluble anti-CD3 (1 μg ml−1), anti-CD28 (5 μg ml−1) and Treg cells (at different ratios with Teff) were cultured in 96-well plates overnight at 37°C. Treg cells were sorted from C57BL/6 mice and activated with anti-CD3, anti-CD28 and IL-2 for 3 days in the absence or presence of simvastatin (2 μM), 25-hydroxycholesterol (0.5 μM ml−1), 22(R)-hydroxycholesterol (5 μM; Sigma), atorvastatin (5 μM; EMD Millipore), lovastatin (5 μM; EMD Millipore), mevalonate (100 μM; Sigma), or certain combinations as specified; vehicle control did not have measurable effects (not shown). Viable Treg cells were purified using Ficoll and incubated with freshly isolated Tefl cells for additional analysis as described earlier.

**Colitis model.** A total of 4 × 105 T eff cells (CD4CD45RB−CD25−) from CD4S1.1 mice were mixed with 2 × 105 wild-type or raptor-deficient Treg cells (from Foxp3Riptorfl/fl or C4dRiptorfl/fl mice), and were transfected intraperitoneally into Rag1l−/− mice. Mice were assessed for clinical signs of colitis weekly and were analysed 8–10 weeks after transfer. Colonos were fixed in 10% (vol/vol) neutral buffered formalin, and sectioned at 4 μm thickness. Colon pathology was assigned scores by an experienced pathologist (P.V.) as described. Lymphocytes were isolated from spleen and mesenteric lymph nodes and analysed by flow cytometry.

**De novo lipogenesis, cholesterol measurement and bioenergetics assays.** Treg cells were activated with anti-CD3, anti-CD28 plus IL-2 for 20 h, and [1-14C]-acetate acid (Perkin Elmer; 4 μCi ml−1) was added for an additional 4 h of culture. For labelling with [U-14C]-glucose (American Radiolabeled Chemicals), Treg cells activated for 20 h were washed with PBS and incubated in glucose-free medium with dialysed FBS and [U-14C]-glucose (4 μCi ml−1) for an additional 4 h of culture. After incubation, cells were collected, washed twice with PBS and lysed in 0.5% Triton X-100. Lipids were extracted by the addition of chloroform and methanol mixture (2:1 vol/vol) with vortexing, followed by the addition of water with vortexing. After centrifugation, the lipid-containing phase (at the bottom) was separated and 14C incorporation was measured using a Beckman LS6500 scintillation counter. Results are normalized to cell number. Cellular cholesterol level was measured using Amplex Red assay (Life Technologies). Briefly, cells were washed with PBS and lysed in the Amplex Red reaction buffer. After 15 min of incubation, cell lysates were centrifuged at maximum speed for 5 min. Fifty micro-litres of supernatant was pipetted into a 96-well tissue culture plate and a 50 μl aliquot of Amplex Red working solution was added to each well. The plate was incubated for 120 min at 37°C, protected from light. Fluorescence was subsequently measured on a fluorescence microplate reader ( Molecular Devices). A cholesterol standard curve was determined for each plate using a cholesterol reference standard. The bioenergetic activities of the ECAR and OCR pathways were measured using the Seahorse XF24-3 extracellular flux analyser as per the manufacturer’s instructions (Seahorse Biosciences).

**Gene expression profiling and gene-set enrichment analysis.** RNA samples from freshly isolated Treg cells from wild-type and C4dRiptorfl/fl mice were analysed using the Affymetrix HT_MG-430_PM GeneTitan peg array, and expression signals were summarized using the RMA algorithm (Affymetrix Expression Console v1.1). Gene-set enrichment analysis within canonical pathways was performed as described.

**RNA and immunoblot analysis.** Real-time PCR analysis was done as described with primers and probe sets from Applied Biosystems, or using the Power SYBR Green Master Mix from Life Technologies. Immunoblots were performed and quantified as described previously, using the following antibodies: p-S6, p-Akt Ser473, p-4E-BP1, p-foxo1, raptor (all from Cell Signaling Technology) and β-actin (Sigma).

**Statistical analysis.** P values were calculated with Student’s t test, Mann–Whitney test, or analysis of variance (GraphPad Prism) as specified in figure legends, with proper post-test analysis performed. Statistical analysis of mouse survival and respective P values were determined using the logrank test. *P < 0.05; **P < 0.01; ***P < 0.001.

31. Yang, K., Neale, G., Green, D. R., He, W. & Chi, H. The tumour suppressor Tsc1 enforces quiescence of naïve T cells to promote immune homeostasis and function. Nature Immunol. 12, 888–897 (2011).

32. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl Acad. Sci USA 102, 15545–15550 (2005).