

# The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2

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The kinase mTOR has emerged as an important regulator of the differentiation of helper T cells. Here we demonstrate that differentiation into the T<sub>H</sub>1 and T<sub>H</sub>17 subsets of helper T cells was selectively regulated by signaling from mTOR complex 1 (mTORC1) that was dependent on the small GTPase Rheb. Rheb-deficient T cells failed to generate T<sub>H</sub>1 and T<sub>H</sub>17 responses *in vitro* and *in vivo* and did not induce classical experimental autoimmune encephalomyelitis (EAE). However, they retained their ability to become T<sub>H</sub>2 cells. Alternatively, when mTORC2 signaling was deleted from T cells, they failed to generate T<sub>H</sub>2 cells *in vitro* and *in vivo* but preserved their ability to become T<sub>H</sub>1 and T<sub>H</sub>17 cells. Our data identify mechanisms by which two distinct signaling pathways downstream of mTOR regulate helper cell fate in different ways. These findings define a previously unknown paradigm that links T cell differentiation with selective metabolic signaling pathways.

The kinase mTOR is an evolutionarily conserved member of the phosphatidylinositol-3-OH kinase (PI(3)K)-related kinase family that has a central role in the regulation of metabolism, protein synthesis, energy balance, proliferation and survival<sup>1</sup>. This kinase senses environmental cues such as amino acids, insulin and growth factors and then integrates such signals to regulate cellular metabolism<sup>2</sup>. It forms the core of two distinct signaling complexes whose activation is regulated differently<sup>3,4</sup>. The complex mTORC1 contains the scaffolding protein Raptor, as well as the subunits mLST8, PRAS40 and Deptor<sup>5</sup>. Activation of mTORC1 is achieved through signaling by the kinases PI(3)K, PDK1 and Akt. This complex promotes phosphorylation of the translational regulators S6K1 and 4E-BP1 and is believed to have a central role in regulating cellular growth and proliferation by modulating metabolism<sup>6</sup>. The second mTOR signaling complex, mTORC2, consists of mLST8, the scaffolding protein Rictor and the subunits mSIN1 and Protor<sup>5</sup>. Rictor is a critical adaptor protein for mTORC2, and Rictor-deficient mice and mouse embryonic fibroblasts have been shown to lack mTORC2 activity<sup>4</sup>. The activity of mTORC2 can be measured by phosphorylation of the hydrophobic motif of Akt at Ser473 and other kinases of the AGC (protein kinases A, G and C) family (SGK1 and PKC- $\alpha$ )<sup>7</sup>. The exact upstream activators of mTORC2 signaling have yet to be fully elucidated. Despite ongoing studies aimed at delineating both upstream activators and downstream effectors of the two respective signaling pathways, the distinct physiological consequences mediated by mTORC1 and mTORC2 have yet to be defined in biological systems.

A central role for mTOR in sensing the immune microenvironment and dictating immune function and differentiation has begun to emerge<sup>8</sup>. CD4<sup>+</sup> T cells that lack mTOR fail to differentiate into effector cells under appropriate skewing conditions<sup>9</sup>. Instead, after activation, mTOR-deficient T cells become Foxp3<sup>+</sup> regulatory cells. This inability to differentiate into effector cells in the absence of mTOR is associated with less activation of the transcription factors STAT4, STAT6 and STAT3 in response to the skewing cytokines interleukin 12 (IL-12), IL-4 and IL-6, respectively. Moreover, mTOR controls the development of CD8<sup>+</sup> memory T cells in part by regulating expression of the transcription factors T-bet and eomesodermin<sup>10,11</sup>. In addition, the PI(3)K-mTOR axis regulates lymphocyte trafficking<sup>12</sup>.

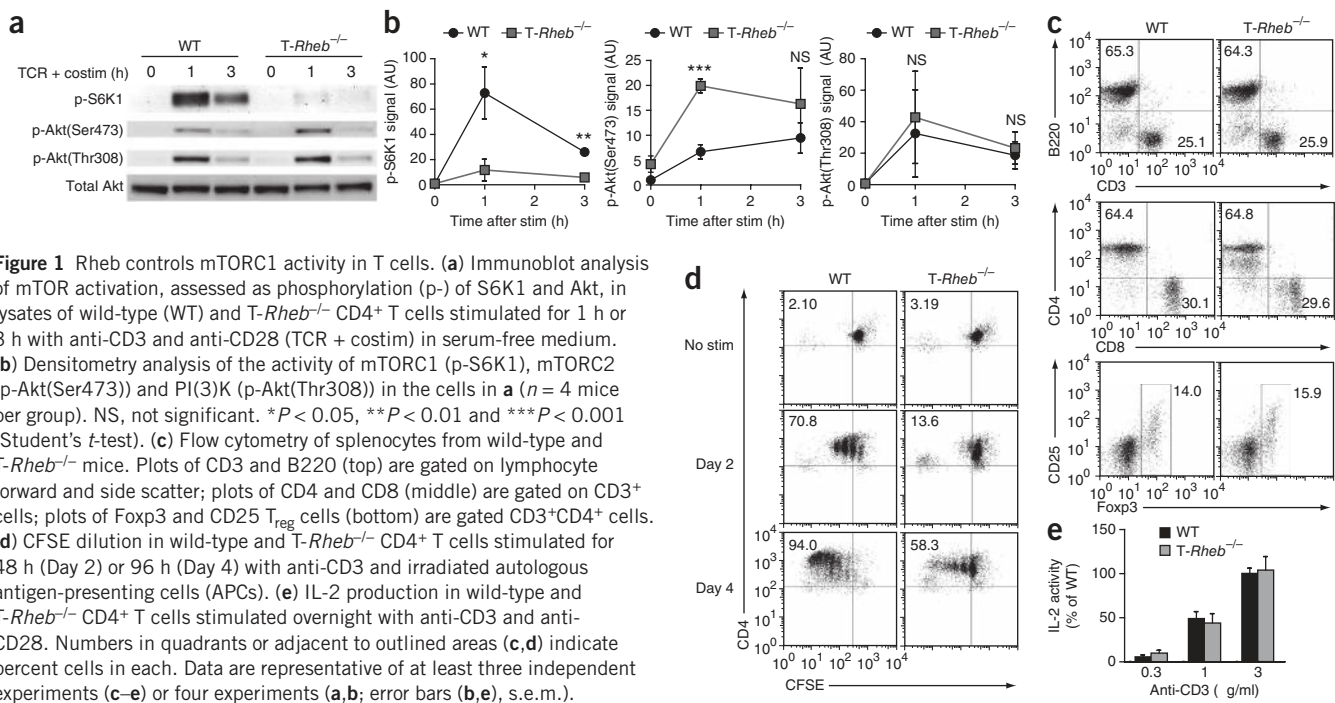
The small GTPase Rheb is isolated from the central nervous system and was originally thought to be a mediator of the GTPase Ras-mitogen-activated protein kinase pathway<sup>13,14</sup>. The mammalian Rheb family consists of two proteins, Rheb1 and Rheb2; both are expressed in T cells, but their function has yet to be elucidated<sup>13,15</sup>. Rheb is a crucial regulator of mTORC1 signaling<sup>16</sup>. The tuberous sclerosis complex (TSC), composed of TSC1 and TSC2, functions as a GTPase-activating protein for Rheb. When TSC is inhibited, the active, GTP-bound form of Rheb interacts with mTORC1 to stimulate its activity<sup>15</sup>.

Despite emerging appreciation for the central role of mTOR in regulating the differentiation of effector T cells, memory T cells and regulatory T cells (T<sub>reg</sub> cells), the downstream signaling pathways involved in regulating these processes have yet to be elucidated. By selectively

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knocking out Rheb in T cells, we demonstrate here that the differentiation of both T helper type 1 (T<sub>H</sub>1) and IL-17-producing helper T (T<sub>H</sub>17) effector cells required mTORC1 signaling, whereas T<sub>H</sub>2 differentiation was preserved. Mice with T cell-specific deletion of Rheb did not mount T<sub>H</sub>1 or T<sub>H</sub>17 responses *in vivo* and were resistant to the development of classical experimental autoimmune encephalomyelitis (EAE). As T cells lacking mTORC1 activity were still able to become T<sub>H</sub>2 cells, we further investigated the role of mTORC2 signaling in T<sub>H</sub>2 development. After deleting mTORC2 signaling in T cells, we found that they were unable to become T<sub>H</sub>2 cells but maintained their ability to differentiate into T<sub>H</sub>1 cells and T<sub>H</sub>17 cells. Our findings

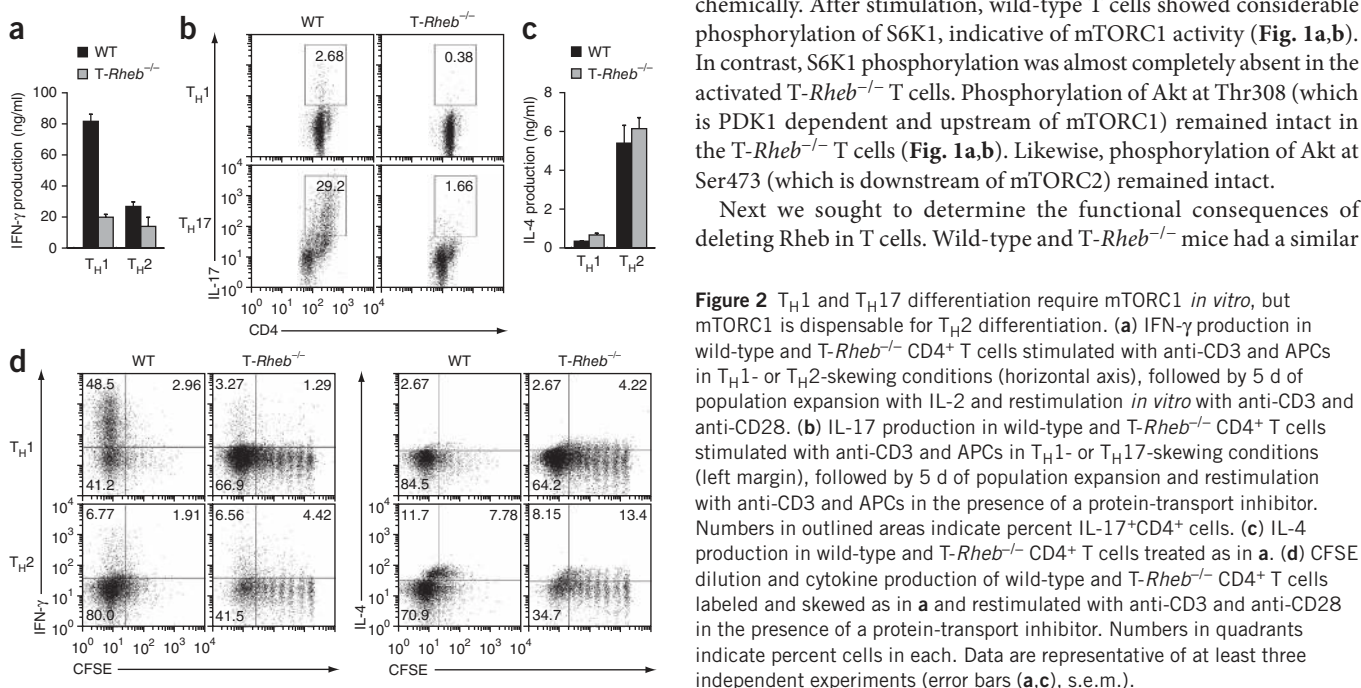
define distinct downstream signaling pathways as the mechanisms by which mTOR regulates the differentiation of helper T cells.

## RESULTS

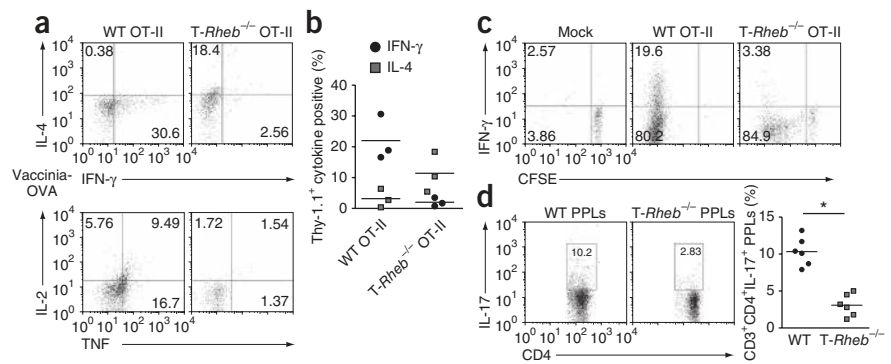
### Rheb regulates mTORC1 activity in T cells

We hypothesized that genetic deletion of Rheb in T cells would inhibit mTORC1 activation. We bred mice with *loxP*-flanked *Rheb* alleles with mice expressing Cre recombinase from the *Cd4* promoter and enhancer regions, which led to deletion of Rheb in T cells in the '*T-Rheb*<sup>-/-</sup>' progeny (**Supplementary Fig. 1**). We stimulated CD4<sup>+</sup> T cells from wild-type and *T-Rheb*<sup>-/-</sup> mice with antibody to CD3 (anti-CD3) and anti-CD28 and assessed the activity of mTORC1 and mTORC2 biochemically. After stimulation, wild-type T cells showed considerable phosphorylation of S6K1, indicative of mTORC1 activity (**Fig. 1a,b**). In contrast, S6K1 phosphorylation was almost completely absent in the activated *T-Rheb*<sup>-/-</sup> T cells. Phosphorylation of Akt at Thr308 (which is PDK1 dependent and upstream of mTORC1) remained intact in the *T-Rheb*<sup>-/-</sup> T cells (**Fig. 1a,b**). Likewise, phosphorylation of Akt at Ser473 (which is downstream of mTORC2) remained intact.

Next we sought to determine the functional consequences of deleting Rheb in T cells. Wild-type and *T-Rheb*<sup>-/-</sup> mice had a similar



**Figure 3** T cells deficient in mTORC1 cannot skew toward  $T_H1$  or  $T_H17$  *in vivo*. (a) Cytokine production in C57BL/6 (Thy-1.2<sup>+</sup>) mice immunized with vaccinia virus expressing ovalbumin and given adoptive transfer of  $1 \times 10^6$  wild-type or *T-Rheb*<sup>-/-</sup> CD4<sup>+</sup> OT-II (Thy-1.1<sup>+</sup>) T cells; splenocytes obtained 4 d later were rechallenged overnight *in vitro* with OVA peptide in the presence of a protein-transport inhibitor. Plots are gated on Thy-1.1<sup>+</sup> cells. TNF, tumor necrosis factor. (b) Cytokine production by cells from mice treated as in a. Each symbol represents an individual mouse ( $n = 3$ ); small horizontal lines indicate the mean.  $P < 0.01$  for IFN- $\gamma$  or 0.05 for IL-4 (Student's *t*-test). (c) Cytokine production in C57BL/6 (Thy-1.2<sup>+</sup>) mice given mock immunization or immunized as in a and given adoptive transfer as in a, except with CFSE-labeled cells. (d) Cytokine production from Peyer's patch lymphocytes (PPLs) isolated from wild-type and *T-Rheb*<sup>-/-</sup> mice ( $n = 6$ ) and stimulated with PMA and ionomycin. \* $P < 0.01$  (Student's *t*-test). Numbers in quadrants (a,c) or outlined areas (d) indicate percent cells in each. Data are representative of at least three independent experiments.



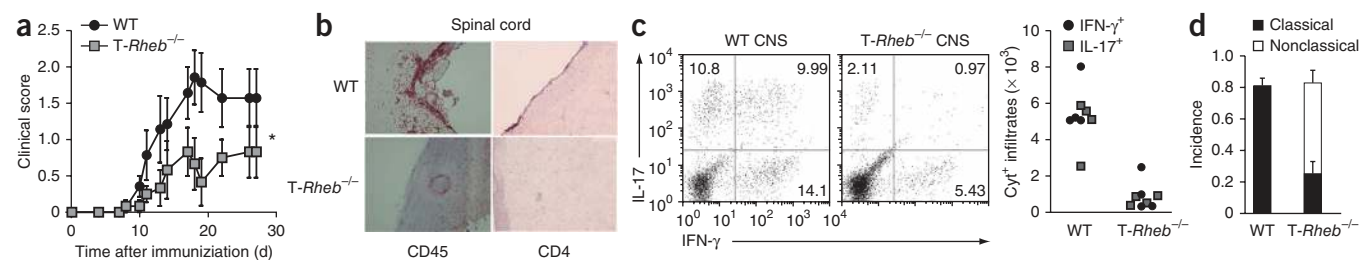
frequency of CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells and B cells (Fig. 1c and Supplementary Fig. 2). In addition, despite the lack of mTORC1 activity, we did not see any difference between wild-type and *T-Rheb*<sup>-/-</sup> mice in the development of Foxp3<sup>+</sup> T cells. Similar to T cells lacking mTOR<sup>9</sup>, T cells from the *T-Rheb*<sup>-/-</sup> mice did not demonstrate a block in proliferation but instead proliferated more slowly than their wild-type counterparts (Fig. 1d). Likewise, IL-2 production was similar in wild-type and *T-Rheb*<sup>-/-</sup> T cells (Fig. 1e), which indicated that T cell antigen receptor (TCR)-induced activation and subsequent expression of IL-2 was not affected by the absence of Rheb. In some systems, mTOR signaling can affect apoptosis. After *in vitro* activation, there was no evidence of greater apoptosis in T cells from *T-Rheb*<sup>-/-</sup> mice, as assessed by staining with annexin V and 7-amino-actinomycin D (Supplementary Fig. 3). Thus, deleting Rheb in T cells specifically eliminates mTORC1 activity while preserving mTORC2 activity. Furthermore, elimination of Rheb in T cells still permits TCR-induced cytokine expression and proliferation.

### $T_H1$ and $T_H17$ differentiation require mTORC1

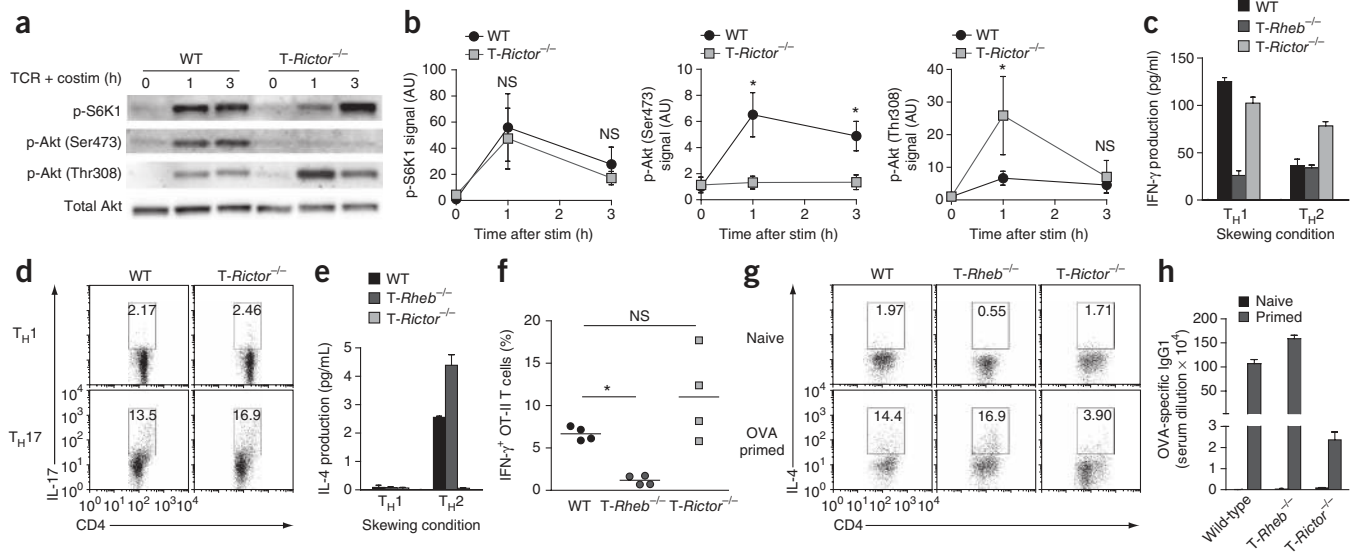
Given that mTOR-deficient T cells fail to differentiate into  $T_H1$ ,  $T_H2$  or  $T_H17$  effector cells, we wanted to determine the effect of Rheb deletion on T cell differentiation. There was no difference between wild-type and *T-Rheb*<sup>-/-</sup> littermates in their naive and memory

T cell compartments, as assayed with freshly isolated lymphocytes (Supplementary Fig. 4). Similar to T cells lacking mTOR, *T-Rheb*<sup>-/-</sup> T cells failed to differentiate into  $T_H1$  cells under the appropriate skewing conditions *in vitro* (Fig. 2a). *T-Rheb*<sup>-/-</sup> T cells also failed to differentiate into  $T_H17$  cells (Fig. 2b). These observations identify mTORC1 signaling as the downstream mediator of mTOR-dependent  $T_H1$  and  $T_H17$  differentiation. In contrast to mTOR-deficient T cells, *T-Rheb*<sup>-/-</sup> T cells were able to differentiate into  $T_H2$  cells (Fig. 2c). Therefore, inhibition of mTORC1 activity via deletion of Rheb selectively inhibited  $T_H1$  and  $T_H17$  differentiation but preserved  $T_H2$  differentiation.

To determine if the defect in  $T_H1$  skewing was linked to the proliferative defect in Rheb-deficient T cells, we labeled wild-type and Rheb-deficient T cells with the cytosolic dye CFSE, cultured them in  $T_H1$ - or  $T_H2$ -skewing conditions and assessed their cytokine production after rechallenge. Rheb-deficient T cells did proliferate *in vitro*, although at lower rates. Furthermore, Rheb-deficient T cells that divided several times under  $T_H1$ -skewing conditions still did not make interferon- $\gamma$  (IFN- $\gamma$ ) in amounts similar to those of wild-type cells (Fig. 2d). Furthermore, the slightly greater IL-4 production observed did not directly correlate with the number of cell divisions. Thus, the inability of Rheb-deficient T cells to differentiate into  $T_H1$  cells was not secondary to their lower rate of proliferation.



**Figure 4** *T-Rheb*<sup>-/-</sup> mice do not develop EAE but instead develop an alternative autoimmune disease. (a) Disease progression in wild-type and *T-Rheb*<sup>-/-</sup> mice ( $n = 7$ ) immunized with MOG and complete Freund's adjuvant to induce EAE. \* $P < 0.01$  (one-way analysis of variance). (b) Immunohistochemistry of CD45 and CD4 in sections of the central nervous system from wild-type mice, frozen at the height of disease. (c) Cytokine production in samples from the central nervous system, allowed to 'rest' for 16 h in unsupplemented medium and stimulated with PMA and ionomycin in the presence of a protein-transport inhibitor ( $n = 4$  mice). Plots (left) are gated by CD4 expression and side scatter and numbers in quadrants indicate percent cells in each; each symbol (right) represents an individual mouse.  $P < 0.05$  for IFN- $\gamma$  or 0.01 for IL-17 (Student's *t*-test). (d) Incidence of classical or nonclassical EAE in mice immunized with MOG and complete Freund's adjuvant. (e) Immunohistochemistry of CD45 and CD4 in the cerebellum, assessed as in b. Original magnification (b,e),  $\times 20$ . Data are representative of three independent experiments (error bars (a,d), s.e.m.).



**Figure 5** T cells deficient in mTORC2 cannot skew toward  $T_H2$  but retain  $T_H1$  and  $T_H17$  skewing. (a) Immunoblot analysis of mTOR activation (assessed as in Fig. 1a) in lysates of wild-type and *T-Rictor*<sup>-/-</sup> CD4<sup>+</sup> T cells stimulated for 1 h or 3 h with anti-CD3 and anti-CD28 in serum-free medium. (b) Densitometry analysis of the activity of mTORC1, mTORC2 and PI(3)K (assessed as in 1b;  $n = 4$  mice). \* $P < 0.05$  (Student's *t*-test). (c) IFN- $\gamma$  production in wild-type, *T-Rheb*<sup>-/-</sup> and *T-Rictor*<sup>-/-</sup> CD4<sup>+</sup> T cells stimulated overnight with anti-CD3 and APCs in  $T_H1$ - or  $T_H2$ -skewing conditions, followed by population expansion for 5 d with IL-2 and restimulation *in vitro* with anti-CD3 and anti-CD28. (d) IL-17 production by wild-type and *T-Rictor*<sup>-/-</sup> CD4<sup>+</sup> T cells stimulated with anti-CD3 and APCs in  $T_H1$ - or  $T_H17$ -skewing conditions, followed by population expansion for 5 d and restimulation with anti-CD3 and APCs in the presence of a protein-transport inhibitor. Numbers in outlined areas indicate percent IL-17<sup>+</sup>CD4<sup>+</sup> cells. (e) IL-4 production in cells treated as in c. (f) Cytokine production by splenocytes (treated as in Fig. 3a) from C57BL/6 (Thy-1.2<sup>+</sup>) mice immunized with vaccinia virus expressing ovalbumin and given adoptive transfer of  $1 \times 10^6$  wild-type, *T-Rheb*<sup>-/-</sup> or *T-Rictor*<sup>-/-</sup> CD4<sup>+</sup> OT-II (Thy-1.1<sup>+</sup>) T cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean. \* $P < 0.01$  (Student's *t*-test). (g) IL-4 production in CD4<sup>+</sup> T cells from wild-type, *T-Rheb*<sup>-/-</sup> and *T-Rictor*<sup>-/-</sup> mice immunized with alum (Naive) or ovalbumin plus alum (OVA primed) and boosted after 14 d; splenocytes were stimulated 48 h with OVA protein (100  $\mu$ g/ml) with protein-transport inhibitor present for the final 8 h. Numbers in outlined areas indicate percent IL-4<sup>+</sup>CD4<sup>+</sup> cells. (h) OVA-specific serum immunoglobulin G1 (IgG1) antibody titers in the mice in g. Data are representative of four (a, b) or two (f) experiments or at least three independent experiments (c–e, g, h; error bars (b, c, e, h), s.e.m.).

Next we sought to confirm *in vivo* the results reported above. Infection with vaccinia virus leads to a robust  $T_H1$  response. We obtained T cells from wild-type and *T-Rheb*<sup>-/-</sup> mice crossed onto the OT-II TCR–transgenic background specific for ovalbumin (OVA) and adoptively transferred the cells into wild-type C57BL/6 mice<sup>17</sup>. We infected the recipient mice with vaccinia virus expressing ovalbumin and evaluated clonotypic T cells 5 d later for expression of IFN- $\gamma$  and IL-4. As expected, wild-type OT-II cells differentiated into  $T_H1$  cells with robust production of IFN- $\gamma$ , IL-2 and tumor necrosis factor but little IL-4 (Fig. 3a, b). In contrast, *T-Rheb*<sup>-/-</sup> clonotypic T cells failed to robustly produce IFN- $\gamma$  but produced substantial IL-4. To assess the role of proliferation in this *in vivo* system, we labeled OT-II cells with CFSE. Cells transferred into host mice not infected with virus failed to proliferate or produce cytokines after rechallenge, whereas wild-type T cells transferred into immunized mice proliferated *in vivo* and produced IFN- $\gamma$  (Fig. 3c). However, Rheb-deficient T cells, even those undergoing multiple rounds of proliferation, failed to produce IFN- $\gamma$ . The addition of an IL-4-neutralizing antibody to the culture conditions failed to restore IFN- $\gamma$  production (data not shown). These data demonstrate that selective deletion of mTORC1 signaling leads to enhanced  $T_H2$  differentiation. Thus, selective inactivation of mTORC1 in T cells did not lead to T cell tolerance but instead promoted differentiation down an alternative effector pathway.

We also assessed the ability of Rheb-deficient T cells to differentiate into  $T_H17$  cells *in vivo*.  $T_H17$  cells develop *in vivo* in the gut in response to pathogens. After brief stimulation with the phorbol ester PMA and ionomycin *in vitro*, there were considerably fewer *in vivo*-differentiated CD3<sup>+</sup>CD4<sup>+</sup>  $T_H17$  cells in the Peyer's patches of *T-Rheb*<sup>-/-</sup> mice than in those of wild-type mice (Fig. 3d). Overall, these data show that

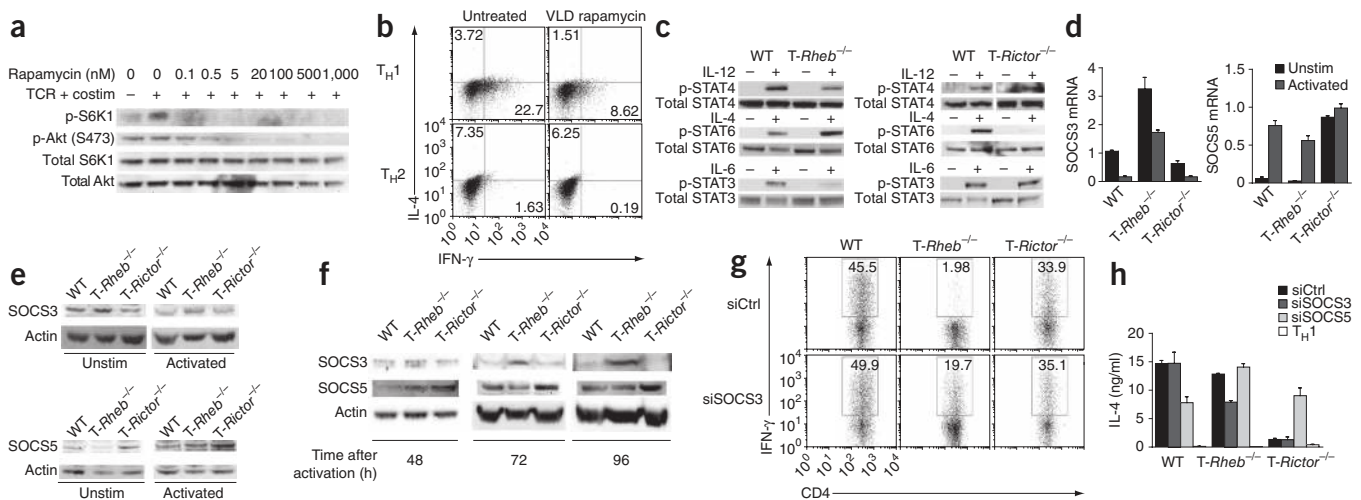
$T_H1$  and  $T_H17$  differentiation are specifically regulated by mTORC1 signaling *in vivo* and *in vitro* but  $T_H2$  differentiation is not.

### *T-Rheb*<sup>-/-</sup> mice are resistant to EAE

Because both  $T_H1$  and  $T_H17$  cells are linked to the promotion of autoimmune diseases and IFN- $\gamma$  and IL-17 participate in promoting the pathology seen in EAE, we determined the susceptibility of the *T-Rheb*<sup>-/-</sup> mice to this experimental model<sup>18</sup>. After immunization with myelin oligodendrocyte glycoprotein (MOG) peptide of amino acids 35–55, wild-type mice developed clinical EAE (Fig. 4a), whereas *T-Rheb*<sup>-/-</sup> mice had lower clinical scores, associated with less leukocytic infiltration in the spinal cord and less population expansion of  $T_H1$  and  $T_H17$  cells (Fig. 4b, c). However, nearly 60% (on average) of MOG-immunized *T-Rheb*<sup>-/-</sup> mice developed 'nonclassical EAE' (Supplementary Video 1), which is characteristic of  $T_H2$  responses to MOG (Fig. 4d). MOG-specific  $T_H2$  cells or IFN- $\gamma$ -deficient cells can induce a neurological disorder characterized by ataxia (not paralysis) and infiltration of the cerebellum with cells of the immune response<sup>19</sup>. *T-Rheb*<sup>-/-</sup> mice had more leukocytic infiltration, including CD4<sup>+</sup> cells in the cerebellum (Fig. 4e), and T cells isolated from these mice produced more IL-4 and IL-5 in response to MOG peptide *in vitro* (data not shown). Thus, mTORC1-deficient T cells were neither 'immunodeficient' nor unable to traffick to the CNS. Therefore, selective deletion of mTORC1 signaling leads to enhanced  $T_H2$  differentiation *in vivo*, even under strongly  $T_H1$ - and/or  $T_H17$ -promoting conditions.

### $T_H2$ differentiation requires mTORC2 signaling

We further bred mice with loxP-flanked Rictor alleles with mice expressing Cre recombinase from the *Cd4* promoter and enhancer



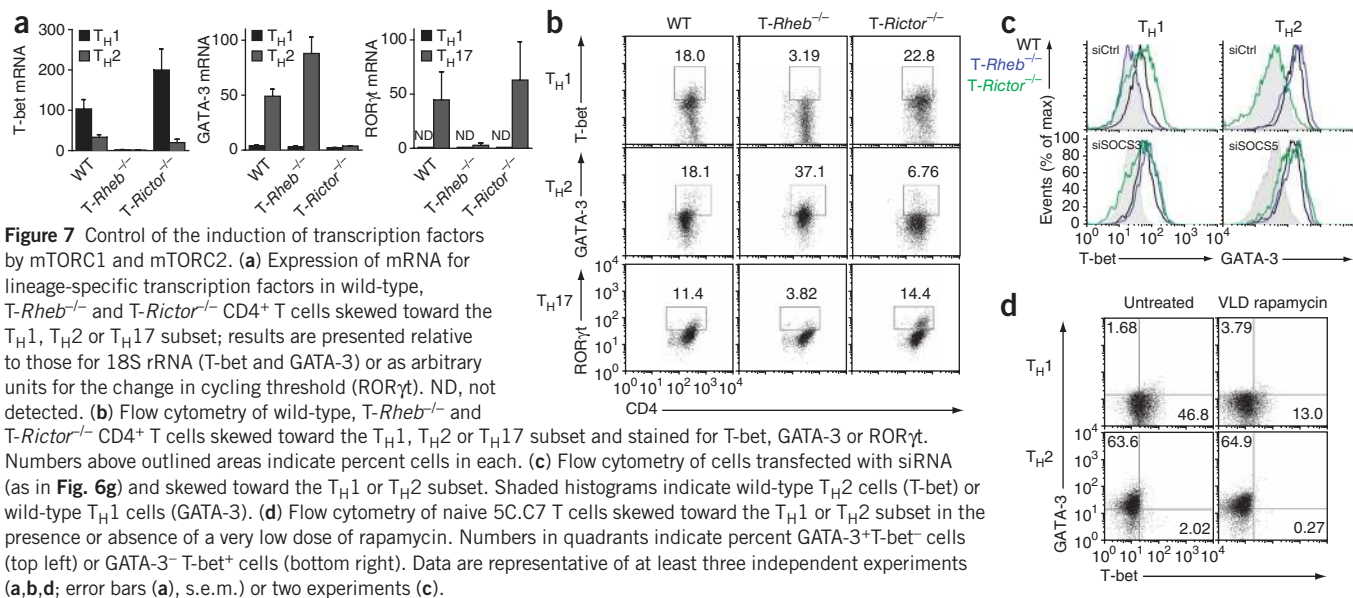
**Figure 6** Both mTORC1 and mTORC2 influence cytokine signaling by inhibiting SOCS proteins differently. **(a)** Immunoblot analysis of mTOR activation (assessed as in **Fig. 1a**) in lysates of wild-type, naive T cells stimulated for 8 h with anti-CD3 and anti-CD28 plus various doses of rapamycin (above lanes) in serum-containing medium. **(b)** Cytokine production by wild-type 5C.C7 T cells stimulated for 48 h with pigeon cytochrome c peptide in *T*<sub>H1</sub>- or *T*<sub>H2</sub>-skewing conditions in the presence (VLD rapamycin) or absence (Untreated) of a very low dose of rapamycin (100 pM) and washed, followed by population expansion for 5 d in fresh drug and IL-2, then restimulation with anti-CD3 and anti-CD28. Numbers in quadrants indicate percent IL-4<sup>+</sup>IFN-γ<sup>+</sup> cells (top left) or IL-4<sup>-</sup>IFN-γ<sup>+</sup> cells (bottom right). **(c)** Immunoblot analysis of STAT phosphorylation in lysates of freshly isolated wild-type, *T-Rheb*<sup>-/-</sup> and *T-Rictor*<sup>-/-</sup> CD4<sup>+</sup> T cells resuspended for 1 h in serum-free medium and stimulated for 30 min with IL-12, IL-4 or IL-6. **(d)** SOCS3 and SOCS5 mRNA in wild-type, *T-Rheb*<sup>-/-</sup> and *T-Rictor*<sup>-/-</sup> CD4<sup>+</sup> T cells left unstimulated (Unstim) or activated overnight with anti-CD3 and anti-CD28 (Activated); results are normalized to those of 18S rRNA and are presented relative to those of wild-type unstimulated cells (for SOCS3) or stimulated cells (for SOCS5). **(e)** Immunoblot analysis of SOCS3 and SOCS5 in cells treated as in **d**. **(f)** Kinetic analysis of SOCS3 and SOCS5 in lysates of wild-type, *T-Rheb*<sup>-/-</sup> and *T-Rictor*<sup>-/-</sup> CD4<sup>+</sup> T cells stimulated for 48, 72 or 96 h with anti-CD3 and anti-CD28; actin serves as a loading control. **(g)** IFN-γ production in wild-type, *T-Rheb*<sup>-/-</sup> and *T-Rictor*<sup>-/-</sup> CD4<sup>+</sup> T cells transfected by nucleofection with control siRNA (siCtrl) or siRNA specific for SOCS3 (siSOCS3) or SOCS5 (siSOCS5; data not shown), allowed to 'rest' for 16 h and then stimulated for 48 h in *T*<sub>H1</sub>- or *T*<sub>H2</sub>-skewing conditions, followed by population expansion for 48 h in IL-2 and restimulation with anti-CD3 and anti-CD28. Numbers in outlined areas indicate percent IFN-γ<sup>+</sup>CD4<sup>+</sup> cells. **(h)** IL-4 production by the cells in **g**. *T*<sub>H1</sub>, wild-type *T*<sub>H1</sub> cells (negative control). Data are representative of at least three independent experiments (**a-f**) or two experiments (**g,h**; error bars (**d,h**), s.e.m.).

regions (producing *T-Rictor*<sup>-/-</sup> progeny) to determine the effect of selectively eliminating mTORC2 in T cells<sup>20</sup> (**Supplementary Fig. 5**). After stimulation with anti-CD3 and anti-CD28, *T-Rictor*<sup>-/-</sup> T cells had less mTORC2 activity, as determined by phosphorylation of the Akt hydrophobic motif (Ser473). The activity of mTORC1 in these cells was normal, as determined by phosphorylation of S6K1 (**Fig. 5a,b**). In agreement with observations obtained with Rictor-deficient mice<sup>7</sup>, proximal (PI(3)K-PDK1) signaling, assessed as phosphorylation of Akt at Thr308, was intact and consistently greater. *T-Rictor*<sup>-/-</sup> CD4<sup>+</sup> T cells developed normally (with a slightly lower frequency of peripheral CD8<sup>+</sup> cells; **Supplementary Fig. 6**) and produced amounts of IL-2 equivalent to those produced by wild-type T cells (**Supplementary Fig. 7**), which indicated that TCR-induced signaling was intact. *T-Rictor*<sup>-/-</sup> T cells proliferated more slowly after stimulation but had a less severe defect than *T-Rheb*<sup>-/-</sup> T cells had (**Supplementary Fig. 7**). In addition, *T-Rictor*<sup>-/-</sup> mice did not have more activation-induced apoptosis (**Supplementary Fig. 3**) and had normal proportions of CD4<sup>+</sup> T cells in the naive compartment (**Supplementary Fig. 8**).

*T-Rictor*<sup>-/-</sup> T cells were able to develop into *T*<sub>H1</sub> and *T*<sub>H17</sub> cells under the appropriate skewing conditions *in vitro* (**Fig. 5c,d**) but failed to differentiate down the *T*<sub>H2</sub> pathway (**Fig. 5e**). To exclude the possibility that the presence of previously activated cells may have altered skewing conditions in our system, we sorted naive (CD62L<sup>hi</sup>CD44<sup>lo</sup>) CD4<sup>+</sup> cells from wild-type, *T-Rheb*<sup>-/-</sup> and *T-Rictor*<sup>-/-</sup> mice and skewed them with plate-bound anti-CD3 and soluble anti-CD28. As seen with bulk CD4<sup>+</sup> T cells,

*Rheb*-deficient cells failed to differentiate into *T*<sub>H1</sub> or *T*<sub>H17</sub> cells, and *Rictor*-deficient cells failed to differentiate into *T*<sub>H2</sub> cells (**Supplementary Fig. 9**). TCR signaling strength can affect *T*<sub>H1</sub> and *T*<sub>H2</sub> responses<sup>21</sup>. Specifically, low concentrations of peptide can impair IFN-γ production and create a bias toward the *T*<sub>H2</sub> response. To assess the effect of strength of peptide signaling on the differentiation of *Rheb*-deficient and *Rictor*-deficient T cells, we stimulated wild-type, *Rheb*-deficient and *Rictor*-deficient OT-II T cells under conditions of widely varying doses of OVA peptide as described before<sup>22</sup>. However, even at a dose of 100 μM OVA peptide, there was no evidence of more IFN-γ production from *Rheb*-deficient T cells (**Supplementary Fig. 10**). In contrast, *Rictor*-deficient T cells produced *T*<sub>H1</sub> cytokines even at exceedingly low doses of peptide.

Consistent with the *in vitro* data, *T-Rictor*<sup>-/-</sup> T cells had robust production of IFN-γ in response to infection with vaccinia virus (**Fig. 5f**). *T-Rictor*<sup>-/-</sup> mice had normal susceptibility to EAE (**Supplementary Fig. 11**) associated with numbers of IFN-γ- and IL-17-producing cells in response to MOG peptide equivalent to those in wild-type mice. To assess *T*<sub>H2</sub> responses *in vivo*, we primed and boosted wild-type (C57BL/6), *T-Rheb*<sup>-/-</sup> and *T-Rictor*<sup>-/-</sup> mice with OVA in alum as an adjuvant to elicit a *T*<sub>H2</sub> response. *T-Rictor*<sup>-/-</sup> mice had less induction of IL-4-secreting OVA-specific T cells than did wild-type or *T-Rheb*<sup>-/-</sup> mice (**Fig. 5g**) and failed to generate antigen-specific immunoglobulin G1 antibodies (which are dependent on *T*<sub>H2</sub> cell help; **Fig. 5h**). Thus, mTORC2 regulates the ability of helper T cells to differentiate into *T*<sub>H2</sub> cells.



### Distinct STAT activation by mTORC1 and mTORC2

Next we determined if we could reproduce the results reported above in wild-type primary T cells by pharmacological inhibition of mTOR activity. Biochemical analysis of T cells showed that rapamycin inhibited mTORC2 activity<sup>23</sup> (Fig. 6a). However, very low doses of rapamycin (100–500 pM) selectively inhibit mTORC1 in T cells. Rapamycin at such a very low dose specifically inhibited T<sub>H</sub>1 differentiation by naive wild-type T cells activated under either T<sub>H</sub>1- or T<sub>H</sub>2-skewing conditions (Fig. 6b), consistent with the findings obtained with Rheb-deficient T cells.

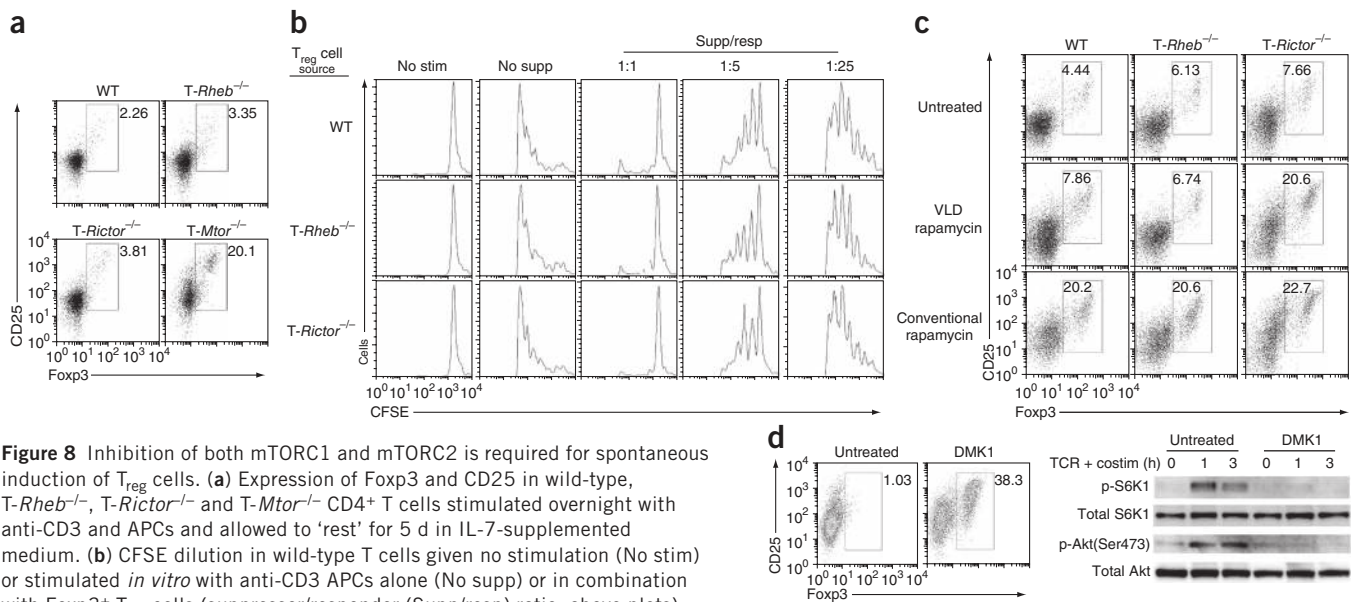
It is known that T cells that lack mTOR cannot differentiate into effector cells, in part because they have diminished responses to helper T cell-skewing cytokines<sup>9</sup>. We exposed freshly isolated T cells from wild-type, *T-Rheb*<sup>-/-</sup> and *T-Rictor*<sup>-/-</sup> mice to skewing cytokines and assessed STAT activation. Consistent with their inability to become T<sub>H</sub>1 or T<sub>H</sub>17 cells, *T-Rheb*<sup>-/-</sup> T cells showed less phosphorylation of STAT4 and STAT3 in response to IL-12 and IL-6, respectively (Fig. 6c, left). However, *T-Rheb*<sup>-/-</sup> T cells demonstrated enhanced STAT6 phosphorylation in response to IL-4 (a T<sub>H</sub>2-promoting cytokine). *T-Rictor*<sup>-/-</sup> T cells showed intact IL-12- and IL-6-induced activation of STAT4 and STAT3, whereas IL-4-induced activation of STAT6 was diminished (Fig. 6c, right). These findings indicate that the ability of mTORC1 and mTORC2 to regulate the differentiation of effector T cells differently is due in part to their ability to regulate STAT activation differently. In response to different cytokines, Rheb-deficient T cells showed less IL-23- and IFN-β-induced activation of STAT4 and less IL-10- and IL-21-dependent activation of STAT3 (data not shown). Rictor-deficient T cells showed less STAT6 activation in response to IL-13 (data not shown).

Studies have shown that mTOR influences STAT activation<sup>9,24,25</sup>. To determine how distinct STAT proteins are regulated differently in T cells, we examined the expression of cytokine receptors on Rheb- and Rictor-deficient T cells. Wild-type, Rheb-deficient and Rictor-deficient T cells all had equivalent expression of the receptors IL-12Rβ1, IL-4R and IL-6R (Supplementary Fig. 12), which indicated that mTOR did not regulate cytokine receptor expression.

STAT activation is partially regulated by the expression of inhibitory SOCS proteins, which can dephosphorylate Jak kinase-dependent residues on STAT proteins in different ways. We assessed the expression patterns of SOCS3 and SOCS5 in the Rheb- and Rictor-deficient T cells. Consistent with published findings<sup>26–28</sup>, wild-type T cells expressed SOCS3 mRNA and protein at baseline and downregulated its expression after activation (Fig. 6d,e), whereas SOCS5 expression was low in naive T cells and increased with activation<sup>29,30</sup>. In resting Rheb-deficient T cells, expression of SOCS3 mRNA and protein was much higher, and these cells retained higher expression after activation (Fig. 6d,e), whereas SOCS5 expression was similar to that in wild-type cells. Rictor-deficient T cells produced SOCS3 at baseline (albeit in smaller amounts than the wild-type cells produced) and this expression decreased after activation, whereas SOCS5 expression was markedly upregulated in the resting cells and remained higher after activation (Fig. 6d,e). Thus, at baseline and after activation, SOCS3 and SOCS5 are regulated differently in Rheb-deficient and Rictor-deficient T cells.

SOCS protein expression is regulated dynamically over time. To examine the expression patterns of SOCS proteins at later time points, we activated T cells for 48, 72 or 96 h. At these time points, SOCS3 protein remained more abundant in Rheb-deficient T cells, whereas wild-type and Rictor-deficient cells downregulated SOCS3 (Fig. 6f). Furthermore, whereas wild-type and Rheb-deficient cells consistently upregulated SOCS5 after activation, Rictor-deficient T cells had much more SOCS5 at later time points.

To prove that the larger amounts of SOCS3 in the Rheb-deficient T cells and of SOCS5 in the Rictor-deficient T cells had functional importance, we transfected freshly isolated wild-type, Rheb-deficient and Rictor-deficient CD4<sup>+</sup> T cells with small interfering RNA (siRNA) specific for SOCS3 and SOCS5. This resulted in substantial knockdown of SOCS3 and SOCS5 mRNA (Supplementary Fig. 13). In T cells stimulated in T<sub>H</sub>1 and T<sub>H</sub>2 differentiation conditions, knockdown of SOCS3 mRNA resulted in more IFN-γ production in Rheb-deficient T cells (Fig. 6g), whereas knockdown of SOCS5 mRNA in Rictor-deficient T cells resulted in a greater ability to produce IL-4 (Fig. 6h). Such observations demonstrate that SOCS proteins regulate T<sub>H</sub>1, T<sub>H</sub>17 and T<sub>H</sub>2 differentiation in the Rheb- and Rictor-deficient T cells.



**Figure 8** Inhibition of both mTORC1 and mTORC2 is required for spontaneous induction of T<sub>reg</sub> cells. **(a)** Expression of Foxp3 and CD25 in wild-type, T-Rheb<sup>-/-</sup>, T-Rictor<sup>-/-</sup> and T-Mtor<sup>-/-</sup> CD4<sup>+</sup> T cells stimulated overnight with anti-CD3 and APCs and allowed to 'rest' for 5 d in IL-7-supplemented medium. **(b)** CFSE dilution in wild-type T cells given no stimulation (No stim) or stimulated *in vitro* with anti-CD3 APCs alone (No supp) or in combination with Foxp3<sup>+</sup> T<sub>reg</sub> cells (suppressor/responder (Supp/resp) ratio, above plots) generated from wild-type, T-Rheb<sup>-/-</sup> and T-Rictor<sup>-/-</sup> CD4<sup>+</sup> T cells (with TGF- $\beta$  and IL-2); T<sub>reg</sub> cells were enriched by CD25 magnetic separation, and Foxp3 expression was confirmed by intracellular staining. **(c)** Expression of Foxp3 and CD25 in wild-type, T-Rheb<sup>-/-</sup> and T-Rictor<sup>-/-</sup> CD4<sup>+</sup> T cells stimulated with anti-CD3 and APCs in the presence of a very low dose or conventional (500 nM) dose of rapamycin and allowed to 'rest' for 5 d in IL-7-supplemented medium (with drug). **(d)** Expression of Foxp3 and CD25 (left) by wild-type T cells stimulated with anti-CD3 and APCs in the presence (DMK1) or absence (Untreated) of DMK1, followed by population expansion for 5 d in the presence of IL-2 and drug, and immunoblot analysis (right) of mTOR activation in lysates of wild-type T cells stimulated for 1 or 3 h with anti-CD3 and anti-CD28 in serum-free medium with or without DMK1. Numbers adjacent to **(a,d)** or in **(c)** outlined areas indicate percent CD25<sup>+</sup>Foxp3<sup>+</sup> cells. Data are representative of at least three independent experiments.

### Transcription factor expression in T-Rheb<sup>-/-</sup> and T-Rictor<sup>-/-</sup> T cells

Next we sought to determine if the transcription factors T-bet, ROR $\gamma$ t and GATA-3, which have critical roles in the development of T<sub>H</sub>1, T<sub>H</sub>17 and T<sub>H</sub>2 cells, were selectively affected by deletion of Rheb or Rictor. Rheb-deficient T cells failed to fully upregulate T-bet mRNA and protein under T<sub>H</sub>1 conditions and ROR $\gamma$ t under T<sub>H</sub>17 conditions while expressing abundant GATA-3 under T<sub>H</sub>2 conditions (Fig. 7a,b). In fact, Rheb-deficient cells had higher expression of GATA-3 than did wild-type cells, consistent with the enhanced ability of Rheb-deficient cells to phosphorylate STAT6 and produce IL-4 *in vivo*. In T-Rictor<sup>-/-</sup> T cells, T-bet and ROR $\gamma$ t were transcribed and translated under T<sub>H</sub>1 and T<sub>H</sub>17 conditions, respectively, but GATA-3 was not expressed under T<sub>H</sub>2 conditions (Fig. 7a,b). In T cells stimulated in T<sub>H</sub>1 and T<sub>H</sub>2 differentiation conditions, knockdown of SOCS3 mRNA resulted in higher T-bet expression in Rheb-deficient T cells (Fig. 7c), whereas knockdown of SOCS5 mRNA in Rictor-deficient T cells resulted in higher GATA-3 expression (Fig. 7c). Furthermore, wild-type T cells exposed to a very low dose of rapamycin (which inhibits T<sub>H</sub>1 development but not T<sub>H</sub>2 development) had lower T-bet expression but not lower GATA3 expression in response to the appropriate differentiating conditions (Fig. 7d). Thus, in the absence of the nutrient-sensitive signaling pathways mediated by mTOR, the ability to respond to cytokines is diminished and T cells fail to properly upregulate lineage-specific transcription factors.

### T<sub>reg</sub> cell development in the absence of mTORC1 and mTORC2

It is known that mTOR-deficient T cells differentiate down a Foxp3<sup>+</sup> T<sub>reg</sub> cell pathway and that inhibition of mTORC1 alone is not sufficient for the generation of T<sub>reg</sub> cells<sup>9</sup>. Rapamycin can enhance the generation of inducible T<sub>reg</sub> cells<sup>31–33</sup>. To determine the specific effect of mTORC1

and mTORC2 on T<sub>reg</sub> cell differentiation, we cultured wild-type, T-Rheb<sup>-/-</sup>, T-Rictor<sup>-/-</sup> and T-Mtor<sup>-/-</sup> T cells *in vitro* under activating conditions (in the absence of exogenous transforming growth factor- $\beta$  (TGF- $\beta$ )) and then assessed the generation of Foxp3<sup>+</sup> T cells. The elimination of neither mTORC1 signaling nor mTORC2 signaling alone led to more Foxp3<sup>+</sup> cells (Fig. 8a). Notably, however, inducible T<sub>reg</sub> cells (made with TGF- $\beta$  and IL-2) from wild-type, T-Rheb<sup>-/-</sup>, T-Rictor<sup>-/-</sup> mice all seemed to inhibit T cell function equally (Fig. 8b).

To further test the hypothesis that both mTORC1 and mTORC2 must be inhibited to generate T<sub>reg</sub> cells, we activated wild-type, T-Rheb<sup>-/-</sup> and T-Rictor<sup>-/-</sup> T cells in the presence of a conventional dose of rapamycin (500 nM, which inhibits mTORC1 and mTORC2) or a very low dose of rapamycin (500 pM, which inhibits only mTORC1). Wild-type T cells differentiated into Foxp3<sup>+</sup> T cells at the conventional dose of 500 nM (Fig. 8c). T-Rheb<sup>-/-</sup> T cells required the mTORC2 inhibition induced by the conventional dose. In contrast, T-Rictor<sup>-/-</sup> T cells require only the very low dose of rapamycin to differentiate into T<sub>reg</sub> cells. Finally, the mTOR-specific kinase inhibitor DMK1 (which acutely inhibits both mTOR downstream signaling pathways)<sup>34</sup> promoted the generation of T<sub>reg</sub> cells from naive wild-type T cells, consistent with our data obtained with mTOR-, mTORC1- and mTORC2-deficient T cells (Fig. 8d). Thus, inhibition of both mTORC1 and mTORC2 is required for the generation of T<sub>reg</sub> cells in the absence of exogenous TGF- $\beta$ .

### DISCUSSION

In this report we have defined precise and reciprocal roles for signaling by mTORC1 and mTORC2 in regulating the differentiation of CD4<sup>+</sup> helper T cells. Our data have ascribed tissue-distinct physiological functions to mTORC1 and mTORC2. Thus, mTORC1 signaling promotes T<sub>H</sub>1 and T<sub>H</sub>17 differentiation, mTORC2

signaling promotes  $T_H2$  differentiation, and inhibition of mTOR leads to  $T_{reg}$  cells. Preliminary studies in our laboratory have failed to demonstrate differences in the activation of mTORC1 and mTORC2 under  $T_H1$ - and  $T_H2$ -skewing conditions. Therefore, we hypothesize that it is the differences in expression and activation of downstream substrates and signaling pathways that confer on mTORC1 the ability to regulate  $T_H1$  and  $T_H17$  differentiation and confer on mTORC2 the ability to regulate  $T_H2$  differentiation.

Our studies provide a critical link between metabolism and T cell differentiation. It is known that mTORC1 signaling integrates growth factors, energy, oxygen and amino acids to promote processes necessary for cell growth<sup>2,5</sup>. In addition, in T cells, the PI(3)K-Akt-mTORC1 axis is activated by engagement of the coreceptor CD28 and signaling via the IL-2 receptor<sup>35</sup>. IL-7 promotes mTOR activity, helping to prevent atrophy in T cells<sup>36</sup>, whereas IL-4-induced mTOR activity prevents apoptosis<sup>37</sup>. IL-12 and IFN- $\gamma$  promote the sustained activation of mTORC1 activity in CD8<sup>+</sup> T cells<sup>11,38</sup>. Relatively little is known about the precise role of mTORC2 signaling in regulating cellular function<sup>5</sup>. The ability of mTORC2 to regulate  $T_H2$  differentiation provides potential clues for determining the specific role of mTORC2 in other cell types.

The small GTPase Rheb has been identified as an activator of mTORC1 (ref. 15). Our data have demonstrated that Rheb signaling has an important role in regulating T cell differentiation. The same block in differentiation is seen in T cells treated with a very low dose of rapamycin, T cells deficient in mTOR or T cells deficient in Rheb, which supports the hypothesis that the ability of Rheb to direct differentiation is achieved through mTORC1 activation. Furthermore, mTORC1 signaling seems to be both necessary and sufficient for  $T_H1$  and  $T_H17$  differentiation, because Rictor-deficient T cells still maintain their ability to become  $T_H1$  and  $T_H17$  effector cells.

Rictor-deficient T cells generated by crossing of mice expressing Cre from the distal promoter of the gene encoding the kinase Lck (dLck-iCre) and mice with loxP-flanked *Rictor* alleles have been studied<sup>39</sup>. Consistent with our study, that Rictor deletion inhibited  $T_H2$  differentiation<sup>39</sup>. However, the generation of fewer  $T_H1$  cells (albeit modest compared with  $T_H2$  inhibition) was also reported<sup>39</sup>. The cause of these divergent observations remains unclear. The differences between the two models in their Cre expression might result in subtle differences in T cell development and could affect helper T cell differentiation. Additionally, we found that Rictor-deficient T cells had a greater propensity to differentiate to  $T_H17$  cells than did wild-type cells. In addition, there seem to be differences in signaling in the Rictor-deficient T cells used in each study. Notably, unlike T cells from our mice, which demonstrated robust phosphorylation of Akt Thr308 and subsequent S6K1 activation, T cells in the other study showed less signaling upstream of mTOR<sup>39</sup>, which could also impair mTORC1 signaling. We suggest that when the signaling defects are restricted downstream of mTORC2 signaling, there is a selective block in  $T_H2$  development but not in  $T_H1$  development. Thus, it is possible that the Rictor-deficient T cells in the previously published report<sup>39</sup> act more like mTOR-deficient T cells.

Rapamycin promotes the generation of  $T_{reg}$  cells in the absence of exogenous TGF- $\beta$ <sup>40-42</sup>. The ligand for the T cell-inhibitory receptor PD-1 has been shown to promote inducible  $T_{reg}$  cells by inhibiting mTOR activation<sup>43</sup>. In addition, local depletion of essential amino acids can promote the generation of  $T_{reg}$  cells by inhibiting mTOR activity<sup>44</sup>. We have shown here that signaling from both mTORC1 and mTORC2 must be inhibited for skewing of cells down the  $T_{reg}$  cell pathway. Our genetic studies were complemented by a

pharmacological approach. The simultaneous inhibition of mTORC1 and mTORC2 by an mTOR kinase inhibitor was a potent inducer of  $T_{reg}$  cells in the absence of exogenous TGF- $\beta$ , which indicates that drugs in this class can act as potent agents inducing immunosuppression and tolerance.

Both mTORC1 and mTORC2 can promote cell differentiation in other tissues. Cerebral cortical development is dependent on mTORC1, whereas terminal differentiation of myoblasts is mediated by mTORC2 (refs. 45,46). In this model, mTORC2 signaling inactivates the Rho-associated kinase ROCK1, which in turn promotes activation of the transcription factors Foxo1 and Foxo3a. We have demonstrated a similar role for signaling by mTORC1 and mTORC2 in regulating distinct tissue-specific transcription factors. It has been shown that rapamycin promotes the differentiation of CD8<sup>+</sup> memory cells<sup>10</sup>. Thus, by inhibiting T-bet expression, rapamycin not only inhibits effector functions (such as IFN- $\gamma$  production) but also promotes the expression of eomesodermin, which in turn promotes the generation of memory T cells<sup>11</sup>. The decrease in helper T cell-specific transcription factors is due to a decrease in specific STAT activation in response to cytokines. Although the IL-12-, IL-6- and IL-4-induced phosphorylation of STAT4, STAT3 and STAT6 was not completely lost in the mTORC1- and mTORC2-deficient T cells, the diminished response contributed to the observed differentiation failure. The ability of mTOR to regulate STAT3 phosphorylation has been described in cancer and neuron activation; however, the precise mechanism for this regulation is unclear<sup>24,25,47</sup>. We have demonstrated that in T cells, differences in the expression of SOCS3 and SOCS5 contribute to the STAT-activation defects. Along these lines, mTORC2 inhibits the activity of Foxo transcription factors<sup>7,48</sup>. Foxo proteins control expression of the transcription factor KLF2, which in turn promotes SOCS5 expression<sup>49</sup>. Preliminary studies have shown that Rictor-deficient T cells have more KLF2, consistent with the observed increase in SOCS5 in these cells (data not shown). We propose a model in which mTORC1 and mTORC2 activate distinct signaling programs, the net sum of which is to promote the differentiation of specific effector cells. Future studies should identify the mTORC1- and mTORC2-specific targets that control differentiation.

## METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

*Note: Supplementary information is available on the Nature Immunology website.*

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## AUTHOR CONTRIBUTIONS

G.M.D. did research, helped design experiments and wrote the paper; K.N.P. assisted with *in vivo* experiments and biochemistry; A.T.W. assisted with EAE induction and central nervous system isolation and did immunohistochemistry; E.H. assisted with very-low-dose rapamycin experiments; D.J.M. synthesized the mTOR kinase inhibitor; M.R.H. helped design experiments and contributed reagents; B.X. and P.F.W. generated the original mouse line with loxP-flanked *Rheb* alleles; and J.D.P. designed experiments, oversaw research and wrote the paper.

## COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.



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## ONLINE METHODS

**Mice.** Mice were kept in accordance with guidelines of the Johns Hopkins University Institutional Animal Care and Use Committee. C57BL/6, *Cd4-Cre* and OT-II mice from Jackson Laboratories were, where applicable, bred to Thy-1.1 backgrounds; 5C.C7 mice were from Taconic Farms. Mice with *loxP*-flanked *Rheb* alleles were generated in the laboratory of P.F. Worley. Mice with *loxP*-flanked *Rictor* alleles were a gift from M. Magnuson. Mice with *loxP*-flanked *Mtor* alleles were a gift from S.C. Kozma.

**Antibodies and reagents.** The following antibodies for flow cytometry were from BD Biosciences: anti-B220 (RA3-6B2), anti-CD3 (2C11), anti-CD4 (RM4-5), anti-CD8 (Ly-3), anti-CD25 (PC61), anti-GATA-3 (L50-823), anti-IFN- $\gamma$  (XMG1.2), anti-IL-2 (JES6-5H4), anti-IL-4 (11B11), anti-IL-5 (TRFK5), anti-IL-17A (TC11-18H10) and antibody to tumor necrosis factor (MP6-XT22). Anti-IL-5 (TRFK5), anti-ROR $\gamma$ t (AFKJS-9), anti-T-bet (eBio4B10) and anti-Foxp3 (FJK-16s) were from eBioscience. Stimulatory anti-CD3 (2C11) and anti-CD28 (37.51), as well as neutralizing anti-IFN- $\gamma$  (XMG1.2) and anti-IL-4 (11B11) were purified from hybridoma supernatants prepared 'in-house'. Neutralizing anti-IL-12p40 (C17.8) was from eBioscience. Antibody to S6K1 phosphorylated at Thr421 and Ser424 (9204), anti-S6K1(202), antibody to Akt phosphorylated at Ser473 (9271) or Thr308 (244F9), anti-Rheb1 (4935), anti-Rictor (2140), antibody to phosphorylated STAT6 (9361), anti-STAT6 (9362), antibody to phosphorylated STAT3 (D3A7) and anti-STAT3 (9132) were from Cell Signaling Technologies. Anti-Akt (H-136), antibody to phosphorylated STAT4 (sc-101804), anti-STAT4 (C-20), anti-SOCS3 (H-103) and anti-SOCS5 (M-300) were from Santa Cruz Biotechnologies. Vaccinia virus expressing ovalbumin was a gift from C. Drake. Cytokines were from Peprotech. Rapamycin was from LC Labs. DMK1 (compound 401 in ref. 34) was synthesized at Johns Hopkins. ON-TARGET siRNA reagents for control and SOCS3- or SOCS5-specific RNA-mediated interference were from Dharmacon. Nucleofection reagents were from Lonza.

**Immunoblot analysis.** Immunoblot analysis was done essentially as described<sup>50</sup>. Magnetically purified CD4<sup>+</sup> T cells were stimulated in serum-free medium with anti-CD3 (1  $\mu$ g/ml), anti-CD28 (2  $\mu$ g/ml) and antibody to hamster immunoglobulin G1 (0.75  $\mu$ g/ml; G94-56; BD Biosciences), then were collected and lysed. For immunoblot analysis of STAT proteins, cells were kept in cytokine-free medium for 1 h, then were stimulated for 30 min with IL-12 (10 ng/ml), IL-4 (1 ng/ml) or IL-6 (10 ng/ml). For immunoblot analysis of SOCS proteins, cells were allowed to 'rest' for 2 h and then were lysed or stimulated with plate-bound anti-CD3 (5  $\mu$ g/ml) and soluble anti-CD28 (2  $\mu$ g/ml).

**Enzyme-linked immunosorbent assay.** IL-2, IFN- $\gamma$  and IL-4 were analyzed by enzyme-linked immunosorbent assay as described by the manufacturer (eBioscience).

OVA-specific antibodies were analyzed by enzyme-linked immunosorbent assay in Nunc-Immuno plates (eBioscience) coated overnight at 4 °C with 100  $\mu$ l OVA (100  $\mu$ g/ml in PBS) and blocked for 1 h at 25 °C with 5% (wt/vol) BSA (Roche). Wells were washed several times and serum diluted in 5% (wt/vol) BSA was added for 2 h. Wells were washed and then were incubated for 1 h with biotinylated anti-mouse immunoglobulin G1 (A85-1; BD Biosciences) diluted to a concentration of 0.5  $\mu$ g/ml in 5% (wt/vol) BSA. Wells were washed and then were incubated for 30 min with streptavidin-conjugated horseradish peroxidase. Wells were washed and then were incubated for 5 min with tetramethylbenzidine substrate or until the reaction approached saturation. Linear regression analysis was used to determine titer.

**T cell stimulation and skewing.** Unless otherwise stated, T cells were stimulated with anti-CD3 (5  $\mu$ g/ml) and, where appropriate, anti-CD28 (2  $\mu$ g/ml). APC-free systems used anti-CD3 diluted in PBS for coating of flat-bottomed plates. PMA (phorbol 12-myristate 13-acetate) and ionomycin (Sigma) were used at a concentration of 50 ng/ml and 500 ng/ml, respectively.

T cells were skewed with irradiated APCs and anti-CD3 and then washed, followed by population expansion for 5 d, then were rechallenged with plate-bound

anti-CD3 and soluble anti-CD28 or with fresh APCs and anti-CD3. Sorted naive cells were skewed with plate-bound anti-CD3 and anti-CD28 and rechallenged likewise. T cells skewed after nucleofection were stimulated for 48 h, followed by population expansion for 48 h for less cell loss. Skewing conditions were as follows: T<sub>H</sub>1, IL-12 (5 ng/ml), IFN- $\gamma$  (100 ng/ml) and anti-IL-4 (100  $\mu$ g/ml), with IL-2 (1 ng/ml) during the rest period; T<sub>H</sub>2, IL-4 (1 ng/ml), anti-IL-12 (100  $\mu$ g/ml) and anti-IFN- $\gamma$  (100  $\mu$ g/ml), with IL-2 (1 ng/ml) during the rest period; T<sub>H</sub>17, TGF- $\beta$  (10 ng/ml), IL-6 (10 ng/ml), anti-IFN- $\gamma$  (100  $\mu$ g/ml) and anti-IL-4 (100  $\mu$ g/ml); T<sub>reg</sub>, TGF- $\beta$  (5 ng/ml) and IL-2 (1 ng/ml).

**Intracellular staining.** Brefeldin A (GolgiPlug; BD Biosciences) or monensin (GolgiStop; BD Biosciences) was used for cytokine staining. Cell surfaces were stained, then cells were fixed and made permeable with BD Cytofix/Cytoperm and then stained for cytokines. Intracellular staining of transcription factors was done without stimulation, with the eBioscience Foxp3 Fixation/Permeabilization kit. Gates were set appropriately with unstimulated controls and voltages were set on the basis of isotype-matched control antibodies.

**Vaccinia infection.** C57BL/6 Thy1.2<sup>+</sup> host mice were immunized with 2  $\times$  10<sup>6</sup> plaque-forming units of vaccinia virus expressing ovalbumin and were given adoptive transfer of 1  $\times$  10<sup>6</sup> to 2  $\times$  10<sup>6</sup> CD4<sup>+</sup>Thy1.1<sup>+</sup> OT-II T cells from wild-type or T-*Rheb*<sup>-/-</sup> mice. Then, 4 d after transfer, splenocytes were collected and rechallenged overnight with OVA peptide (50  $\mu$ g/ml; Anaspec).

**OVA sensitization.** OVA (Sigma-Aldrich) was adsorbed by gentle shaking for 30 min onto Imject Alum adjuvant (Pierce) to a final concentration of 100  $\mu$ g/ml. Mice were primed with 200  $\mu$ l intraperitoneally and boosted 14 d later. On day 18, serum and spleens were collected. Serum was analyzed for antibodies and spleens were stimulated 48 h with OVA (100  $\mu$ g/ml).

**EAE.** Mice on the C57BL/6 background were immunized subcutaneously with 150  $\mu$ g MOG peptide (Genway) with incomplete Freund's adjuvant (Thermo Scientific) supplemented with *Mycobacterium tuberculosis* (4 mg/ml; Difco). Mice received 200  $\mu$ g pertussis toxin (List Biological Laboratories) intravenously on both day 0 and day 4. Mice were assigned scores as follows by a researcher 'blinded' to sample identity: 0.5, flaccid tail; 1, partial hindlimb paralysis; 2, full hindlimb paralysis; 3, partial forelimb paralysis; 4, full forelimb paralysis; 5, moribund. Mice were killed after they achieved a score of 4 or higher or at the end of the time course. The central nervous system was collected and either was frozen or infiltrates were isolated on Percoll (GE Healthcare). Infiltrates were placed in culture medium for 24 h, then stimulated for 8 h with PMA and ionomycin.

**In vitro suppression.** T<sub>reg</sub> cells were generated from wild-type, T-*Rheb*<sup>-/-</sup> and T-*Rictor*<sup>-/-</sup> mice *in vitro* with TGF- $\beta$  and IL-2, then CD25<sup>+</sup> cells were isolated (Miltenyi-Biotec) and analyzed for Foxp3 expression by flow cytometry. Wild-type T cells were labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester; Invitrogen) and then were stimulated with APCs (at a ratio of 1:10, T cells/APCs) and anti-CD3 (1  $\mu$ g/ml). According to calculations from Foxp3 staining, various numbers of T<sub>reg</sub> cells were added to the stimulations to achieve the desired ratio. Suppression was assessed 72 h later by analysis of CFSE dilution by wild-type naive T cells.

**RNA-mediated interference.** T cells were isolated by CD4<sup>+</sup> negative selection and allowed to 'rest' in medium for 2 h. Cells were transfected with pools of control siRNA or SOCS3- or SOCS5-specific siRNA (200 pM; Dharmacon) with the Amaxa T cell Nucleofection kit (Lonza). T cells were resuspended at a density of 20  $\times$  10<sup>6</sup> cells per ml in T cell nucleofection solution, added to siRNA, transfected by nucleofection with the X-001 program and immediately transferred into 1.5 ml T cell Nucleofector medium. T cells were washed after 16 h and stimulated in normal culture medium.

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## Corrigendum: The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2

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In the version of this article initially published, the Discussion section cited a published study of mice with conditional deletion of *Rictor* in T cells without specifying which *Lck* promoter was used to drive the expression of Cre recombinase. The distal *Lck* promoter (dLck) was used. The error has been corrected in the HTML and PDF versions of the article.