ONLINE METHODS

Mice

Mice were kept in accordance with the Johns Hopkins University Institutional Animal Care and Use Committee guidelines. C57/BL6, CD4-Cre, and OT-II mice were purchased from Jackson laboratories and, where applicable, bred to Thy-1.1 backgrounds. 5C.C7 mice were purchased from Taconic Farms. Rheb floxed mice were generated in the lab of P.F. Worley (Johns Hopkins

University, Department of Neuroscience). Rictor floxed mice were a gift from M. Magnuson (Vanderbilt University). mTOR floxed mice were a gift from S.C. Kozma (University of Cincinnati).

Antibodies and reagents

Antibodies for flow cytometry and immunohistochemistry were purchased from BD Biosciences, with the exception of RORγT, T-bet, and Foxp3 antibodies. Anti-CD3 (2C11), anti-CD28 (37.51), and anti-IFN-γ (XMG1.2) were purified from hybridoma supernatants. Anti-IL-12p40 and anti-IL-4 were purchased from Invitrogen. Antibodies to pS6K1(T421/S424), S6K1, pAkt(S473), pAkt(T308), Rheb1, Rictor, pSTAT6, STAT6, pSTAT3, and STAT3 were purchased from Cell Signaling Technologies. Antibodies to Akt, pSTAT4, STAT4, SOCS3, and SOCS5 were purchased from Santa Cruz Biotechnologies. Vaccinia-OVA was a gift from C. Drake (Johns Hopkins University). Cytokines were purchased from Peprotech. Rapamycin was purchased from LC Labs. The mTOR kinase inhibitor DMK1 (compound 401 in [34]) was synthesized at Johns Hopkins. siRNA reagents (ON-TARGET siRNA) for control, SOCS3, and SOCS5 RNAi were purchased from Dharmacon. Nucleofection reagents were from Lonza.

Immunoblotting

Immunoblotting was performed essentially as described[50]. Magnetically purified $CD4^+$ T cells were stimulated in serum-free media with anti-CD3 (1 μg/ml), anti-CD28 (2 μg/ml), and anti-hamster-IgG1 (0.75 μg/ml), then harvested and lysed. For STAT immunoblots, cells were kept in cytokine-free media for 1 h, then stimulated with IL-12 (10 ng/ml), IL-4 (1 ng/ml), IL-6 (10 ng/ml) for 30 minutes. For SOCS immunoblots, cells were rested for 2 h and lysed or stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml).

ELISA

ELISA for IL-2, IFN-γ, and IL-4 was performed as detailed by the manufacturer (eBioscience). ELISA for OVA-specific antibodies was performed using Nunc Immuno-ELISA plates (eBioscience) coated with 100 μL of OVA (100 μg/ml in PBS) overnight at 4 deg and blocked with

5% BSA for 1h at RT. Wells were washed several times, and serum diluted in 5% BSA was added for 2 h. Wells were washed and incubated with biotinylated anti-murine IgG1 diluted to 0.5 μg/ml in 5% BSA for 1 h. Wells were washed and incubated with streptavidin-conjugated HRP for 30 minutes. Wells were washed and incubated with TMB substrate for 5 minutes, or until the reaction approached saturation. Linear regression analysis was used to determine titer.

T cell stimulations and skewing

Unless otherwise stated, T cells were stimulated with anti-CD3 at 5 μg/ml, and, where appropriate, anti-CD28 at 2 μg/ml. APC-less systems used anti-CD3 diluted in PBS to coat flat-bottomed plates. PMA and ionomycin were used at 50 ng/ml and 500 ng/ml, respectively.

T cells were skewed using irradiated APCs and anti-CD3, washed and expanded 5 days, then rechallenged with either plate-bound anti-CD3 and soluble anti-CD28, or fresh APCs and anti-CD3. Sorted naive cells were skewed using plate-bound anti-CD3 and anti-CD28, and rechallenged likewise. T cells skewed after nucleofection were stimulated for 48 h, and expanded for 48 h to reduce cell loss.

T_H1: IL-12 (5 ng/ml), IFN-γ (100 ng/ml), anti-IL-4 (100 μg/ml); IL-2 (1 ng/ml) during the rest period

T_H2: IL-4 (1 ng/ml), anti-IL-12 (100 μg/ml), anti-IFN-g (100 μg/ml); IL-2 (1 ng/ml) during the rest period

T_H17: TGF-β (10 ng/ml), IL-6 (10 ng/ml), anti-IFN-g (100 μg/ml), and anti-IL-4 (100 μg/ml)

T_{reg}: TGF-β (5 ng/ml), IL-2 (1 ng/ml)

Intracellular staining

Staining for cytokines was performed using brefeldin A (GolgiPlug, BD Biosciences) or monensin (GolgiStop, BD Biosciences). Cells were surface stained, then fixed and permeabilized using BD Cytofix/Cytoperm, then stained for cytokines. Intracellular staining of transcription factors was performed without stimulation, using the eBioscience Foxp3 Fixation/Permeabilization kit. Gates

were set as appropriate using unstimulated controls and voltages were set based on isotype controls.

Vaccinia infection

C57/BL6 Thy1.2^{+} host mice were immunized with 2 x 10^{6} PFU Vaccinia-OVA and adoptively transferred with 1-2 x 10^{6} CD4^{+}Thy1.1^{+} OT-II T cells from wild-type or T-*Rheb*$^{-/-}$ mice. Four days after transfer, splenocytes were harvested and rechallenged with OVA peptide (50 μg/ml) overnight.

OVA sensitization

Ovalbumin (OVA) (Sigma-Aldrich) was adsorbed onto Imject Alum adjuvant (Pierce) at a final concentration of 100 μg/ml by gently shaking for 30 minutes. Mice were primed with 200 μL intraperitoneally and boosted 14 days later. On day 18, serum and spleens were harvested. Serum was analyzed for antibodies and spleens were stimulated 48 h with 100 μg/ml OVA.

Experimental Autoimmune Encephalomyelitis

B6-background mice were immunized with 150 μg of MOG peptide with Incomplete Freund's Adjuvant supplemented with 4 mg/ml *Mycobacterium tuberculosis* subcutaneously. Mice received 200 μg of pertussis toxin intravenously on both day 0 and day 4. Mice were scored blindly as such: 0.5=flaccid tail, 1=partial hind limb paralysis, 2=full hind limb paralysis, 3=partial forelimb paralysis, 4=full fore limb paralysis, 5=moribund. Mice were sacrificed upon scoring 4 or higher, or at the end of the timecourse. CNS was harvested and either frozen or infiltrates isolated on Percoll. Infiltrates were placed in culture media for 24 h, then stimulated with PMA and ionomycin 8 h.

In vitro suppression

T_{reg} were generated from wild-type, T-*Rheb*$^{-/-}$, and T-*Rictor*$^{-/-}$ mice *in vitro* using TGF-β and IL-2, CD25 isolated (Miltenyi-Biotec), and analyzed for Foxp3 expression by flow cytometry. Wild-type

T cells were CFSE labeled and stimulated with APCs (1:10 ratio) and 1 μg/ml anti-CD3. Using calculations from Foxp3 staining, varying numbers of T_{reg} were titrated into the stimulations to achieve the stated ratios. Suppression was read 72 h later by analyzing CFSE dilution of the wild-type naïve T cells.

RNA interference

T cells were isolated using $CD4^+$ negative selection and rested in media 2 h. Cells were transfected with 200 pM control, SOCS3, or SOCS5 siRNA pools (Dharmacon) using the Amaxa T cell nucleofection kit (Lonza). Briefly, T cells were resuspended at 20 x 10^6/ml in T cell nucleofection solution, added to siRNA, nucleofected using the X-001 program, and immediately transferred to 1.5 ml of T cell nucleofector media. T cells were washed after 16 h and stimulated in normal culture media.