METHODS

Mice. Mice that express floxed *fur* alleles ($fur^{f/f}$) [7] were backcrossed six times with C57/BL6 mice. C57/BL6, CD4-Cre, $RAG2^{-/-}$ and $TCR\alpha^{-/-}$ mice all on C57/BL6 background were from Taconic. CD4-Cre mice were bred with $fur^{f/f}$ animals to generate T cell specific furin knockout (CD4cre-$fur^{f/f}$). For *in vitro* conversion assays CD4cre-$fur^{f/f}$ animals bearing Foxp3-GFP transgene (from Dr. M. Oukka, Harvard Medical School) were used. All mice were housed under pathogen free conditions in accordance with the NIH Animal Care and Use Committee.

Flow cytometry. For surface staining, cells were resuspended in FACS staining buffer (PBS + 1% FBS) containing 2 $\mu g\ ml^{-1}$ Fc block (BD Pharmingen) and stained on ice for 15-30 minutes using indicated antibodies from BD Pharmingen or biotinylated anti-LAP antibody and goat-IgG from R&D Systems. Intracellular Foxp3 was assessed by intracellular staining according to manufacturer's instructions, using antibody and buffers from eBiosciences. For intracellular cytokine stainings cells were activated for 4 h with phorbol myristate acetate and ionomycin and golgiplug (BD Pharmingen) was added to cultures after 2 h. Intracellular staining was done with intracellular cytokine staining kit (BD Pharmingen). Cells were analyzed using a FACS Calibur or FACS Canto instrument (BD Pharmingen) and data were analyzed with Flow-Jo software (Treestar).

Cell purification and culture. For quantification of furin deletion efficiency and induced Treg culture, $CD4^+$ and $CD8^+$ cells were purified by positive selection using magnetic beads (Miltenyi Biotech). Cells were activated for 3 days with plate-bound anti-CD3 and

CD28 antibodies (5 μg ml^{-1} in PBS), and further expanded for 3 additional days in complete 10% FBS RPMI supplemented with IL-2 (50 U ml^{-1}). For Th1 polarization, initial activation of $CD4^+$ cells was done in the presence of IL-12 (10 ng ml^{-1}) and anti-IL-4 antibody (10 μg ml^{-1}). For induced Treg culture $CD4^+$ cells were activated with plate-bound anti-CD3 10 μg ml^{-1} and soluble anti-CD28 2 μg ml^{-1} antibodies, IL-2 100 ng ml^{-1} and TGFβ-1 5 ng ml^{-1}. For suppression assays and microarray analysis $CD4^+$ cell were first enriched with a $CD4^+$ T cell negative selection kit (Miltenyi) followed by flow cytometry sorting for $CD4^+CD25^-CD45Rb^{hi}$ and $CD4^+CD25^+$ or $CD4^+CD44^{low}CD62L^+$ cells using a MoFlo cell sorter (Dako). For *in vitro* conversion assay $CD4^+$Foxp3-GFP^+ and $CD4^+$Foxp3-GFP^- cells were purified by flow cytometry.

Western blot and RT-PCR. Western blotting was performed as described[2] using actin (Chemicon), furin (Santa Cruz) or activated TGFβ-1 (sc-146, Santa Cruz) antibodies. Total RNA was isolated with RNAeasy kit (Qiagen) and reversed transcribed cDNA synthesis kit (Applied Biosystems). *Fur* exon two specific RT-PCR was performed using ABI PRISM 7700 Sequence Detection System as described previously[7].

Cytokine and antibody measurements. Mesenteric lymph node T cells (1 x 10^6 ml^{-1}) were activated for 48 h with plate-bound anti-CD3 (10 μg ml^{-1}) and soluble anti-CD28 (2 μg ml^{-1}) (BD Pharmingen). Cytokine levels in supernatants or sera were determined with IL-13 and IL-17 ELISA (R&D Systems) or with mouse Th1/Th2 cytokine or inflammation Cytometric Bead Array kits (BD Pharmingen). Serum circulating anti-DNA and ANA antibodies and IgG1, IgG2 and IgE immunoglobulins were determined with

ELISA kits from Alpha Diagnostic International. To determine TGFβ-1 production, FACS-sorted $CD4^+CD25^-$ and $CD4^+CD25^+$ were first stimulated for 48 h with anti-CD3 (10 μg ml^{-1}) and soluble anti-CD28 (2 μg ml^{-1}) in complete RPMI 10% FBS supplemented with IL-2 (50 U ml^{-1}), rested 24 h in complete medium, washed twice with PBS and finally re-stimulated in RPMI +3% FBS +1% Nutridoma (Roche) supplemented with IL-2 50 U ml^{-1} for another 48 h. TGFβ-1 was measured in the supernatants using a multispecies TGFβ-1 ELISA kit (Invitrogen) or ELISA specific for mature/active TGFβ-1 (eBiosciences); TGFβ-1 concentration in the final stimulation medium was measured and subtracted as background.

Microarray analysis. Total RNA from wild-type- and furin-deficient $CD4^+CD44^{low}CD62L^+$ cells was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Approximately 500 ng of RNA was labeled using a MessageAmp II Biotin Enhanced kit (Ambion) and hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix) according to the manufacturers' protocols. Gene expression values were determined using GeneChip Operating Software (GCOS) v1.1.1. Data were analyzed using GeneSpring software GX 7.3.1 (Agilent Technologies). Gene expression was normalized across each chip as well as across all experiments. Genes with an average expression value below 50 and those flagged absent in all samples were deleted from subsequent analysis.

Isolation of gut intraepithelial and lamina propria lymphocytes. To isolate intraepithelial lymphocytes, Peyer's patches were removed and mouse small intestines

were cut into 1-3 cm segments. Gut pieces were placed in RPMI 2% FBS with constant stirring and incubated for 20 minutes at 37°C. After incubation, the gut suspension was strained though a sterile strainer, and flow-through was collected in a beaker on ice. To ensure optimum IEL yield, gut pieces were placed into a 50 ml tube containing 15 ml serum-free RPMI, shaken vigorously for 30 seconds, and strained as above and further filtered through a 70 μM cell strainer. Cells were sedimented for 10 min at 1500 rpm 4°C. The cell pellet was re-suspended in 30% Percoll and centrifuged at 1600 rpm at RT for 20 min. The supernatant was discarded and the pellet containing IELs were collected for analysis. Isolation of lamina propria lymphocytes was performed as described previously[22].

***In vitro* suppression assay.** Varying numbers of wild-type or furin deficient $CD4^+CD25^+$ T regulatory cells were cultured in 96-well round-bottom plates with 5 x 10^4 wildtype effector $CD4^+CD25^-$ T cells along with 5 x 10^4 $CD90^+$ -depleted, irradiated splenocytes used as antigen-presenting cells [23]. Cells were stimulated with 0.5 μg ml^{-1} CD3 antibody (BD Biosciences) for 72 h at 37°C and 5% CO_2. Cultures were pulsed with [^{3}H]TdR at 1 μCi/well for the last 16 h of culture. The experiment was performed three times in triplicate.

***In vivo* suppression assay.** *In vivo* suppression assays were done as previously described[16]. Briefly, $TCR\alpha^{-/-}$ mice were injected intravenously with 3 x 10^5 FACS sorted wild-type or furin deficient naïve $CD4^+CD25^-CD45Rb^{hi}$ cells with or without 1.5 x 10^5 wild-type or furin deficient $CD4^+CD25^+$ T regulatory cells (five mice per group). Mice

were monitored weekly for weight loss and signs of disease, and sacrificed on week 10. Total mesenteric lymph node cells were isolated and counted; T cell numbers and ratios of $CD4^+Foxp3^+$ cells were determined with flow cytometry. Experiment was repeated three times. For proliferation and IFNγ production assessment $RAG2^{-/-}$ mice were reconstituted with CFSE-labeled naïve $CD4^+CD25^-CD45Rb^{hi}$ cells alone or with $CD4^+CD25^+$ T regulatory cells (3 mice per group). Congenic markers CD45.1 and CD45.2 were used to distinguish between transferred cells. Mice were analyzed on day seven for effector T cell proliferation and cytokine production by flow cytometry.

In vitro conversion assay. $CD4^+FoxP3^+$ T regulatory cells were activated for 4 days with plate-bound anti-CD3 and IL-2 (100 U ml^{-1}). The activated Treg cells were then co-cultured for an additional 4 days with CFSE-labeled wild-type of furin-deficient $CD4^+Foxp3^-$ responder cells in the presence of splenic dendritic cells at a 5:5:1 ratio ($CD4^+Foxp3^+$: $CD4^+Foxp3^-$:splenic dendritic cells), anti-CD3 (2 μg ml^{-1}) and IL-2 (100 U ml^{-1}) as indicated. Cytokine-induced conversion of wild-type or furin-deficient $CD4^+Foxp3^-$ effectors was investigated in the absence of Treg cells, but in the presence of dentritic cells and exogenous TGFβ-1 (5 ng ml^{-1}). $CFSE^+$ T cells were analyzed for Foxp3 expression using flow cytometry.

Statistical analysis. P values were calculated using Student's t-test; error bars in graphs represent s.e.m.