**Supplemental methods**

**Cell preparation**

For iTreg generation, splenic CD4+ Tcon cells from ATG5 FLC, WT FLC, Atg7\(^{fl/fl}\) Foxp3\(^{cre}\) or WT Foxp3\(^{cre}\) mice were purified by MACS beads (CD4) positive selection according to the manufacturer’s protocols (Miltenyi Biotec) and sorted based on 7AAD\(^{-}\)CD45.1\(^{-}\)CD45.2\(^{+}\)CD4\(^{+}\)CD25\(^{-}\) expression for cell from FLC mice and based on 7AAD\(^{-}\)CD4\(^{+}\)YFP\(^{-}\) for Atg7\(^{fl/fl}\) Foxp3\(^{cre}\) or WT Foxp3\(^{cre}\) mice. Twenty-four–well plates were coated with anti-CD3 (2C11, 10 mg/ml) and anti-CD28 (N3751, 1 mg/ml) antibodies (Abs). CD4 Tcon were seeded at 0.5 or 1x 10⁶ cells/well and resuspended in 1 ml IMDM with 10% FBS, 2 mM L-glutamine, 50 mM 2-ME, 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate, supplemented with 100 U/ml recombinant human IL-2 (Aldesleukin), 10ng/ml TGF-b1 (BioLegend), and 100 ng/ml rapamycin (Invitrogen). 1ml of medium was added on day 3, and cells were harvested on day 5 for analysis or further studies.

To analyze cytokine production, splenic cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h at 37°C and brefeldin A (1 mg/ml) was added for the last 3 hours to examine cytokine production.

**Suppression assay**

In round bottom 96 wells plate, 5x10⁴ CFSE or violet dye stained splenic effector T cells (sorted CD4\(^{+}\) CD25\(^{-}\) T cells from Ptprca\(^{a}\) mice) were stimulated with an anti-CD3 Ab (2C11,1 mg/ml) and with 12.5x10⁴ DC (MHCII\(^{+}\) CD11c\(^{+}\)) isolated from spleen of C57BL/6 and purified using Optiprep density gradient (Elitechgroup, Australia) following by a MACS beads (CD11c) positive selection on columns according to the manufacturer’s protocols (Miltenyi Biotec). Cells were incubated for 5 days with sorted 7AAD\(^{-}\)CD4\(^{+}\) YFP\(^{+}\) Treg
from $Atg7^{fl/fl}$:Foxp3$^{cre}$ mice, 7AAD$^{neg}$CD4$^+$ YFP$^+$ Treg WT Foxp3$^{cre}$ mice or 7AAD$^{neg}$CD4$^+$ RFP$^+$ Treg Foxp3-RFP mice (ratio 1/1) or with Atg5$^{-/-}$ or WT iTreg generated in vitro from FLC mice (ratio 1/4) in complete IMDM media. Effector T cell proliferation were assessed by flow cytometry analysis of CFSE or violet dye dilution in CD4$^+$ CD45.1$^+$ T cells.
Supplemental Figures

Figure S1

A) Flow cytometry analysis of WT and Atg5−/− iTreg generated in vitro from sorted splenic CD45.2+CD45.1−CD4+CD25− of WT and ATG5−/− FLC mice. Frequency (%), absolute number (#) and FoxP3 intensity (geometric mean) of iTreg (n = 4-3 from 3 independent experiments).

B) Flow cytometry analysis of WT and Atg7−/− iTreg generated in vitro from sorted splenic CD4+YFP− of Atg7−/−Foxp3cre+ (Atg7−/−) or WT -Foxp3cre+ (WT) mice. Frequency (%), absolute number (#) and FoxP3 intensity (geometric mean) (n = 2 from 2 independent experiments). Data are shown as mean ± SEM. Abbreviations: Induced Treg (iTreg), autophagy-related gene (Atg), Fetal Liver Chimera (FLC).

Figure S1. Efficient in vitro generation of autophagy deficient iTreg.
Figure S2. Autophagy in Treg is required to control effector T cell activation.

(A) Frequency (%) of CD4^+FoxP3^{neg} and (B) frequency (%) and absolute number (#) of CD4^+FoxP3^{neg}CD25^+ cells gating on CD4^+CD8^-CD3^+ T cells. (C) Representative histogram and diagram of frequency (%) of Ki67^+ cells in CD4^+FoxP3^{neg} T cells. (D) Representative dot plot and frequency (%) of CD62L^+CD44^-, CD62L^-CD44^+ in CD4^+FoxP3^{neg} T cells. (E) Absolute number (#) of CD4^+FoxP3^{neg} Tcon (CD4) and CD8^+FoxP3^{neg} Tcon (CD8) and frequency (%) of IFN\(\gamma^+\), IL-17^+ and IL-10^+ cells in FoxP3^{neg} CD4^+ and CD8^+ T cells from in vitro restimulated splenocytes of Atg7^{flo/flo}-Foxp3cre^+ (Atg7^-/-) and WT-Foxp3cre^+ (WT) mice (male and female) (n = 6 from 2 independent experiments). Data are shown as mean ± SEM. Statistical significance was determined using an unpaired 2-tailed Mann-Whitney U test (*p<0.05; **p<0.01; ***p<0.001). Statistical analyses were performed using GraphPad Prism Version 6.01 software.

Abbreviations: conventional T cells (Tcon), autophagy-related gene (Atg).
Figure S3

A) Ex vivo Treg

Nil

WT

Atg7/-

B) iTreg

Nil

WT

Atg5/-

CFSE

Figure S3. Autophagy deficient Treg are suppressive in vitro.

A) Representative histograms of proliferation (violet dye dilution) and percentage of inhibition of proliferation of violet dye stained stimulated splenic effector T cells (sorted CD4+ CD25reg T cells) incubated 5 days with sorted 7AADnegCD4+ YFP+ Treg from (Atg7fl/fl-Foxp3cre) (Atg7+/−) mice or 7AADnegCD4+ RFP+ Treg from Foxp3-RFP mice or WT-Foxp3cre+ (WT) (ratio 1/1) (n=2 from 2 independent experiments). B) Representative histograms of proliferation (CFSE dilution) and percentage of inhibition of proliferation of CFSE stained and stimulated splenic effector T cells (sorted CD45.1+CD4+CD25reg) incubated 5 days with Atg5/− and WT iTreg generated in vitro from FLC mice (ratio 1/4) (n=2 from 2 experiments).

Abbreviations: 7-Aminoactinomycin D (7-AAD), Yellow fluorescent protein (YFP), autophagy-related gene (Atg), Red Fluorescent Protein (RFP), Carboxyfluorescein succinimidyl ester (CFSE), Induced Treg (iTreg), Fetal Liver Chimera (FLC).
**Figure S4**

(A-C) Cytometry analysis of Atg7\(^{+/−}\) and WT mixed chimera generated by injecting BM from Atg7\(^{fl/fl}\)-Foxp3\(^{cre}+(CD45.2^+)\) (Atg7\(^{−/−}\)) or WT-Foxp3\(^{cre}+(CD45.2^+)\) (WT) mice with equal number of BM from congenic (CD45.1\(^+\)CD45.2\(^+)\) mice into irradiated syngenic Ptpc\(^a\) mice (CD45.1\(^+)\) (n = 5). (A) Absolute number of total donor cells CD45.1\(^+\)CD45.2\(^+\) and CD45.1\(^−\)CD45.2\(^+\) in spleen of WT and Atg7\(^{−/−}\) groups. Absolute number of Treg CD4\(^+\)FoxP3\(^+\) from congenic (CD45.1\(^+\)CD45.2\(^+)\) donor cells or from Atg7\(^{fl/fl}\)-Foxp3\(^{cre}+(CD45.2^+)\) or WT-Foxp3\(^{cre}+(CD45.2^+)\) donor cells in spleen (B) or BM (C) of recipients. Data are shown as mean ± SEM. Statistical significance was determined using an unpaired 2-tailed Mann-Whitney U test (*p<0.05; **p<0.01; ***p<0.001). Statistical analyses were performed using GraphPad Prism Version 6.01 software. Abbreviations: autophagy-related gene (Atg).

**Figure S5**

**Figure S5. CD25 expression on TIGIT\(^+\) and TIGIT\(^{neg}\) Treg in spleen and BM of C57BL/6 mice.** (A) Overlay of histograms representing CD25 expression on TIGIT\(^+\) or TIGIT\(^{neg}\) FoxP3\(^+\) CD4\(^+\) Treg of C57BL/6 mice using intracellular staining to stain FoxP3.