Supplemental document

Supplemental methods

Cell preparation

For iTreg generation, splenic $CD4^+$ Tcon cells from ATG5 FLC, WT FLC, *Atg7*$^{fl/fl}$-*Foxp3cre*$^+$or *WT-Foxp3cre*$^+$ mice were purified by MACS beads (CD4) positive selection according to the manufacturer's protocols (Miltenyi Biotec) and sorted based on $7AAD^{neg}CD45.1^{neg}CD45.2^+CD4^+CD25^{neg}$ expression for cell from FLC mice and based on $7AAD^{neg}CD4^+YFP^{neg}$ for *Atg7*$^{fl/fl}$-*Foxp3cre*$^+$or *WT-Foxp3cre*$^+$ 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 10^6 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, 5x10^4 CFSE or violet dye stained splenic effector T cells (sorted $CD4^+$ $CD25^{neg}$ T cells from $Ptprc^a$ mice) were stimulated with an anti-CD3 Ab (2C11,1 mg/ml) and with 12.5x10^4 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 $7AAD^{neg}CD4^+$ YFP^+ Treg

from *Atg7*$^{fl/fl}$-*Foxp3cre*$^{+}$ mice, 7AAD^{neg}CD4^{+} YFP^{+} Treg *WT Foxp3cre*$^{+}$ 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

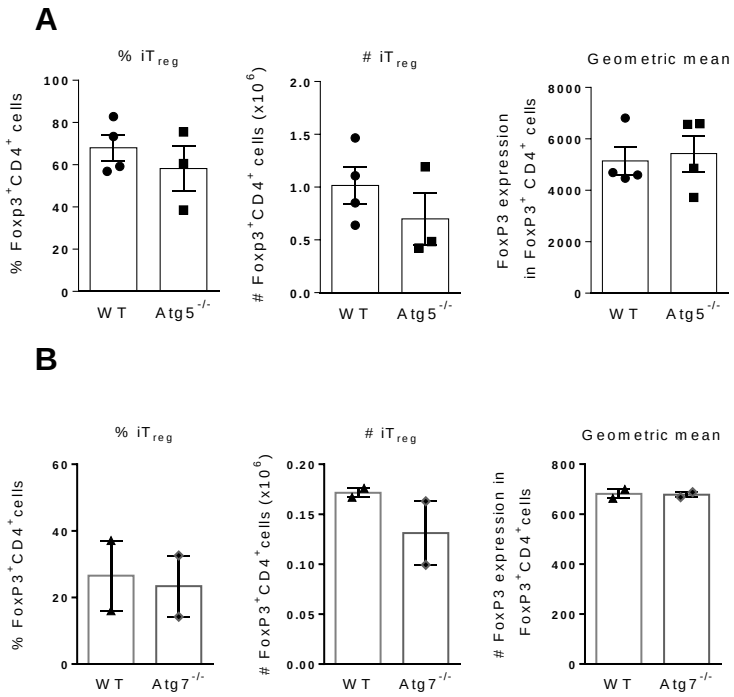

Figure S1. Efficient in vitro generation of autophagy deficient iTreg.

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^{neg} of Atg7$^{fl/fl}$-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 S2

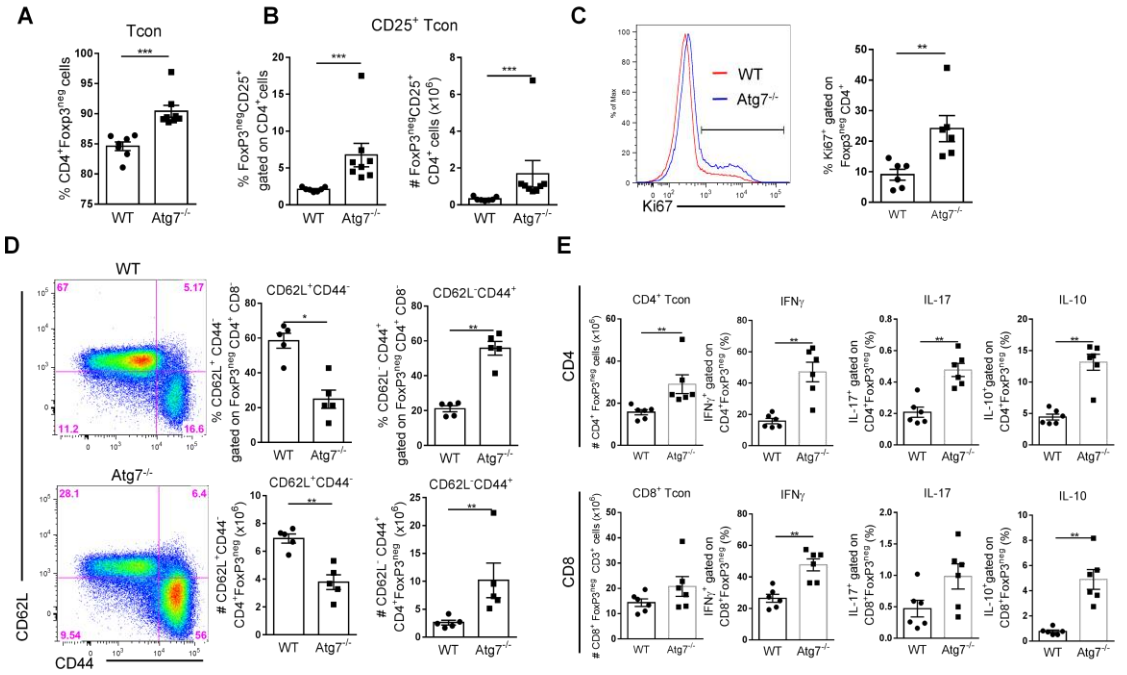

Figure S2. Autophagy in Treg is required to control effector T cell activation.

(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 $IFN\gamma^{+}$, $IL\text{-}17^{+}$ and $IL\text{-}10^{+}$ cells in $FoxP3^{neg}$ $CD4^{+}$ and $CD8^{+}$ T cells from in vitro restimulated splenocytes of *Atg7$^{fl/fl}$-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

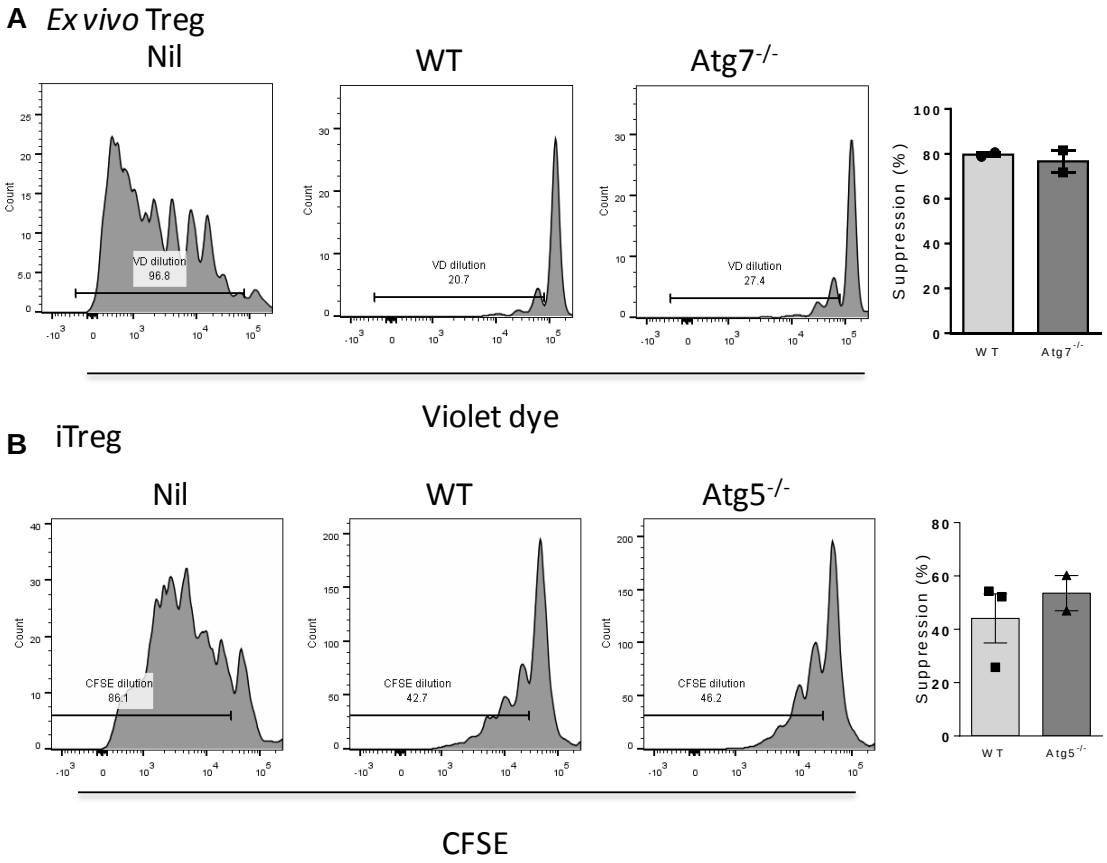

Figure S3. Autophagy deficient Treg are suppressive in vitro.

A) Representative histograms of proliferation (violet dye dilution) and percentage of inhibition of proliferation of violet dye stained stimulated splenic effector T cells (sorted $CD4^+$ $CD25^{neg}$ T cells) incubated 5 days with sorted $7AAD^{neg}CD4^+$ YFP^+ Treg from *(Atg)7*$^{fl/fl}$*-Foxp3cre*$^+$ ($Atg7^{-/-}$) mice or $7AAD^{neg}CD4^+$ 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 $CD45.1^+CD4^+CD25^{neg}$) 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

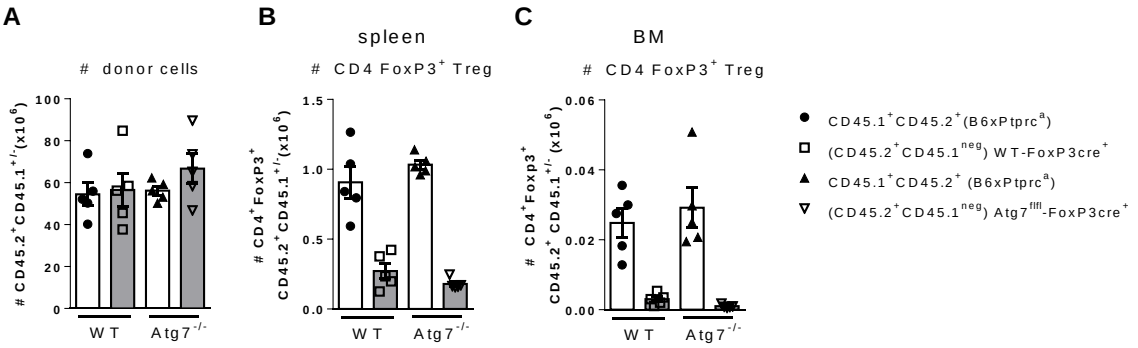

Figure S4. Reduced reconstitution of Cre^{+} Treg compare to Cre^{neg} Treg

(A-C) Cytometry analysis of *Atg7*$^{-/-}$ and WT mixed chimera generated by injecting BM from *Atg7*$^{fl/fl}$*-Foxp3cre*$^{+}$ (CD45.2^{+}) (Atg7$^{-/-}$) or *WT-Foxp3cre*$^{+}$ (CD45.2^{+}) (WT) mice with equal number of BM from congenic (CD45.1^{+} CD45.2^{+}) mice into irradiated syngenic Ptprc^{a} mice (CD45.1^{+}) (n = 5). (A) Absolute number of total donor cells CD45.1^{+} CD45.2^{+} and CD45.1^{neg} 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*$^{fl/fl}$*-Foxp3cre*$^{+}$ (CD45.2^{+}) or *WT-Foxp3cre*$^{+}$ 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

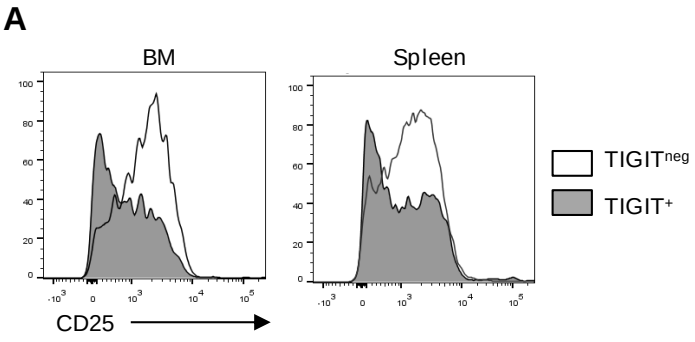

Figure S5. CD25 expression on TIGIT^{+} and TIGIT^{neg} Treg in spleen and BM of C57BL/6 mice. (A) Overlay of histograms representing CD25 expression on TIGIT^{+} or TIGIT^{neg} FoxP3^{+} CD4^{+} Treg of C57BL/6 mice using intracellular staining to stain FoxP3.